Characterizing the influence of different factors on acid resistance phenotypes of a panel of *Escherichia coli* salami validation strains

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Agricultural Science

Author: Santiago Ramirez Cuevas

University of Tasmania

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DECLARATION

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THESIS ABSTRACT

Acid resistance of *Escherichia coli* (*E. coli*) is relevant to food safety, as certain foods rely on acidity for preservation and pathogen inactivation. In Australia, food borne disease outbreaks associated with pathogenic *E. coli* and involving salami and other fermented food products have been well documented. The ability of pathogenic *E. coli* to survive through the food chain, together with its low infectious dose, is hypothesized to be partly due to its ability to resist acid challenge. In the last two decades numerous laboratories worldwide have worked to understand and characterize *E. coli* acid resistance. These laboratories have reported that the mechanisms used by *E. coli* against acid challenge are yet to be completely understood and many knowledge gaps exist. Careful analysis of the current published data on the *E. coli* acid resistance reveals substantial differences in the way experiments are conducted. These include differences in media, acid type and pH values. The hypothesis and central argument of this thesis is that slight variations in the parameters used for *in vitro* acid challenges might have a significant effect on the acid resistance profiles of *E. coli* cells. More specifically this project focuses on demonstrating that slight variations in the parameters for an acid challenge experiment can vary its outcome and ultimately the interpretation and application of the experiment results. The document's central argument is the contribution of knowledge regarding the response of *E. coli* cells to *in vitro* acid challenge.

The acid response of a panel of 5 *E. coli* salami validation strains and two controls was studied using a proteomic gel-based approach. Proteomic analysis revealed time-dependent protein expression differences following acid challenge. It was

concluded however that such approach was too coarse to reveal differences in protein expression profiles that may be associated with differences in the acid resistance phenotypes amongst *E. coli* strains studied.

In order to obtain a better understanding of the phenotypes of the salami validation strains, a series of small experiments were performed. These experiments investigated the effects that storage, acid adaptation, type of acid used; salt and temperature have on the ability of *E. coli* strains to tolerate acid challenge. An interesting finding from these experiments was the fact that a significant percentage of cells in cultures challenged with acid may enter a viable but non-culturable state following acid challenge. Using fluorescent microscopy, it was demonstrated that following acid exposure the number of viable cells using plate counts as an indicator was considerably less than when assessed using fluorescent microscopy.
In addition, it was also demonstrated that the inclusion of 1% glucose to the growth media, increased the capacity of cultures to withstand acid challenge when compared with cultures grown without the addition of glucose.

Subsequently, two *E. coli* strains (O157:H7 and an Australian salami validation *E. coli* strain) were tested for their ability to tolerate acidic conditions, in broth and in semi-solid agar. In this study a blend of carrageenans was developed to assess survival of *E. coli* in an acidic semi-solid environment. The novel blend allowed for a homogeneous semi-solid-agar to remain stable at pH 4.5 following the addition of acid. Studies revealed that survival of both strains were affected by the physical state of the challenge media. Bacterial cells, regardless of pathogenicity, appeared to have

a higher acid resistance when challenged in the semi-solid media than when challenged in broth.

A panel of 12 *E. coli* strains (pathogenic and non-pathogenic) was challenged utilizing four different media (BHI, TSB, NB and MM), four different salt concentrations (0.5%, 3.5%, 8.5% and 12.5%) and three different acid treatments varying in pH values (2.4 to 4.6) and L-lactic acid concentrations (0.42 to 3.7 g/L). Results demonstrated that the acid resistance profiles within the panel of strains significantly varied across the treatments. Of the components that altered acid resistance, NaCl and media type had the largest effect. In particular, BHI broth appeared to have the greatest protective effects across all treatments. Buffer composition altered the amount of acid required to reach a set pH level and ultimately the amount of free acid in the solution. In some cases individual strains that appeared to be acid resistant under a certain set of conditions displayed no ability to resist acid under another.

The acid resistance of the pathogenic *E. coli* O157:H7 (Sakai, ATCC BAA-460) was characterized on a genetic level using microarray analysis. Genetic expression profiles of exponential phase cells (acid sensitive), stationary phase cells (acid resistant) and stationary phase cells challenged with acid in the presence or absence of 3.5% NaCl were analyzed. Transcriptome analysis revealed the presence of previously identified acid-resistance-associated genes for stationary phase cultures prior to and following acid challenge. Transcripts for the challenged cultures revealed the presence of a large quantity of up-regulated genes of bacteriophage origin. These results suggest that the given challenge conditions were capable of

triggering the activation of the bacteriophage inserted genome. Such activation might lead to toxin production or the promotion of genes that might be advantageous for intestinal colonization.

The hypothesis of this thesis was supported by the results. The outcome of various acid challenges was altered with changes of the experimental parameters. The experimental data demonstrated that the parameters of *in vitro* acid challenge experiments have an independent effect on the acid resistant phenotypes of *Escherichia coli* cells. *E. coli* strains that might appear resistant to acid challenge within a panel of strains under one condition, might appear to be acid-sensitive if the conditions of the challenge are changed. The data also demonstrated that changes of the physical state of the challenge media (liquid versus semi-solid) can have a direct effect on acid survival of. While at a genetic level, there significant changes were observed when NaCl is introduced in an acid challenge.

The results suggest that the interaction of environmental factors play an important role in setting acid resistance levels of *E. coli* strains. These observations argue the closer attention must be paid to the parameters used in acid challenge experiments as variations on experimental outcomes may arise due to the possible secondary effects of secondary variables.

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"There is such a thing as food and such a thing as poison. But the damage done by those who pass off poison as food is far less than that done by those who generation after generation convince people that food is poison"

Paul Goodman (1911-1972) American author, poet and critic.

CHAPTER PREFACE:

This chapter summarizes the relevance of food safety and its impact on human health. In parallel it highlights the importance of food safety research and the necessity to make foods safer. Since this project focuses on the *E. coli* stress response associated with salami manufacture, both themes are also introduced. The main aim of this chapter is to provide the reader with background information for the following experimental chapters.

1.1. Food-borne disease

Food-borne disease can be defined as a disease caused by agents that enter the body upon the consumption of food or drink. Although due to their nature and complexity food-borne diseases are difficult to define, it is believed that to this date there are more than 250 different types. It has been estimated that in 1995 alone up to 1.8 million people died from diarrheal disease, with a great proportion linked to food-borne disease (WHO, 2009). Food-borne diseases are the leading causes of illness and death in developing countries, while in developed countries, they have been estimated to affect up to one-third of the population each year (WHO, 2009). The most common food borne diseases are those caused by the bacteria *Salmonella*, *Campylobacter* and pathogenic *E. coli* (particularly the O157:H7 serotype) and by a group of viruses referred to as Norwalk or Norwalk-like (CDC, 2005). The prevalence of *Salmonella*, *Campylobacter*, and *E. coli* in food is partly due to their intrinsic presence in the intestinal tracts of many animals, some of which are routinely consumed by humans. However, the presence of these and other pathogens in food is not exclusive to those that contain such animal products, as environmental cross-contamination can readily occur. For example, many food borne related outbreaks have been reported from the ingestion of non-animal related food sources, such as an outbreak in the United States involving lettuce contaminated with a virulent *E. coli* O157:H7 strain, which is normally a commensal of cattle. The strain was found to be present in high numbers, after the lettuce was irrigated with contaminated water (Hilborn *et al.*, 1999). At a much smaller scale, many cross-contamination-food-borne associated outbreaks have

been reported to occur in restaurants, catering companies and ordinary households (Rangel *et al.*, 2005).

1.2. Food borne disease trends

To this date, the incidence of food-borne disease has become a major trans-national concern; it is not only on the rise but has a major negative economic impact and on public health. The increment of food-borne disease cases can be linked to the expanding globalization and urbanization trends (Alterkruse et al, 1997; Bettcher., 1997). In the past few decades more and more food products are being mass-produced, meaning that more food is prepared by less people. Thus the effects of a contamination event in mass manufactured food products can be multiplied due to centralized food processing and distribution systems. For example, the biggest Salami factory in central Europe owned by the Pick – Delhus group in Hungary has the capability of manufacturing an amazing annual figure of 20,000 tons of fermented meat product (Anon., 2004). Taking those output figures as an example one can only imagine the monetary and health impact should contamination of a single batch with a food-borne microorganism was to occur. Due to globalization consumer demands for a great variety of products and the availability of all-year-round seasonal products have increased. In consequence, there has been a large increase in the amount of food products imported and exported by many nations. A factor contributing to the emergence and persistence of food pathogens is these rapid movements of agricultural and livestock products amongst states and countries. Through time this phenomenon has increased the exposure of humans to unknown agents, including bacteria, which

assist in the rapid propagation of such due to low immunity (Pina et al, 2005). In addition, there has been a rise in the involvement of environmentally resistant and host-adapted pathogen species which are now becoming difficult to control with traditional food preservation methods (Alterkruse et al 1997). As a consequence it is imperative for global food safety that, food preservation methods are constantly revised so that they evolve at a faster rate than the resistant mechanisms of such adapted pathogens.

In summary, the factors that contribute to pathogen emergence can be summarized in three major categories. 1. Environmental, which include, climate changes (temperature and drought), deforestation and dam constriction. 2. Food related factors, witch include, changes in food production and distribution, processing modifications (decrease levels of NaCl and fat), alteration to packaging and theuse of antibiotics. 3. Consumer-related factors, which include rapid urbanization, increase international travel and changes in eating habits (Samelis & Sofos, 2002). It has been speculated that the emergence of foodborne pathogens is due to their genetic makeup, which allows them to adapt and evolve to different challenges, environmental and antimicrobial challenges. The exposure of the pathogens to sublethal doses of antibiotics, chemicals and adverse environmental factors has provided selective pressure for the resistant organisms to prevail in mix populations within the food chain (Pina et al 2005)

In terms of food safety such changes have had many repercussions as the standards of food safety and hygiene practices are not the same from country to country (Käferstein *et al.*, 1997). In economical terms this can also have a great impact. For example, some countries might choose to import food products from one nation instead of another simply based on their food safety practices. To continue the example of the Pick-

Delhus group, the manufacturer was unable to export to the US for many months due to a ban imposed due to concerns over Hungarian inspection techniques (Anon., 2004). The latest issue is also of relevance to Australia, since many countries prefer to import Australian products over those from other countries based on its reputation of being "safe". On the other hand, many products from various countries are not allowed to enter Australian soil due to the strict food regulations imposed by the Australian Regulatory bodies such as the Australian Quarantine and Inspection Services (AQIS) and Food Standards Australia and New Zealand (FSANZ).

1.3. Food borne disease in Australia

Although Australia is known for its excellent food safety and quality standards, food borne illnesses are ubiquitous phenomena. In Australia, an estimated 5.4 million cases of food-borne gastroenteritis are believed to occur each year (FoodNet, 2008). Of these cases about 80% is estimated to be caused by either, pathogenic *E. coli*, *Salmonella*, *Campylobacter* or by Norwalk-viruses. Each year it is estimated that about 1.2 million people visit the doctor, with an estimate of 2.1 million days of work loss each year (WHO, 2009). In addition it is estimated that about 300,000 antibiotic prescriptions are prescribed as a result of food-borne illness each year (CDC, 2005). Such phenomena are not only of economic concern but can also contribute substantially to the emergence of bacterial antibiotic-resistance (Magee *et al.*, 1999). In Australia a number of outbreaks have been linked to the consumption of salami and other fermented meats (Cameron, 1995; CDC, 1995). One of the most serious fermented meat related outbreak occurred in 1995 in South Australia, resulting in the

hospitalization of several people and the death of a child (Cameron, 1995). As result FSANZ implemented a specific set of requirements to the Food Standards Code concerning the manufactures of salami and other fermented meats; formerly known as uncooked comminuted fermented meats (UCFM) (FZANZ, 2009). In fact this particular event is associated with the origin of this project, where Meat and Livestock industries (MLA) joined forces with CSIRO Food Science Australia in order to implement research in relation to the safe manufacture of salami. The basis of this venture was to develop a set of salami validation strains whereby the safety of salami manufacturing could be more accurately assessed. In 2000, some of the work that arose from this collaboration was published by Paul Vanderlinde and Lesley Duffy (Duffy *et al.*, 2000).

1.4. Salami

In "worldwide terms", salami refers to a type of cured sausage that is fermented and dried. The word salami is derived from the word "sale" (Latin/ Italian for salt), and the Italian word "ame" referring to various. Although originating from Italy, salami manufacture has rapidly spread to many regions of the world, and subsequently many regional styles have been developed (Roper, 2009). In general salami manufacture involves three main steps, preparation, fermentation and maturation (Incze. K., 1998; Nightingale et al., 2006).

In the first step all the ingredients are mixed and the starter culture (starter cultures discussed in detail below) is introduced. Although the recipes vary for each style, amongst the most common ingredients are: ground beef or pork or a mix of beef and

pork, salt, pepper, sugar and other spices together with certain additives such as sodium nitrate. Once mixed ingredients are stuffed into casings and they are allowed to ferment. The fermentation parameters between manufacturers and different types of salami readily vary (Getty et al, 2000). During fermentation, also known as "curing", the casings are placed in a controlled atmosphere, with an average humidity range between 90 and 95% and a temperature range between 15 °C and 30°C; conditions which promote the growth of the starter culture (Lucke, 1994; Metaxopoulos *et al.*, 1981; Hammes., & Hertel, C., 1998; Ravyts *et al.*, 2008 Getty et al, 2000). The fermentation process can vary from 17 h to 36 h, and upon completion the salami enter what is known as maturation process. The maturation process also known as the "drying process" in which the product dries within a controlled atmosphere with a relative humidity ranging as low as 65% to 85% and an average temperature of 13°C (Bacus, 1986; Lucke, 1985). During the maturation the flavours are enhanced and the water activity is gradually reduced. The maturation process is designed to prevent further replication of the starter culture. Following this process, the salami product is then ready for human consumption without the need of cooking (Metaxopoulos *et al.*, 1981; Ravyts *et al.*, 2008).

In microbiological and food safety terms salami is classified as a ready to eat uncooked product or UCFM, and its safe manufacture can be seen as an intricate play of hurdles and systems that synergize ensuring the inactivation of pathogens (Nightingale *et al.*, 2006; Ravyts *et al.*, 2008 Lucke , 1994). Salami manufacture involves the use of a combined hurdle effect of antimicrobial substrates and conditions that prevent bacterial replication and survival. During the preparation process a starter culture is introduced to the mixture of raw meat (which carries beef or pork or both), spices and other agents. Starter cultures are added for the inhibition of the growth of undesirable

microorganisms, both enteric pathogens and spoilage organisms (Metaxopulus *et al.*, 2002) The inhibition of undesirable microorganisms by the starter culture is mainly due to competitive inhibition of nutrients and the generation of by-products such as acids, peroxides, enzymes and or bacteriocins (Lucke, 1994; Hammes.& Hertel, 1998 Ravyts *et al.*, 2008) . Starter cultures in salami mixtures can vary from product to product and they are usually chosen by the manufacturers to produce a desired effect on the product. Some of the commonly used starter cultures are: a range of *Lactobacillus* species, *S.canosus*, *S. xylosus*, *Laeuconostoc mesenteroides* (Dicks, *et al* 2004; Hammes.& Hertel, 1998; ; Lucke, 1994). The drying process also assists in the inactivation of pathogens by restricting the availability of water, which hinders bacterial growth and survival (Nightingale *et al.*, 2006; Ravyts *et al.*, 2008). In relevance to this thesis it must be acknowledged that it was believed, and perhaps it still is, that the presence of salt in the salami mixture is considered as an additional hurdle. However, as seen in some of the following chapters the presence of salt under certain conditions can actually augment the acid tolerance of *E. coli* and perhaps other potential pathogens.

1.5. Salami and food-borne disease

In the past salami manufacturers relied entirely on the intrinsic properties of the manufactured salami sausages (low water activity, low pH etc.). However, when resistant pathogens such as *Salmonella, Listeria monocytogenes* and *E. coli* O157:H7 are initially present in high numbers, salami manufacture may reduce the numbers but may not ensure that the final product is pathogen free (Gianfranceschi *et al.*, 2006;

Glass & Doyle, 1989; Junttila, 1989; Bacus, 1996). For this reason FSANZ mandated a number of requirements for uncooked fermented comminuted meat products, which must be followed by manufacturers. One of these is the ability to demonstrate that their process can achieve a 3-log reduction in generic *E. coli* prior to leaving the manufacturer's control (FSANZ regulatory code; www.foodstandards.gov.au/thecode). Since there is no heating involved, manufacturers must assure that the combined hurdles applied in their manufacturing process are effective in the inactivation of pathogens. The presence of pathogens in the end product would result in an increase likelihood of a food borne associated outbreak.

Other nations have different standards for the validating the safe manufacture of fermented meats, including salami. For example in the United States, salami manufacturers must assure that their process is capable of achieving a 5-log reduction of *E. coli O157:H7*. In 2001, the United States department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) mandated the lethal standards for ready to eat meats to ensure the reduction of 6.5 log CFU/g of viable *Salmonella* and a 5.0 log CFU/g for *E. coli* O157:H7 together with a zero-tolerance policy for the detection of L. monocytogenes (USDA-FSIS, 2001).

As a consequence certain types of salami, which do not undergo long periods of maturation and for which fermentation conditions do not reach low pH, the 5-log reduction is very difficult to achieve. In this country the manufacturers must add an additional heat treatment to the salami manufacturing practice. A 3 log reduction, is therefore more achievable and thus it is easier for salami manufacturers in Australia to comply with the manufacturing practices than it is for the US manufacturers. This is an important factor as it is this variations in the regulations for food manufacture products that may assist in contributing to the emergence of pathogens world-wide. For

example, in certain countries in Europe, the sale and distribution of home made RTE cured meats is still a common practice and thus reduction standards might not be adequately monitored, leading to a potential exposure of pathogens to sub-lethal conditions, which may contribute to microbial adaptation strategies (Samelis and Sofos, 2002).

1.6. Salami validation strains

Complementary to the FSANZ regulations, in a venture project between CSIRO Food Science Australia (FSA) and Livestock Industries, a panel of five non-pathogenic *E. coli* strains (known as the salami challenge/validation strains) was developed in order to validate the salami manufacturing process. The research project, led by Paul Vanderlinde at FSA, involved the investigation of the survival of a panel of 65 *E. coli* strains (pathogenic and non-pathogenic) within an *in vitro* salami model. The five most robust non-pathogenic strains were selected as the salami validation strains, also named salami challenge strains (EC1604, EC1605, EC 1606, EC 1607, EC 1608). These salami challenge strains had the surprising ability to survive in the *in vitro* model for up to 28 days while other strains (including some outbreak strains like O157:H7 Sakai) managed to survive the challenge for 15 days or less (Duffy *et al.*, 2000). The challenge *in vitro* model consisted in HCl-acidified broth (pH 4.5) in water bath challenge The conclusions of this project suggested the potential use for these strains for validating salami manufacturing in plants without posing a risk of cross-contamination. During this thesis, these five salami challenge strains are used in order

to gain a better understanding of the overall *E. coli* stress response for both pathogenic and non-pathogenic strains.

In general, there is a tendency within the scientific community to believe that pathogenic *E. coli* are better equipped with mechanisms to cope with environmental stresses such as acid stress when compared to their non-pathogenic counterparts (Arnold & Kaspar, 1995; Benjamin & Datta, 1995; Doyle, 1991). Hence most current research regarding the acid stress response involves in one way or another pathogenic strains, particularly the *E. coli* O157:H7 serotypes.

1.7.*E. coli* O157: H7, and *E. coli* K-12 strains

The pathogenic strains of the *E. coli* subgroup known as EHEC (enterohemorrhagic *Escherichia coli*) particularly the O157:H7 serotypes are a worldwide threat to public health. These strains have been implicated in many outbreaks causing a range of food borne associated diseases. In severe cases their ingestion may cause hemorrhagic colitis and might cause death if haemolytic uraemic syndrome is developed (Kaper *et al.*, 2004 Doyle et al, 1997). In the United States alone, an estimated 75,000 cases of O157:H7 infection have been calculated to occur each year (CDC, 2005). Amongst the most severe outbreaks worldwide have been the 1982 hamburger outbreak in the United States and the Sakai outbreak in Japan. The latter involved close to 10,000 cases and resulted in 12 deaths due to lunches distributed to several facilities, previously prepared at one location (Michino *et al.*, 1999). Strains isolated from these two outbreaks were isolated and are now used as reference strains in laboratories across the world. These two strains are known as EDL933 (Bell *et al.*, 1994) and "the

Sakai" strain (Michino *et al.*, 1999), both of which are investigated throughout this thesis as positive controls for biological comparisons against other pathogenic and non-pathogenic *E. coli* strains. The genome sequences for both of these strains are now publically available (Hayashi *et al.*, 2001; Perna *et al.*, 2001) and gene annotation has been refined over the last several years (Daubin & Ochman, 2004; Keseler *et al.*, 2005; Lerat & Ochman, 2004). The laboratory strain K-12 is perhaps the most commonly used *E. coli* strain for research world wide. Since first isolated decades ago, *E. coli* K-12 has now become one of the most used tools in studying the basic principles of biology. The entire genome of K-12 was first sequenced in 1997 by Blattner and his colleagues (Blattner *et al.*, 1997).

The *E. coli* genome has a single circular chromosome with large variations in genome sizes and numbers of protein coding genes occurring in different strains (Table 1.1). These differences are mainly due to the independent integration of numerous DNA segments, for example phage genomes. Some of these introduced segments encode virulence determinants (such as Shiga toxins) thus representing distinct pathogenicity islands that confer the specific virulence patterns of EPEC and uropathogenic (UPEC) strains (Chen *et al.*, 2006; Shaikh & Tarr, 2003; Zhou *et al.*, 2001).

Table 1.1. Comparison of genomes from different *E. coli* strains with different virulence attributes

Strain no.	Genome size	ORFs	Protein-encoding	RNA-encoding	Pseudo-genes*	Virulence**
K12: MG1655	4639675	4603	~4245	188	~170	None
Sakai: OH157:H7	5498450	5502	~5152	141	~209	EHEC
EDL933 OH157:H7	5528445	5476	~5140	128	~208	EHEC
CFT73	5231428	5379	~5113	116	~150	UPEC

*Based on data from (Daubin & Ochman, 2004; Lerat & Ochman, 2004).

**EHEC – enterohaemorrhagic *E. coli* ; UPEC – uropathogenic *E. coli* .

1.8. Acid resistance of *E. coli*

Most enteric bacteria including non-pathogenic and pathogenic *E. coli* are neutrophiles, which grow best at close to neutral pH. However, these organisms are also capable of growing in media with an acidic pH. In the case of *E. coli*, it is capable

of surviving in extreme acidic environments, nearly as well as *Helicobacter pylori*, an organism known for its ability to resist acid stress in the human gastric environment (Richard & Foster, 2004). It is this ability to adapt to extreme environments that gives *E. coli* a particular importance in food safety research together with its previous association with food-borne outbreaks.

1.8.1. pH homeostasis

E. coli regulates its internal pH at 7.4 to 7.8 during growth over the external pH range of 4.7 to 9.0; the general permissive growth range for *E. coli* species (Hickey & Hirshfield, 1990; Slonczewski *et al.*, 1981). As for all cells, *E. coli* cell survival depends on the ability to maintain ions at different concentrations across the cell membrane. Differences in proton (H^+) concentrations assist many metabolic functions including ATP generation. The difference between the concentrations of ions across the membrane produces an protonmotive force. The protonmotive force across the membrane is generated by the chemical difference in proton concentration ΔpH, and the differences in electrical charge $\Delta\varphi$. It is this force that dictates if (H^+) get drawn or repelled into or out of the cell. The ability of the *E. coli* cell to survive alkaline or acidic environments is therefore dependant on the cells internal ability to regulate this protonmotive force (Foster 1994, Goodwin et al, 1987)

The mechanisms of pH homeostasis in *E. coli* have proven remarkably elusive.; electron transport components pump hydrogen ions and contribute to the proton gradient, but their role in regulating internal pH is partly unknown.(Foster, 2004). The principal proton pumps that form part of the electron transport systems and ATP

hydrolysis assist in maintaining a proton gradient across the cytoplasm (Zilberstein, 1984). When there is an excessive proton concentration in the cytoplasm and the cell is being subjected to an acidic stress, its survival is dependent on its ability to regulate the internal pH by utilizing the proton pumps together with other acid resistance mechanisms (discussed in detail below).

Another factor that may also assist in cell survival during acid challenge is the composition of the cell membrane itself. It has been demonstrated that survival to acid challenge can be an influenced by the presence of cyclopropane fatty acids (CFAs) in the cell membrane (Ying-Ying & Cronan, 1999. Brown et al, 1997). Cyclopropane fatty acids are a major component of bacterial species, including *E. coli* (Grogan and Cronana, 1997). These acids are formed during the stationary growth phase by the action of CFA synthase, which activity is mostly rpoS dependent. Therefore, strains with *rpoS* mutations and reduced CFA synthase activity have been demonstrated to be more acid sensitive than strains with normal *rpoS* activity (Hsin-Yi et al, 2003). It has been hypothesized that the presence of CFAs might decrease membrane proton permeability and or somewhat actively increase proton efflux activity (Ying-Ying & Cronan, 1999, Hsin-Yi, 2003).

Hydrogen ions (pH) alone however, are not solely responsible for growth inhibition or survival of *E. coli* species. It has been shown that the type of acid present in the cytoplasm variably affects survival. Studies have demonstrated that the capacity of weak acids as food preservatives is related to their ability to reduce the cytoplasmic pH and their intrinsic inhibitory effects (Salmond, 1984). Weak acids permeate freely across the *E. coli* membrane; therefore their inactivation effect is partially dependent

on their concentration. The higher the number of molecules in the cytoplasm the lower the pH reduction is (Salmond, 1984). Surival studies using various types of organic acids have revealed differences in challenge survival. In the work of Cheng *et al* (2002) acid adapted E. coli cells were demonstrated to have better tolerance to acetic acid followed by lactic acid and propionic acid. In their work propionic acid demonstrated to have the most inactivation effect. In contrast, the authors found that for non-adapted cultures lactic acid had the most lethal effect. They results suggest that intrinsic mechanisms that assist in survival to acid challenge are affected by the type of organic acid present in the cytoplasm. Various studies have demonstrated the efficiency of acids to affect survival include specific effects on cellular membranes, enzymes, the ATP proton pumping mechanisms and facilitated transport of the acid molecules (Alakomi et al, 2000; Diez-Gonzales et al, 1997; Stratford, 1998). For example, it has been cited that lactic acid affects bacterial survival due to iron chelation and the inhibition of lactate dehydrogenase (Shelef, 1994).

The work of Buchanan and Edelson (1996) demonstrated that in general lactic acid had the most lethal effect on nine *E. coli* strains when compared to HCL, acetic, citric and malic acids. Although results varied amongst the strains, a combined trial revealed that the inactivation effect was as follows: lactic > acetic > citric > malic > HCL. With HCL having a considerably less inactivation effect than all other organic acids (Buchanan and Edelson, 1996). The reason why organic acids have a greater effect than inorganic acid is because weak acids are able to accumulate as intracellular anions (Alkomi et al, 2000; Foster, 1999). Organic acids are able to permeate the cytoplasmic membrane in a protonated form and deprotonate based on the pH of the cytoplasm and the pK_a (Alakomi et al, 2000; Foster, 1999).

1.8.2. Acid resistance systems

With technology development scientist have been able to study the response of *E. coli* cells to environmental stresses at a molecular and genetic level. In the past fifty years novel molecular techniques have made it possible to study the expression of mechanisms that play distinctive roles in *E. coli* metabolism and survival as part of responses generated by external stressors such as temperature, osmolarity, acid, amongst others. It is believed that molecular techniques evolved shortly after World War II by a small group of scientist that experimented using radioisotopes to elucidate metabolic pathways in *E. coli* cultures (Roberts et al, 1955). It was radioisotopic studies that elucidated the synthesis of the major building blocks of protein and nucleic acids. These discoveries were followed by the identification of the DNA helix which then lead to the understanding of DNA replication, protein synthesis and gene expression (Watson and Crick, 1953). To date several molecular mechanisms and genes involved in the *E. coli* stress mechanism have been identified. Several studies have focused on studying the molecular mechanisms of the *E. coli* acid stress response the majority have mainly used microarray techniques and mutant studies (Cheville et al, 1996, Weber et al, 2005, Benjamin and Datta, 1995, Masuda and Church 2002).

A major regulator, *rpoS,* which has shown to cause a 2-fold expression (down- or up-shift) of at least 576 genes upon entry into stationary phase (Weber *et al*, 2005). *E. coli* r*poS* mutant strains upon entry to stationary phase shown decrease tolerance to acid, heat and NaCl challenge when compared with non-*rpoS* mutants (Weber *et al*, 2005).

Molecular analysis studies have revealed that over 30 *E. coli* proteins are acid inducible (Cheville et al, 1995). However, their presence and quantity is dependent on their growth and challenge conditions (Audia *et al.*, 2001; Stancik *et al.*, 2002). Their functions are hypothesized to belong to the acid resistance systems due to their

involvement in metabolic functions, for example involvement of general stress mechanisms including DNA and membrane repair, and biochemical reactions that neutralize pH through proton utilization (Audia *et al.*, 2001; Gajiwala & Burley, 2000; Stim & Bennett, 1993) Of the most thoroughly studied acid resistance systems are the amino acid decarboxylase based systems which function by consuming intracellular protons through decarboxylation reactions. To date *E. coli* is believed to possess four main acid resistance systems (Foster, 2004; Hersh *et al.*, 1996; Iyer *et al.*, 2003). These systems have been categorized as Acid Resistance systems 1, 2, 3 & 4 (AR1, AR2, AR3 & AR4). The AR1 system is also known as the oxidative system and is induced upon entry into the stationary growth phase. This system is glucose-repressed and does not require the presence of amino acids in the immediate environment in order to function. The other three systems require the presence of exogenous amino acids. The AR2 system depends on the presence of glutamate, while the AR3 and AR4 systems depend on the presence of arginine and lysine, respectively. Out of these four systems, the AR2 system is the most studied, perhaps because it offers the most protection against acid. Below, these systems are explained further.

The AR1 system

Out of the four systems the *E. coli* AR1 system is the least understood (Foster, 2004). It is stationary phase dependent and it is best expressed when cells grow in a rich media. This system has been proven to be heavily dependent on the sigma factors, better known as RpoS and the global regulatory protein CRP or cAMP receptor protein. It must be stressed however, that both RpoS and CRP are not exclusive to the AR1 system. For example, it has also been found that the general stress sigma factor,

RpoS, is strongly induced when *E. coli* cells encounter various types of stresses such as starvation, hyperosmolarity, low pH and non-optimal temperatures (Weber *et al.*, 2005).

The expression of the AR1 system can be further induced when *E. coli* cells are grown to stationary phase in a mildly acidic environment such as pH 5.5. The later is also considered as acid pre-adaptation or in most instances as the induction of the acid tolerance response (ATR). In an extensive study by Weber *et al* (2005), it was demonstrated that a large proportion of the *E. coli* genes (10%) are under direct or indirect control of RpoS, playing a major role in the *E. coli* stress response (Weber *et al.*, 2005). Thus, RpoS is somewhat considered to be the master regulator of the *E. coli* general stress response.

The AR2 system

In contrast with the AR1 system, the AR2 system is perhaps the most understood. Accounting to this feature is the fact that it has been proven to offer the greatest protection against acidification, thus increasing its focus for research. This system is governed by glutamate decarboxylase, an enzyme of the *gadA* and the *gadB* genes together with the glutamate/γ-aminobutyric acid (GABA) antiporter, *gadC*. In simple terms, as the internal pH drops, GadA and GadB convert intracellular glutamate to GABA, which is then transported out of the cell by GadC in conjunction with glutamate uptake. However, in reality, it has been found that the expression of this system is highly complex and its regulation is dependent on other transcriptional regulators. Amongst these are: RpoS, Crp, GadE, GadX, GadW, PhoP, EvgA and

YdeO amongst others (Foster, 2004; Richard & Foster, 2007). Of these regulators perhaps the most crucial is GadE since it acts as the principal activator of *gadA*/*gadB*. In fact, most of the other regulators of transcription of *gadA*/*gadB* act through the regulation of GadE (Foster, 2004).

The AR3 and AR4 systems

The AR3 and AR4 systems operate in a very similar way to the glutamate-dependent system (AR2). However, as previously mentioned these systems are less robust in terms of providing acid resistance (Castanie-Cornet & Foster, 2001b). The arginine-dependant system (AR3), consumes excess-internal protons through the decarboxylation of arginine to agmatine. This reaction is facilitated by the enzyme arginine decarboxylase of the *adiA* gene. For the AR4 system, the facilitator enzyme CadA and its antiporter CadB are of the genes *cadAB*. This system consumes internal protons via the decarboxylation of lysine to cadaverine (Gong *et al.*, 2003).

2. Chapter 2- Proteomic profiling of a panel of *E. coli* Australian salami-validation challenge strains

"The important thing in science is not so much to obtain new facts as to discover new ways to think about them"

William Bragg (1890-1971) Physicist.

CHAPTER PREFACE

This chapter was the first attempt to characterize the acid stress response of the *E. coli* salami validation strains. Using a global proteomic gel-based approach, the main aims of the experiment were to observe protein expression profiles that were exclusive to each strain prior to and following acid challenge. It was hypothesized that the protein expression profiles of the salami validation strains would reveal a distinctively similar pattern that would differ to that of the control strains. The rationale for the experimental design was to obtain protein expression profiles for cultures challenged at pH 3.5. However, it must be noted that at this point in time the type of acid used in the challenge media was not believed to influence *E. coli* acid resistance in the extent shown in later chapters.

ABSTRACT

The safe consumption of salami and other ready-to-eat cured meats relies heavily on the adequate implementation of hurdles throughout its manufacture. Inadequate implementation of hurdles may result in the persistence of *Escherichia coli* and other food-borne pathogens throughout the salami manufacturing chain and potentially harm consumers. The molecular characterization of the mechanisms involved in the persistence of pathogenic strains under salami manufacturing conditions is of relevance. As part of complying with the FZANZ code (Standard 4.2.3 & 1.6.1/2), salami manufacturing must be validated in situ.

FZANZ standard code states that an uncooked comminuted fermented meat (UCFM) must be produced in accordance with a food safety management system which: has een verified and audited to ensure the number of *E. coli* organisms in the final UCFM comply with the microbiological limits in Standard 1.6.1 in the Code. Standard 1.6.1. states that all comminuted fermented meat which has not been cooked during the production process must have a microbiological count of Coagulase-positive staphylococci/g of $10^{3;}$, E. coli/g of 3.6 and Salmonella/25 g of 0.

To prevent possible pathogen cross-contamination between the validation step and the standard salami manufacturing process, non-pathogenic *E. coli* is used as a model organism. In the present study, the panel of non-pathogenic *E. coli* salami-validation strains was used to study the influence of salami processing on the cellular proteome to better understand strain-specific responses to acid stress. A total of five *E. coli* strains and two K-12 controls were grown to stationary phase and challenged at pH 3.5 using HCl. Proteome extractions were made prior to (T0), four (T4) and twenty-four (T24) hours following the challenge. Global proteomic expression profiles were analyzed

using 2D Gel-Electrophoresis. Significant protein expression differences between the three different time frames were observed for most strains. However, most of the identified spots were too miniscule to be accurately extracted from the 2D gels. Two of the validation strains were used as model organisms and 12 protein spots were submitted for identification to the Australian Proteome Analysis Facility. Spots were identified using Nano-Liquid Chromatography, matrix assisted laser desorption ionization (MALDI) mass spectrometry and ion nano flow electro-spray followed by MS/MS. The processed proteomic data was used to search the NCBI non-redundant protein database. High scores in the database searches indicated likely matches. Results summaries for likely matches followed with identifications given and all sequences were shown with the N-terminus of the peptide on the left. Only 5 spots resulted in positive protein identifications. Three proteins had a positive identification with the *E. coli* proteins H-NS, GpmA and GrcA. Two proteins, a putative heat shock protein and a ribosome recycling factor protein with positive hits to *Salmonella* and *Shigella* species respectively, were also identified. Unique protein expression profiles were observed for all strains. No commonly expressed proteins that differentiated the acid resistant strains from the acid sensitive controls were not found.

2.1. Introduction

A number of food-borne outbreaks related to salami and similar cured meat products have been reported to date (CDC, 1995; Messier *et al.*, 1989; Williams *et al.*, 2000). Such prevalence might be associated with the way in which salami is prepared, i.e. inadequate fermentation time and drying temperature coupled with the presence or introduction of pathogens to the raw product (Tilden *et al.*, 1996). When correctly implemented the salami manufacturing processes are able to inactivate most pathogens including pathogenic *E. coli* which may be present or introduced in the raw product. In terms of food safety, amongst the most crucial steps of salami manufacturing is the fermentation process. During fermentation the salami batter pH is acidified by the starter culture (i.e. *Lactobacillus species*). In general the pH reduction after fermentation reaches pH values of 4.8 to 4.2 (Pidcock *et al.*, 2002). *Lactobacillus*-based starter cultures lower the pH through the production of lactic acid, either as D- or L- isoforms or both (Harsa, 2004). In terms of bacterial inactivation this process is important as replication and survival of *E. coli* is reduced by low pH. In conjunction to lowering the final external pH, the final concentration of lactic acid (organic acid) in the mixture also impacts on the survival of *E. coli* (Bearson *et al.*, 1997). It has been demonstrated that organic acids, when dissociated and undissociated, can reduce the internal pH of bacterial cells more efficiently than inorganic acids thus having greater antimicrobial effects. (Breidt *et al.*, 2004; Buchanan & Edelson, 1999b). In addition to acid production, the introduction and growth of the starter culture have a double bacteriocidal effect through the production of bacteriocins and through competitive inhibition (Ravyts *et al.*, 2008). The addition of salt to salami batter not only serves to

enhance taste but is also is considered as a synergistic hurdle for salami preservation (Clavero & Beuchat, 1996). However, it has also been reported that at certain concentrations salt can have a protective effect against acid stress (Chapman *et al.*, 2006; Jordan & Davies, 2001).

Salami maturation follows fermentation in this process the salami is allowed to dry at between 13 and 17 °C for an average period of twenty days (Bacus, 1997; Luke, 1985) . During the maturation process the water activity (a_w) of the salami is reduced from 0.989 to 0.916. From a microbiological point of view, the maturation process may assist in bacterial inactivation by the low temperature coupled with the low Aw (Bacus, 1997; Getty et al, 2000; Nightingale *et al.*, 2006). In addition, the reduction of Aw enhances the concentration of solutes such as NaCl, bacteriocins, organic acids and other antimicrobial agents such as nitrates that might be included in the salami mixture (Nightingale *et al.*, 2006).

Salami, according to Australian standards (FSANZ standards 4.2.3 and 1.6.1) is classified as a ready-to-eat-product thus it does not require further cooking. It is required that ready to eat products are free of pathogens as their presence in the post-manufacturing-state represents a risk of illness for consumers, in particular for infants, the elderly and the immune-compromised (FSANZ). In Australia, the biological safety of salami manufacturing is validated as specified by FSANZ, which states that salami manufacturers are required to demonstrate that their process is capable of achieving a 3-log reduction in non-pathogenic *E. coli* (www.foodstandards.gov.au/thecode). These non-pathogenic strains are generally referred as salami validation challenge strains. In Australia, certain manufacturers (names not disclosed for privacy reasons) utilize a

panel of non-pathogenic *E. coli* strains that has been selected for the validation of their salami manufacture. These strains have demonstrated to exert a higher or similar resistance to the stresses in salami than a range of pathogenic strains, including those with the serotype O157:H7 (Duffy *et al.*, 2000). The panel of strains was selected in the work of Duffy *et al* (2000), in their work a total of 65 strains (pathogenic and non-pathogenic were tested) were challenged *in vitro* using parameters that mimic salami manufacturing. Five of the most robust non-pathogenic strains were selected and assigned for salami-manufacturing validation .

In this experimental work, a panel of these five strains as well as two acid-sensitive controls, including a K-12 strain, were used to investigate the effects of the proteome at a global scale following acid challenge (pH 3.5). Proteome extractions were analyzed using 2D-Gel electrophoresis. Differentially expressed protein spots were selected following analysis with PD-Quest (Biorad®) and submitted for further protein identification analysis. A total of 5 proteins were positively identified. Perhaps the most relevant was H-NS, a histone-like protein that has a major regulatory role and which may have influence on acid resistance (Laurent-Winter *et al.*, 1997).

2.2. Methods

2.2.1. Strains

Five salami-validation strains chosen from the work of Duffy *et al* (2000) were used in this experiment and were assigned the following codes: EC1604, EC1605, EC1606, EC1607, and EC1608. A non-pathogenic *E. coli* K-12 strain and Sal4, an acid sensitive strain also obtained from Duffy *et al* (2000), were used as acid sensitive controls.

2.2.2. Culture growth and protein extractions preparation:

Cultures were grown in duplicate under moderate aeration in 10ml of 20mM Tris-HCl BHI pH 7 until their optical density at 600nm reached 1. Absorbance measurements were taken using the absorbance apparatus (Pharmacia LKB, Novaspec II). Thereafter, 600µl of those cultures were used to inoculate 600ml of pre-warmed 50mM Tris-HCl BHI pH 7. Based on preliminary growth curve data, (graphs not shown) the cultures were grown for 17h under moderate aeration to ensure that late stationary phase was reached.

After 17h the cultures were concentrated at 9000rpm in 200ml aliquots. One of the aliquots was used for preparing protein extractions (see below) whilst the remaining two were re-suspended in pre-warmed 400ml of 50mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, BHI pH 3.5 and placed under moderate aeration at 37° C. Four hours following the acidic media re-suspension a further 200ml were concentrated and used for preparing protein extractions. The remaining 200ml of each culture were used for protein extraction preparations 24hrs re-suspension in the acidic

media. The extraction time points were based on data obtained from inactivation curves using standard plate counts (results not shown) and epiflourecent experiments (shown in next chapter). Following the initial 4 hrs the inactivation rates were very miniscule (no statistical differences), in contrast the inactivation rates were very significant 24hrs following re-suspension.

2.2.3. Protein extraction

Proteome preparations were obtained by sonicating 2ml cell suspensions (Bio-Rad Lysis Buffer) with an approximate bacterial count of 8log (~10x20sec). Sonication was performed using a probe-cell sonifier (Microson XL2000), all solutions were submerged in ice-water throughout the entire sonication process. Thereafter, proteins were separated from debris through centrifugation at 30,000 rpm at 4°C (Beckman Optima LE-80K). Protein solutions underwent endonuclease treatment with a final concentration of 100µg/µl DNase and 25µg/µl RNase for 30min at 25°C. Treated samples were then solubilised using Bio-Rad's Rehydration sample Buffer (Bio-Rad. 163-2106). Samples were then centrifuged at 5,000 rpm (Beckman GS-6KR) and carefully decanted to a new Eppendorf tube, assuring the pellet was not transferred.

2.2.4. Quantitative and Qualitative protein assays

Quantitiative analysis of total protein for each duplicate sample was assessed using the Bio-Rad RC DC Protein Assay kit following the exact specifications of the experimental manual. Qualitative analysis of the extractions was also assessed by loading 3 mg/ml into an equilibrated pre-cast Criterion Tris-HCl 10% gel, with 26 × 15µl volume wells- (Bio-Rad 345-011). Once loaded, samples were separated via

vertical 1D separation using a linear current with 10 mA for 40min and 50 mA for approximately 3 h. Following separation, gels were fixed in a 7% acetic acid, 10% methanol solution for 15 min. Gels were gently rinsed with distilled water and placed in a stain solution containing: 17% ammonium sulfate, 3% phosphoric acid, 1g of Coomassie Blue G-250 (AppliChem A3480), 34% methanol and distilled water for 24 h. Gels were then rinsed with distilled water until spots were clearly visible with minimal background color. Gels were scanned with a calibrated densitometer GS-800 (Bio-Rad) and visually inspected for protein degradation (i.e. smearing present or not clearly defined lines).

2.2.5. Horizontal First Dimension (1D) separation

Aliquots of 250µg of the solubilized protein solutions were absorbed into a 4-7pI immobilized 11cm pH gradient (IPG) (Bio-Rad 163-2015). Once absorbed proteins were separated by their iso-electrical charge in the first dimension (1D), using a progressively stepped low to high current with a resistance of 50 µA (steps: 100 V, 2 h; 300 V, 3hrs; 1000V, 2 h;2000 V, 1 h; 3500 V, 12 h). IPGs were then equilibrated with a solution containing: 6M urea in 2% SDS.

2.2.6. Vertical Second Dimension (2D) Separation and identification

Following equilibration, each IPG was placed on top of equilibrated pre-cast Criterion Tris-HCl gel 8-16%, 11 cm IPG well (Bio-Rad 345-0041). Proteins were separated by their molecular weight by applying a constant current of 200 volts for 58-63 min. Once completed the pre-cast gels were removed from their casing and gently rinsed and submerged in pure distilled water for 10 min. Thereafter, each gel was fixed, stained,

and scanned as described above. Gels were stored in an air-tight refrigerated environment before further use.

2.2.7. Protein Identification Analysis

Scanned images for each duplicate gel were studied and grouped using PD-Quest (Bio-Rad). Briefly, each duplicate group was amalgamated into a replicate group and normalized accordingly. Background noise was removed using the algorithms within the software package using the Gaussian Model approach. Spots were matched and grouped using an internal 99% Student t-test ($p < 0.01$). Relevant spots were subjected to further qualitative and quantitative analysis.

2.2.8. Spot extraction

Following analysis, each of the selected spots was carefully extracted from the corresponding gels by using a sterile 20µl wide-pipette-tip. Each gel-spot was placed in a sterile Eppendorf tube and labeled accordingly. Samples were placed in dry ice and sent to the Australian Proteome Analysis Facility (APAF) for further analysis.

2.2.9. Spot identification

MALDI TOF/TOF

Samples FSA1 to FSA10 were subjected to a 16 h tryptic digest at 37°C. Thereafter a 1 µL aliquot from each sample was spotted onto a plate with 1 µL of matrix (α-cyano-4-hydroxycinnamic acid, 4 mg/mL in 70% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) and allowed to air dry. Matrix assisted laser desorption ionisation (MALDI) mass

spectrometry was performed with an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 750 to 3500 Da. The data was exported in a format suitable for submission to the database search program Mascot (Matrix Science Ltd, London, UK). High scores in the database search indicated a likely match, confirmed or qualified by operator inspection. Positive identification took into consideration the percentage sequence coverage, how well the masses matched the significant peaks in the MS spectra, the number of missed cleavages (if missed cleavages are present their location in the sequence is critical) and how well the MW and pI of the identified protein match.

LC MS/MS

Samples, FSA11 and FSA12 (which are the same as samples FSA2 and FSA4), were destained and subjected a 16 h tryptic digest at 37°C. The resulting peptides were extracted using 0.1% formic acid for 15 min. The digested peptides were separated by nano-LC using a CapLC system (Waters Corporation, Milford, MA, USA). Samples (2-5μL) were injected onto a micro C18 pre-column for pre-concentration and desalted with 0.1% formic acid at 30μL/min. After a 3 min wash the pre-column was switched into line with the analytical column containing C18 RP silica (Atlantis, 75um x 100mm, Waters Corporation). Peptides were eluted from the column using a linear solvent gradient, with steps, from H_2O: acetonitrile (95:5; + 0.1% formic acid) to H_2O:acetonitrile (20:80, + 0.1% formic acid) at 400nL/min over a 45 min period. The LC eluent was subject to positive ion nanoflow electrospray analysis on a Micromass QTOF Ultima mass spectrometer (Waters-Micromass, Manchester, UK). The QTOF was operated in a data dependant acquisition mode (DDA), with peak deisotope

selection. In DDA mode a TOFMS survey scan was acquired (m/z 400-1990, 1.0s), with the three largest multiply charged ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 4 s (m/z 50-2500). The LC/MS/MS data was processed using Protein Lynx Global Server (Waters-Micromass, Manchester, UK). The processed data was searched using Mascot (Matrix Science, London, UK). Mascot was used to search the NCBI non-redundant protein database. High scores in the database searches indicated a likely match, confirmed or qualified by operator inspection of the spectra and search results. Results summaries for likely matches follow, with identifications given and all sequences were obtained.

2.3. Results

The quality and quantity of protein was visually assessed using duplicate 26-well gels. It was observed that all samples had a similar amount of loaded protein suggesting that the calculated concentrations following sonication were accurate. No apparent smearing was observed for any sample also suggesting that the sonication step and the endonuclase treatments were successful. An example of one of the duplicate gels can be seen in figure 2.1. Successful separation of proteins by their iso-electric charge and their molecular weight (2D) occurred for all duplicate gels in all 7 strains. Proteins within each gel were successfully stained and were scanned and analyzed using PD Quest. The raw images for each gel belonging to each strain prior to (T0) and following challenge (T4 and T24) are shown in figure 2.2.

Analysis using PD Quest, revealed a very small number of significant protein differences between the 3 challenge time frames (T0, T4 and T24) for each strain. In some instances for example, for strains EC1605 and Q358, the significantly different spots found using the computational power of the software, were so physically small that following visual inspections their validity became inconclusive. Thus, there was not enough clarity to be able to discard the possibility of those spots being false positive or "junk-spots". For the remaining strains, the detected protein expression differences between the time frames was only about 1.4% of the total significantly detected spots (an average 800 spots per gel). In addition, some of the significantly detected spots were too small or too close to adjacent spots that their accurate extraction from the gels proved to be very challenging. Due to the possible cross

contamination of adjacent protein spots, it was decided that pursuing further identification analysis was not scientifically viable.

Nevertheless, two strains EC1607 and EC1608 were selected as model organisms and a total of 10 spots were sent for identification analysis to the Australian Proteomic Analysis Facility. Summary of the locations for spot extraction is exemplified in figure 2.3. Only 3 of the samples, FSA1, FSA6 and FSA10, returned with a positive protein match for the proteins H-NS, GPMA and GRCA of *E. coli*, respectively. A different identification approach was attempted for samples FSA2 and FSA4, labeled FSA11 and FSA12 respectively. This approached proved to be somewhat more accurate as 2 out of the 2 samples returned with a positive identification match. However, none of the obtained amino acid sequences had a positive match to the *E. coli* genome. A summary of the FSA samples is shown in table 2.1.

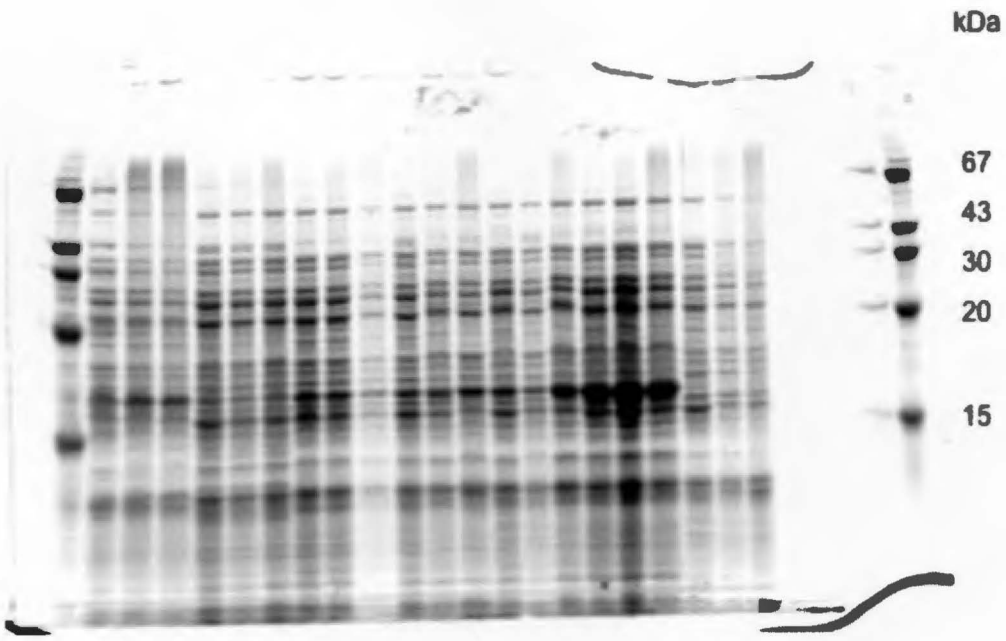


Figure 2.1. Protein Separation- 1D. Vertical 1D protein separation for sonicated samples treated with endonuclease. Protein extractions from left to right of one duplicate set for all the strains loaded and separated using the 2D protein analysis: lane 1 blank control, lanes 2 to 4, EC1604 (T0, T4, T24); lanes 5 to 7, EC1605 (T0, T4, T24); lanes 8 to 10, EC1606 (T0, T4, T24; lanes 11 to 13, EC1607 (T0, T4, T24); lanes 14 to 16, EC1608 (T0, T4, T24); lanes 17 to 19, sal4 (T0, T4, T24); lanes 20 to 22, EC1604 (T0, T4, T24), lanes 23 and 24 blank; lanes 25 to 26 marker and a positive control sample (used to assess gel quality).

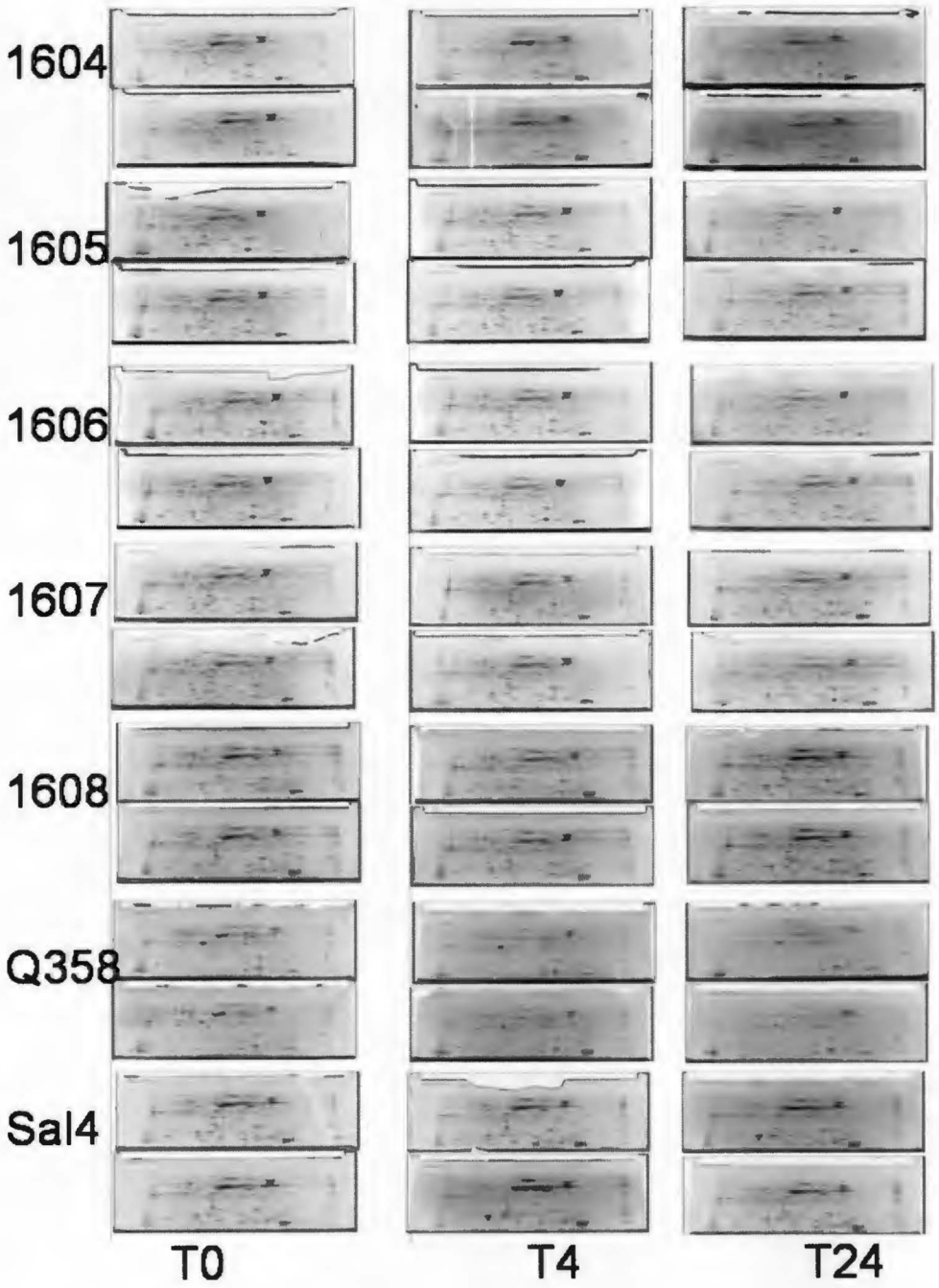


Figure 2.2. Composite image of 42 scanned gel images. Successful protein separation and staining can be observed for all duplicate gels for each strain following acid challenge for 0 h (T0), 4 h (T4) and 24 h (T24). Each image was then grouped filtered and analyzed using PD Quest (Bio-Rad).

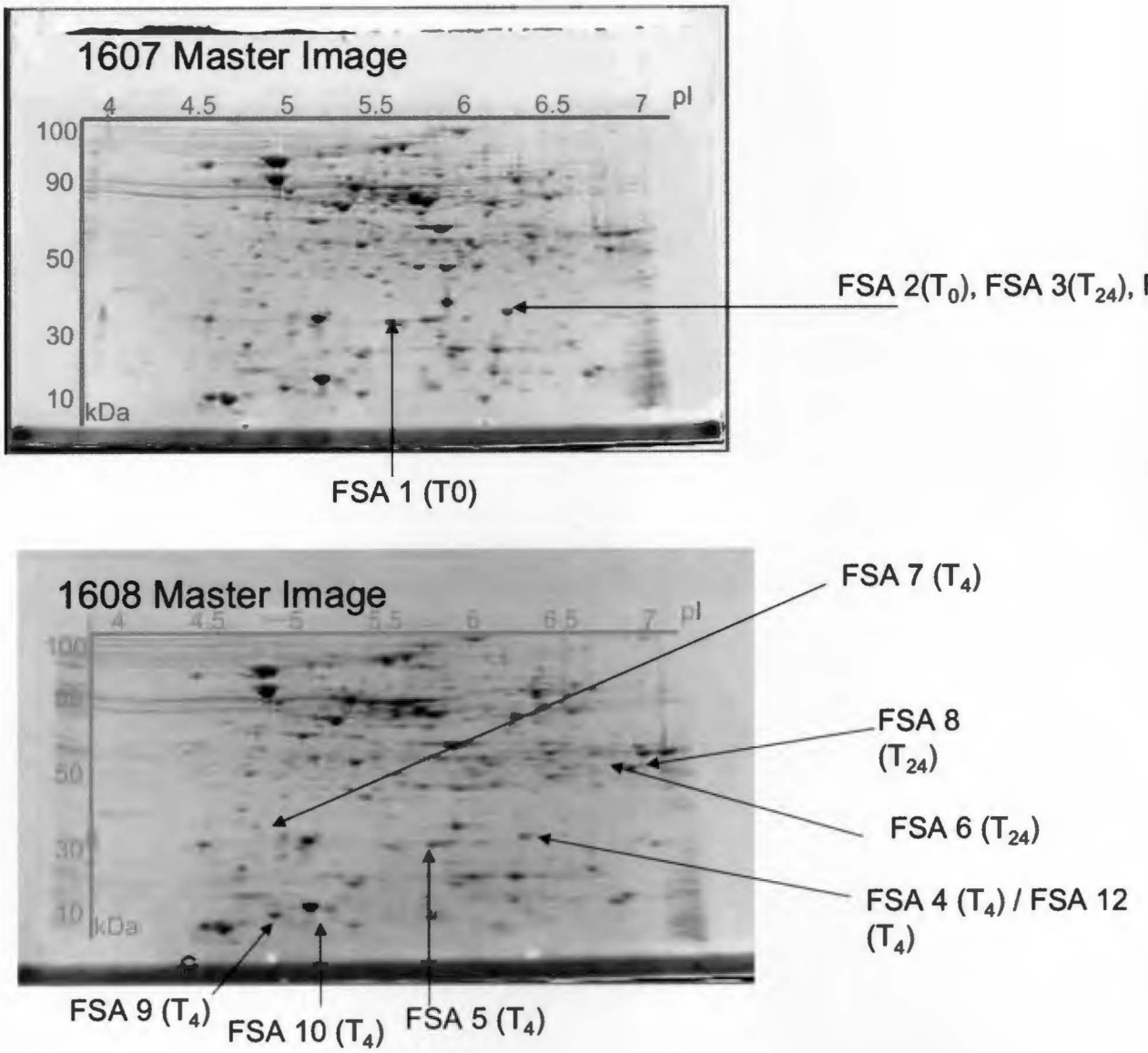


Figure 2.3. Master gel images for strains EC1607 and EC 1608. The image demonstrates the position of extracted protein spots that were subjected to further protein analysis. Each spot was given a code for analysis by the APAF as shown above.

Table 2.1. Summary of extracted spots and identification findings

Sample #	Strain	Treatment	pI*	MW (kDa)	Results
FSA1	1607	T24	5.2	12	HNS- *E. coli*
FSA2	1607	T0	6	15	No significant match
FSA3	1607	T24	6	15	No significant match
FSA4	1608	T4	6	15	No significant match
FSA5	1608	T4	5.3	12	No significant match
FSA6	1608	T24	6.6	35	GPMA_ECO57
FSA7	1608	T4	4.8	28	No significant match
FSA8	1608	T4	6.8	35	No significant match
FSA9	1608	T4	4.8	10	No significant match
FSA10	1608	T4	4.8	8	GRCA_ECO57
FSA11	1607	T0	6	15	HspC2, small heat shock protein
FSA12	1608	T4	6	15	Putative heat shock protein

2.4. Discussion

The qualitative and quantitative analysis results suggested that intracellular proteins from all samples were adequately extracted (Figure 2.1). The 2D gels for each strain in all three time frames, suggested the successful observation for global proteomic expression prior to (T0) and 4h (T4) and 24h (T24) following acid challenge at pH 3.5. PD Quest analysis of each of the protein spots for each strains, revealed acid-induced differential expression across the three challenge time frames. However, as described in the results, these differences were very miniscule (1.4%) and some demonstrating not to be completely scientifically sound. Accounting for this phenomenon could be the fact that the 1D horizontal protein separations by molecular charge was performed using IPG strips ranging from pI 4 to 7. Perhaps the observed protein differences across the challenge time frames could have been better emphasized, if the separation was performed using IPG strips with a narrower pI range. For example, the 4 to 7 pI range could have been split into 4 IPG strips; 3.9 – 5.1, 4.7 – 5.9, 5.5 – 6.7 and 6.3 – 8.3. This would have increase the focusing power of the technique, leading to a better separation of small proteins. In addition, the focusing power of the technique could have been increased by using longer 2D gels, for example from 11cm (used in this work) to 18 cm. According to the Bio-Rad manual the combination of IPGs coupled with a larger gel would have resulted in an average 13-fold focusing power. Similar suggestions can be found in a thorough review by Celis and Gromov (1999) and the work of Lilley et al (2000). . The reason why the latter was not explored further was due to the fact that we were unable to observe large protein expression differences between treatments and thus we decided not to pursue this line of work further due to time and monetary constraints.

A total of 12 protein spots were selected for protein identification as these spots were either not present or had statistically significant expression profiles from those expressed on the controls. It must be noted that, these were not the only spots that differed from the controls; other identified spots were too small to be extract individually. In general, if the smaller spots are believed to be of primary importance and extraction is required, a gel with increased focusing (as mentioned above) power would have to be utilized.

Out of the 10 protein spots subjected to MALDI – TOF/TOF (FSA1 – FSA 10) only 3 samples returned with positive identification, a 33% success rate. In contrast, out of the 2 protein spots subjected to nano-LC MS/MS (FSA11 and FSA12), 2 spots returned with a positive match. In addition samples FSA11 and FSA12 were extracted from the duplicate gel, which were exactly identical to samples FSA2 and FSA4 and which were not detected using MALDI – TOF/TOF but detected using nano-LC-MS/MS. These results suggested that the latter technique was the better approach for protein identification from extracted spots. A similar suggestion is made by Zhang and colleagues who found that LC-MS/MS to be a much more accurate method than MALDI TOF/TOF for the identification of proteins from mammalian cell lines (Zhang *et al.*, 2006). Unfortunately, the identified proteins using LC-MS/MS did not match any known *E. coli* proteins, however the detected amino acid sequences had similarities with *Shigella* and *Salmonella* species (NCBI non-redundant amino acid database) so it is possible they derive from the *E. coli* strains analysed with comparisons limited by the nature of the peptide search engine.

Of the detected proteins perhaps the one of most relevance is H-NS as it plays a major regulatory role in the *E. coli* transcriptome (Laurent-Winter *et al.*, 1997; Nishino & Yamaguchi, 2004). With regards to acid stress, H-NS mutant *E. coli* strains have been demonstrated to possess a higher resistance to acid than their wild-type counterparts. In the work of Hommais *et al* (2004), at least 7 genes previously demonstrated to be involved in *E. coli* acid resistance shown over-expression in a H-NS negative background (Hommais *et al.*, 2004). Amongst these genes were *gadA*, *gadB*, *gltD* and *hdeA*, all of which have previously been demonstrated to play crucial roles in acid resistance of *E. coli*. H-NS mutant cultures were found to survive better sudden low pH challenges than their wild-type counterparts. In this experiment, unlike what was expected, the H-NS protein extracted from the strain EC1607 was only observed 24 h following the challenge. Perhaps accounting for this discrepancy was the fact that under stress conditions H-NS is over-expressed in an attempt to regulate the expression of genes, leading to an energy conservation or survival state. One would think that if acid preservation in *E. coli* is enhanced by the absence of H-NS its over-expression could be counterproductive. However, we must take into consideration that in the work of Hommais *et al.* (2004) the cells were challenged with a pH of 2.5 for 2 hours only, perhaps the expression of H-NS in a more prolonged challenge involving a much milder acidic pH (this experiment) may manifest different physiological responses.

The two other identified proteins extracted from EC1608 were GpmA and GrcA. These proteins were only expressed 24h and 4h following acid challenge respectively. GpmA is a 2, 3-bisphosphoglycerate-dependent phosphoglycerate mutase that is involved in a latter step of glycolysis and perhaps is over expressed in an attempt for cells to generate more ATP which might assist in coping with the acid stress. ATP is needed in many of the reactions that might assist in the elimination of intracellular

protons (Foster, 2004). GrcA (or YfiD) represents the autonomous: glycyl radical cofactor that has been demonstrated to be able to replace an oxidatively damaged pyruvate formate-lyase subunit. This cofactor protein is known to be generally associated with resistance to stress responses (Wagner *et al.*, 2001).

In conclusion, it became apparent that proteomic expressions at a global level were not sufficient in the elucidation of the underlying mechanisms that assist salami challenge strains under study to cope with mineral acid stress. In addition, the challenge strains in this study demonstrated equal or higher resistance to acid when compared to other pathogenic strains. However, it must be noted that such phenotype was observed in a system where the cultures were challenged at 15°C with a high NaCl content. In this work we did not challenge them under the same conditions as we were trying to keep complexity to a minimum. It is considered necessary to better understand the effects on cell physiology (and thus on the effects on the global proteome expression) where acidic conditions comprising a combination of mineral and organic acid are coupled with NaCl .For future gel-based proteomic work however, it would be advisable to increase the focusing power of the analysis by using larger gels and IPG strips with a narrow pI range, alternatively new gel-free approaches are now available allowing for much more comprehensive analysis of the proteome (Gevaert *et al.*, 2007; Ihling & Sinz, 2005).

3. Chapter 3- *E. coli* acid resistance studies: variations in acid resistance phenotypes as a consequence of variations in experimental parameters

"Scientific knowledge is in perpetual evolution; it finds itself changed from one day to the next

Jean Piaget, 1896 – 1980, psychologist and natural scientist.

CHAPTER PREFACE

In the previous chapter very little proteomic differences were found between the salami challenge strains and the sensitive controls. This raised the question whether the *E. coli* challenge stains were truly acid resistant. During the experimental set up, the actual definition regarding *E. coli* acid resistance was also investigated. This chapter summarizes some of the current *E. coli* acid resistance research, emphasizing the variations in experimental parameters that exist in the literature. Due to these variations, a series of experiments were performed to better understand the panel of challenge strains. The knowledge from these experiments together with that of previous literature was integrated and used for further experimental work.

3.1. Introduction

The terms *E. coli* acid resistance, acid adaptation, acid tolerance response and acid shock response can be readily observed in a large array of published literature (Benjamin & Datta, 1995; Buchanan & Edelson, 1999a; Garren *et al.*, 1998; Gorden & Small, 1993; Hill *et al.*, 1995; Lin *et al.*, 1996) The terms *E. coli* acid tolerance and acid shock response have been clearly defined. The acid tolerance response (ATR) is defined as the ability of cultures to resist an acid challenge following a pre-adaptation process, either due to exposure to mild acidic conditions during growth or growth in the presence of fermentable carbohydrates that generates acidic conditions during growth (Buchanan & Edelson, 1996; Leyer *et al.*, 1995). The acid shock response (ASR) is defined as the ability of cultures to resist an acid challenge without an adaptation process; generally the cells are grown in normal conditions and subjected to an acid challenge, typically simulating exposure to stomach gastric acid or an acidic food product (Garren *et al.*, 1998). Research has demonstrated that major phenotypic, genomic and proteomic differences exist between these two responses, in general the ability to resist an acid stress is prolonged during the acid tolerance response as opposed to the acid shock response, which relies more on intrinsic acid resistance (Garren *et al.*, 1998). While the terms ATR and ASR have a clear definition, the terms 'acid resistance' and 'acid stress' are somewhat ambiguous. In many publications these two terms are interchangeably used and each is specific to the experimental parameters of various studies.

The term acid resistance (AR) however, is generally not well defined and it may need some clarification. For example, in the work of Garren *et al.* (1997) *E. coli* cultures

were subjected to acid stress by the exposure to acidic media adjusted using lactic acid. Their acid resistance was measured by the ability of the cultures to remain viable for up to 14 days. In the work of Jordan and Davies (2001) acid-adapted and non-acid adapted *E. coli* cultures cells to media adjusted to pH 3.0 at 30°C for 60 min at different lactate concentrations. In the work of Large *et al.* (2005) acid resistance was assessed by the ability of *E. coli* O157:H7 cultures to resist challenge in media supplemented with and without glutamate and arginine at pH 2.0 and 2.5 following varied growth conditions. In the abovementioned publications, the term 'acid resistance' in *E. coli* research is used to define the ability of cultures to resist a challenge involving any type of acid and any pH value on the acid scale. Small *et al.* (1994) defined *E. coli* 'acid resistance' as the ability of stationary-phase cells to survive extreme low pH. However, they did not define the exact value of the extreme low pH (magnitude of challenge) and the duration of the challenge (survival threshold). Perhaps the term *E. coli* AR can be classified as a response that is intrinsic to individual cultures that may vary under different experimental conditions. Thus, a particular *E. coli* strains cannot be described to have higher AR than other strains until the experimental conditions are clearly defined and thus it is exclusive to the experimental parameters. Having said this, *E. coli* strains that demonstrate enhanced survival to different acid challenges (with varying conditions) could be described to have a generally higher acid resistance than other strains.

Buchanana and Edelson (1996) identified issues in the terminologies, describing that acid ressistance in *E. coli* was initially reported to be limited to a pH-independent, rpoS-associated system in publications such as Foster, 1995 and Lee et al, 1995. While other studies have now elucidated acid resistance mechanisms that are pH-dependent and involve the development of an acid tolerance response (Arnold and Kaspar, 1995)

In addition, most current acid resistance research, including the three publications mentioned above, measure *E. coli* inactivation and assess viability by quantifying the amount of colony forming units (CFU) on recovery media (agar plates). However, in some instances *E. coli* cells subjected to stress could enter a state known as non culturable but viable state (Bloomfield *et al.*, 1998). In this state *E. coli* cells remain viable, with the capacity to replicate under the right conditions, but do not form CFU using standard plating techniques.It is also worth mentioning that the composition of different agars can affect the ability of injured/stressed cells to replicate. Berry and Cutter (1999) demonstrated that the number of CFU were higher when a non selective media (TSA) when compared to a selective media (SMAC). The authors suggested that the use of SMAC could assist in the identification and quantification of stressed cells within a challenge population. In the current thesis, preliminary studies demonstrated that higher number of acid stressed cells were obtained using TYSG. As we were interested in studying the proteomes and transcriptome of challenge cells, excluding injured cells was not in questions

The lack of a true definition for *E. coli* acid resistance has allowed for variations in experimental parameters across laboratories and the generation of ample knowledge regarding this topic. On the other hand however, the lack of standardized parameters for *E. coli* acid resistance research complicates the comparisons of observations made across laboratories. It also makes it difficult to accurately predict acid resistance behaviors of specific *E. coli* strains.

A close observation of published data involving *E. coli* acid resistance from different research groups has brought to our attention the fact there is a lot of variation in the way experiments have been conducted. Variations in experimental parameters across

publications range from differences in pH values, temperature, growth media, acid habituation, challenge media, acidulant type and strains used; all of which has been proven to independently affect the *E. coli* acid resistance phenotypes (see table 3.1.1). Regarding the pH values, it is well known that *E. coli* growth and inactivation can be directly influenced by the pH value of the external environment (Foster, 2004; Gorden & Small, 1993).

In the work of Garren *et al.* (1997), the inactivation of *E. coli* cultures regardless of pathogenicity was directly influenced by temperature (higher survival rates at 25°C than at 32°C), pH (higher survival rates at pH 4 than at pH 3) and acid adaptation (higher survival for acid adapted cells than for non-acid adapted (Garren *et al.*, 1997). In addition, their work also demonstrated that the ability to resist the different challenges varied amongst the different isolates. In the work of Large *et al.* (2005), variation in acid resistance amongst different pathogenic and non-pathogenic *E. coli* strains were observed in various acid challenges. Experiments concluded that strains of the O157:H7 serotype had no superior acid resistance when using three specific acid resistance experiments. However, the authors estimated that more than 60% of the variation observed in one of the treatments was attributed to strain differences. In addition, their work also demonstrated that differences in the amino acid content of the acid challenge media have a direct influence on the acid resistance phenotypes of the *E. coli* strains tested (Large *et al.*, 2005).

Various authors have explored the ability of *E. coli* to resist acid stress using various acidulants such as HCl, lactic acid, acetic acid and citric acid (Breidt *et al.*, 2004). Due to their structural differences and dissociation constants, acidulants have been found to

not only affect the pH of the media but also have an independent effect on *E. coli* inactivation. In the work of Buchanan and Edelson (1999), at pH 3 the calculated molar concentrations of fully dissociated acid for citric, malic, lactic and acetic acids were 13.6, 26.7, 48.4 and 81.8 mM respectively. Their findings suggested that the relative inactivation efficacies of the various acids were strain dependent. In general HCl had the least inactivation efficacy, while lactic acid had the greatest (Buchanan & Edelson, 1999b). In the work of Abdoul-Raouf *et al.* (1993) the order of lethal effectiveness on *E. coli* O157:H7 cultures using different acidulants was acetic acid > lactic acid >citric acid. A summary of the different parameters used in a selected number of acid resistance studies is shown in Table 3.1

The fact that the acid resistance phenotypes of *E. coli* can be directly influenced by the experimental parameters, led us to attempt to gain a better understanding of the ability of the salami validation strains to resist acid under various experimental conditions. The results from 5 independent experiments were used to compare our findings with that of existent literature and allowed for better judgment of our results together with the development of further experiments. The knowledge obtained from this chapter was then used to design, execute and analyze the experimental work shown in chapters 4, 5 and 6 of this thesis.

Table 3.1. Experimental parameter summary for *E. coli* acid resistance studies

E. coli Strain ((s)	Growth conditions (Temp, Media)	habit-uated	Acid(s) used	Media	Supplement (salt, sucrose, other)	Temperature	pH	Reference
6 x O157:H7, O157:H-	TSB+1%G & TSB-G stationary phase. 37°C	Y/N	HCl	BHI		37°C	2.5, 3.0	(Buchanan & Edelson, 1999a)
12, EIEC flora isolates, 1xK12	NA	N	HCl	LB		37°C	2.5	(Gorden & Small, 1993)
5x O157:H7	TSB, 37°C, stationary phase	N	Lactic & HCl	TSB	NaCl (0.5, 1.5, 2.5, 4.5, 6.5, 8.5 and 10.5%),	37°C	3.5, 4.0, 4.5, 5.0, 5.5, 6.0,	(Glass *et al.*, 1992b)
1x STEC O157	TSB+1%G, stationary phase, 37°C	Y	Acetic	NB	NaCl (1, 3 and 8%) and Sucrose (10,20 and 30%)	37°C	3.2, 3.5, 4.0	(Chapman *et al.*, 2006)
2x O157:H7, O111:H11 & O26:H11 +rpos	EG LB. 37°C. stationary phase	N	HCl, MES	LB	Glutamate	37°C	2	(Bhagwat *et al.*, 2005)
1x O157:H7	TSB0.6%YE. 37°C. stationary phase	N	Citric, malic, lactic, mandelic,	TSB0.6%YE		37°C, 25°C, 10°C, 4°C		(Conner & Kotrola, 1995)
1x O157:H7	EG medium. mid-exponential phase, HCl-acid adapted cells	Y/N	Lactate	TPB	NaCl (0, 3,4,5,6,7,8,9%)	30°C	3 , 5,6	(Jordan & Davies, 2001)
11x O157:H7 & 4commensals	LB-MES, mid exponential phase	Y	HCl, MES	EG	Glutamate or arginine	37°C	2,3,4	(Lin *et al.*, 1996)

5x O157:H7 & 2xOutbreak- O157	TSB, 37°C, stationary phase	N	Lactic	TSB	NaCl (0,8.6,16.8%)	37°C, 30°C, 20°C, 5°C	6, 5.4, 4.8	(Clavero & Beuchat, 1996)
13x O157:H7	TSB, 37°C, stationary phase	N	HCl, MES	LB & SMGA	NaCl (1%)	37°C, 25°C, 15°C	5, 1.5	(Cheng & Kaspar, 1998)
1x O157:H7 & 2xcommensals	TSB, 37°C, late log phase	N	Acetic, & formic actic	TSB	NaCl (0, 2% & 4%)	37°C	4.2	(Casey & Condon, 2002)
30x O157:H7, 18xO26:H11, 4x O111:H8, 14x	LBG, EG, LB,MES, BHI+G	Y	HCl	EG	Glutamate or arginine	37°C	2 & 2.5	(Large *et al.*, 2005)
5x O157:H7	TSB, 30°C stationary phase	N	Acetic, citric, lactic	Beef Slurry		30°C, 21°C, 5°C	4.7, 5.0, 5.4	(Abdulraouf *et al.*, 1993)
Various MG1655 mutants	LB, 37°C stationary phase	N	HCl	LB		37°C	2.5	(Masuda & Church, 2003)
2x O157:H7	LB 37°C adapted with: acetic, citric, lactic (pH 6.4 & 5.4)	Y	HCl	SMGA		37°C	1.5	(Yuk & Marshall, 2005)
O157:H7 mutant	TSB, 37°C, stationary phase	N	HCl	SMGA & TSB		37°C, 25°C, 4°C	1.5, 2.5	(Arnold *et al.*, 2001; Arnold & Kaspar,
7x O157:H7	TSB, 35°C, stationary phase	?	HCl	TSB		25°C, 4°C	2, 1.5	(Arnold & Kaspar, 1995)
5x O157:H7	TSB	Y	HCl, Lactic acid	Salami Batter, TSB	Salami Batter	25°C	3.85	(Leyer *et al.*, 1995)

*SMGA = simulated gastric acid; LB = Luria Broth; TSB = tryptic soy broth; MES = morpholinoethane sulfonate (buffer); NA, nutrient agar; EG = *E. coli* minimal media G = glucose

3.2. Experimental Plan

In this chapter, the five experiments were performed independently since each was specifically designed to eliminate doubts that arose from the extensive variations that exist in the experimental parameters of the current literature.

The hypothesis for the first experiment was: The intrinsic acid resistance strength of *E. coli* cultures of the same parent strain is affected by the variance of the parameters of storage and growing conditions across laboratories. The aim for this experiment was to investigate the survival rates of a panel of 5 *E. coli* strains from 4 different stocks following growth in TSB supplemented with glucose (acid adapted) when challenged in BHI-broths at pH 3.5 at 37°C.

The reason behind this experiment in relation to the thesis was to investigate whether the acid resistance phenotypes of the salami challenge strains had being affected by storage and they were not the same as when first identified in the work of Duffy *et al* (2000).

The hypothesis for the second experiment was: The inactivation rate of *E. coli* in an acid challenge is reduced by the presence of glucose during growth, as acid end products of metabolism induce an acid tolerance response. The aim of this experiment was to investigate the survival rates of a panel of 7 *E. coli* strains grown in TSB alone (non-acid adapted) and TSB supplemented with 1% glucose (acid adapted) when challenged in BHI-broths at pH 3.5 at 37°C. The reason behind this experiment was to

confirm whether under these experimental conditions acid resistance was enhanced as this method was used in the work of Duffy *et al* (2000). Most importantly, since these *E. coli* strains had not been well characterized we wanted to confirm if their phenotypic behavior was in unison with previously described literature.

The hypothesis for the third experiment was: The survival of *Escherichia coli* cultures can be affected under experimental conditions by the type of lactic acid used under different NaCl concentrations. The aim of this experiment was to investigate if the use of lactic acid with different isomer conformations in acid challenge studies evoked significant survival differences on 3 *E. coli* strains challenged in five different NaCl concentrations.

The reason behind this experiment was to select the most adequate lactic acid type for use in further experiments. It also allowed us to clarify some doubts that arose when analysing the current literature, as some papers do not identify the type of lactic acid used for *E. coli* acid challenge studies (Abdulraouf *et al.*, 1993; Leyer *et al.*, 1995).

The hypothesis for the fourth experiment was: The inactivation rate of *E. coli* in an acid-salt challenge is influenced by temperature. The aim for this experiment was to investigate the survival rates of one *E. coli* strain (EC1608) at four different salt concentrations when challenged in BHI-broths at pH 4.5 supplemented with lactic acid at 25°C and 37°C.

The reason behind this experiment was to be able to critically evaluate the behavior of the challenge strains compared to the controls at temperatures higher than when first

identified in the work of Duffy *et al.* (2000). In addition we wanted to explore whether the magnitude of inactivation of acid-salt challenge was reduced at lower temperatures.

The hypothesis for the final experiment was: Viable-non-culturable *Escherichia coli* cells can be observed up to 24h following acid challenge with lactic acid (pH 3.5). The aim of this experiment was to investigate whether viable-non-culturable *E. coli* cells are present 4 h and 24 h following acid challenge by using the LIVE/DEAD BacLight (Invitrogen) approach, utilizing the DNA-binding viability stains SYTO-9 and propidium iodide. Normally propidium iodide is excluded from intact cells while SYTO-9 can penetrate intact cells. *E. coli* cells with damaged cell membranes usually become permeabilized (thus under acidic conditions the cells are also likely permanently inactivated) thus they become dual stained with both propidium iodide and SYTO-9. However, the cells fluoresce red due to propidium iodide binding more efficiently to DNA than SYTO-9. *E. coli* cells with undamaged cell membranes (therefore viable/active) will stain green due to SYTO-9 accumulation and propidium iodide exclusion. In some instances following severe stress *E. coli* cells do not replicate on agar plates but still appear to be viable as indicated by the presence of SYTO-9 staining cells. Such cells may have the capacity to recommence divide after cessation of stress conditions i.e. cell resuscitation.

The reason behind this experiment was to corroborate the validity of protein extractions and further RNA extractions from cells that have been subjected to acid stress. For example, in chapter 2, following a 24h acid challenge (pH 3.5) no viable *E. coli* cells were found, however protein extractions from these cultures appeared to be

of excellent quality, showing no significant differences to the extractions from samples showing high numbers of CFU on agar plates.

3.3.Methods

3.3.1. Experiment 1

Prior to commencing the experiments we investigated the storage procedure of the salami challenge strains described in Duffy *et al.* (2000). The strains were grown in standard tryptone soy broth (TSB) (Oxoid CM0876) at 37°C for 24h. Following growth the cultures were stored in Protect® beads (Technical Service Consultants Ltd) following the manufacture's specifications and stored at -80°C or lyophilized using Telsar Cryodos-80 apparatus and following a standard scientific protocol. Prior to lyophilisation 1ml of the grown cultures was mixed with dextran (0.5%), sucrose (7.5%) and sodium glutamate (1%).

With exception for the lyophilized cultures, *E. coli* strains were recovered from -80°C by placing one bead into 10ml of tryptone soy broth (TSB) followed by a 24h incubation period at 37°C. The lyophilized cultures were recovered from a dried state by re-suspending the dried pellet in TSB prior to 48h incubation at 37°C. Following growth, the purity of cultures was checked by plate enumeration and observations of the CFU. Bacterial enumerations were performed by plating 100µl of serially diluted cell solutions onto tryptone yeast soy glucose (TYSG) agar plates followed by 24 h incubation at 37°C. Single CFU from these agar plates were used for the inoculation of each triplicate culture for further growth and challenge experiments. Prior to acid challenge a single CFU of each culture was aseptically inoculated into 10ml of TSB+1% D-glucose and incubated for 17h (to late stationary phase) at 37°C in triplicate. Following growth, cultures were centrifuged at 5000 RPM at room

temperature and re-suspended in 10ml of the challenge media. Brain Heart Infusion (BHI) challenge media was adjusted to pH 3.5 with L-lactic acid. Cultures were challenged in a 37°C water bath and samples were taken for bacterial enumerations (as described above) every 90min for a period of 8h. Cell enumerations were performed as described above.

Statistical analysis

Data was analyzed using Minitab® 14 statistical software (Minitab Inc. Mineapolis, MN, USA). Significant differences of the means of average survivors were determined using one-way analysis of variance. A p value of < 0.05 was regarded as statistically significant. All further references to significant difference indicate $p < 0.05$. (All statistics in this chapter were performed in a similar manner)

Table 3.2. List of strains used for Experiment 1

Strain no.	Assigned Nomenclature	Patho-genic	Detail	Food Science Laboratory
1	EC1604	No	Salami validation strain	Cannon Hill working cultures (CH-1)
2	EC1604	No	Salami validation strain	Cannon Hill lyophilized cultures (CH-2)
3	EC1604	No	Salami validation strain	Werribee (W)
4	EC1604	No	Salami validation strain	North Ryde
5	EC 1605	No	Salami validation strain	Cannon Hill working cultures (CH-1)
6	EC 1605	No	Salami validation strain	Cannon Hill lyophilized cultures (CH-2)
7	EC 1605	No	Salami validation strain	Werribee (W)

8	EC 1605	No	Salami validation strain	North Ryde
9	EC 1606	No	Salami validation strain	Cannon Hill working cultures (CH-1)
10	EC 1606	No	Salami validation strain	Cannon Hill lyophilized cultures (CH-2)
11	EC 1606	No	Salami validation strain	Werribee (W)
12	EC 1606	No	Salami validation strain	North Ryde
13	EC 1607	No	Salami validation strain	Cannon Hill working cultures (CH-1)
14	EC 1607	No	Salami validation strain	Cannon Hill lyophilized cultures (CH-2)
15	EC 1607	No	Salami validation strain	Werribee (W)
16	EC 1607	No	Salami validation strain	North Ryde
17	EC 1608	No	Salami validation strain	Cannon Hill working cultures (CH-1)
18	EC 1608	No	Salami validation strain	Cannon Hill lyophilized cultures (CH-2)
19	EC 1608	No	Salami validation strain	Werribee (W)
20	EC 1608	No	Salami validation strain	North Ryde
21	Sal 4	No	Salami industry isolate	Cannon Hill working cultures (CH-1)
22	MG 1655	No	K-12 laboratory strain	Cannon Hill working cultures (CH-1)
23	Q358	No	K-12 laboratory strain	Cannon Hill working cultures (CH-1)
24	EC2271	Yes	Pathogenic O157:H7	Cannon Hill working cultures (CH-1)

3.3.2. Experiment 2

Cultures were recovered from storage at -80°C as described in experiment 1.

A loop-full of the single CFU was used to inoculate each culture in TSB or TSB+1% glucose (TSB+1%G) in triplicate. Cultures were incubated for 17 h at 37°C. Following growth, cultures were centrifuged at 5000 RPM at room temperature and re-suspended in the challenge media. BHI challenge media was adjusted to pH 3.5 with L-lactic acid. Cultures were challenged in a 37°C water bath and samples were taken every 1 h for a period of 15 h. Cell enumerations were performed as described above

Table 3.3. List of strains used for Experiment 2

Strain no.	Assigned nomenclature	Patho-genic	Detail	Food Science Laboratory
1	EC1604	No	Salami validation strain	Cannon Hill working cultures (CH-1)
2	EC1605	No	Salami validation strain	Cannon Hill working cultures (CH-1)
3	EC1606	No	Salami validation strain	Cannon Hill working cultures (CH-1)
4	EC1607	No	Salami validation strain	Cannon Hill working cultures (CH-1)
5	EC1608	No	Salami validation strain	Cannon Hill working cultures (CH-1)
6	Sal4	No	Salami industry isolate	Cannon Hill working cultures (CH-1)
7	Q358	No	K-12 laboratory strain	Cannon Hill working cultures (CH-1)

3.3.3. Experiment 3

Strains EC1608, Sal4 and Q358 (Table 3.3) were recovered from storage at -80°C as described in experiment 1. A loop-full of the single CFU was used to inoculate each culture in TSB+1% glucose (TSB+1%G) in triplicate. Cultures were incubated for 17 h at 37°C.Following growth; cultures were centrifuged at 5000 RPM at room temperature and resuspended in each challenge media. Each BHI challenge media was adjusted to pH 4.5 with either L-lactic acid or DL-lactic acid and supplemented with 0.5, 3.5, 8.5, 12.5 and 15.5% NaCl at 15°C. A BHI broth culture with 0.5%NaCl with no lactic acid added was used as control. Cultures were challenged in a 15°C water bath and samples were taken every 24h for a period of 15 days. Cell enumerations were performed as described above.

3.3.4. Experiment 4

Strain EC1608 (Table 3.3) was recovered from storage at -80°C as described in experiment 1. A loop-full of the single CFU was used to inoculate each culture in TSB+1% glucose. Cultures were incubated for 17 h at 37°C. Following growth, cultures were centrifuged at 5000 RPM at room temperature and re-suspended in each challenge media. Each BHI challenge media was adjusted to pH 4.5 with L-lactic acid and supplemented with 0.5, 3.5, 8.5, 12.5 and 15.5% NaCl at 25 and 37°C. A BHI broth culture with 0.5%NaCl with no lactic acid added was used as control. Cultures were challenged on a 25°C and a 37°C water bath and samples were taken every 24hrs for a period of 4 Days. Cell enumerations were performed as described above.

3.3.5. Experiment 5

K12-like strain Q358 (Table 3.3) was recovered from storage at -80°C as described in experiment 1. A loop-full of the single CFU was used to inoculate each culture in TSB+1% glucose. Cultures were incubated for 17 h at 37°C. Following growth, cultures were centrifuged at 5000 RPM at room temperature and re-suspended in the challenge media. BHI challenge media was adjusted to pH 3.5 with L-lactic acid. Cultures were challenged at 37°C waterbath. Samples for viability studies were taken prior to and 4h and 24h following the challenge. Plate counts were performed every hour on TYSG plates, using standard dilution and enumeration methods, until no colonies were observed. Viability assays were performed using the LIVE/DEAD Baclight viability assay. For this experiment, each of the selected bacterial samples was stained using the abovementioned stains. For each stain assay, 8ml of each sample was centrifuged at 5000 RPM, the supernatant was discarded and the pellet re-suspended in a 0.85% NaCl solution. The re-suspended cultures were centrifuged at 5000 RPM and the pellet was re-suspended in a 1ml solution containing 0.85% NaCl and 1.5µl of each of SYTO®9 stain solution and 1.5µl propidium iodide stain solution. Samples were left to incubate in the dark for 20min prior to observation with a fluorescent microscope. The epifluorescent microscope used for this study was a Leica™ SP microscope with an attachment for an Olympus off the shelf digital camera. The optical zoom for the photographs was made at 100 × magnification. A long-passed dual emission epifluorescent filter ranging from 495 to 550 (nm) was used this experiment. Photographs were taken within a minute of ignition of the epifluorescent (UV) lamp.

3.4. Results

3.4.1. Experiment 1

Survival variance across cultures of the same strain was observed (Fig. 3.1). In general the Cannon Hill working strains (CH-1, non-lyophilized) demonstrated a higher acid resistance than their respective counterparts. Strain EC1605 from the lyophilized cultures from Cannon Hill laboratory could not be recovered from its dried state. The acid sensitive controls, Sal4 and the 2 K12 strains appeared to be more sensitive than most of the salami validation strains. It is concluded from these results that the working strains from the FSA Cannon Hill laboratory showed the least log reduction counts when compared to most strains and thus could be considered at least in a preliminary sense comparatively acid resistant.

3.4.2. Experiment 2

A direct relationship between survival and the presence of 1% glucose was observed. Inactivation for most strains occurred at a higher rate for cultures grown with TSB when compared to those grown in TSB supplemented with 1% glucose (Fig. 3.2). Interestingly the robustness of some strains varied depending on the growth parameter. For example, Sal4 showed a higher percentage of survivors (when compared to the other strains) following challenge when grown in the presence of glucose, but showed the most sensitivity when grown in TSB alone.

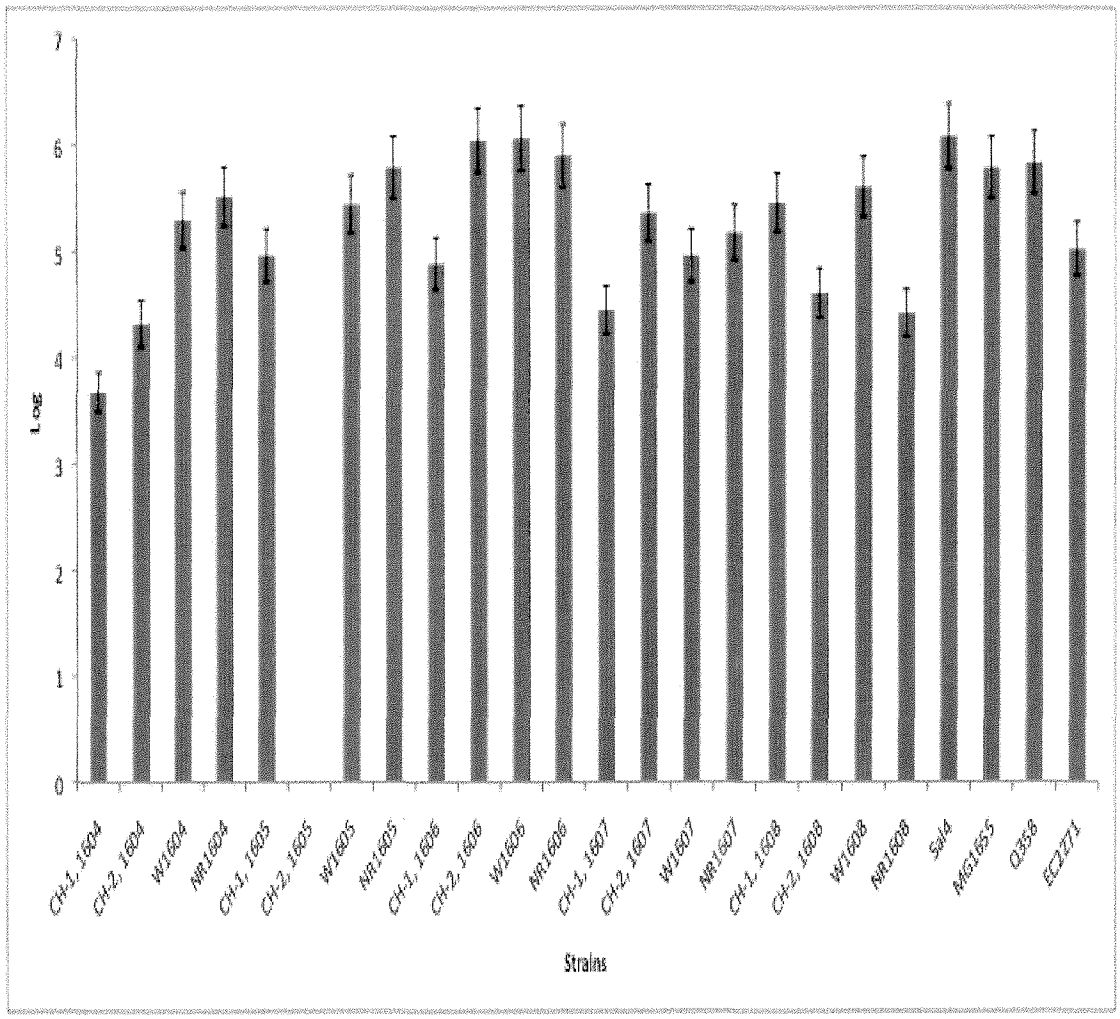


Figure 3.1. Comparison of acid resistance phenotypes amongst stocks from different laboratories that included working (actively growing) strains and those freshly retrieved from storage (see Table 3.1 for explanation of strain designations).

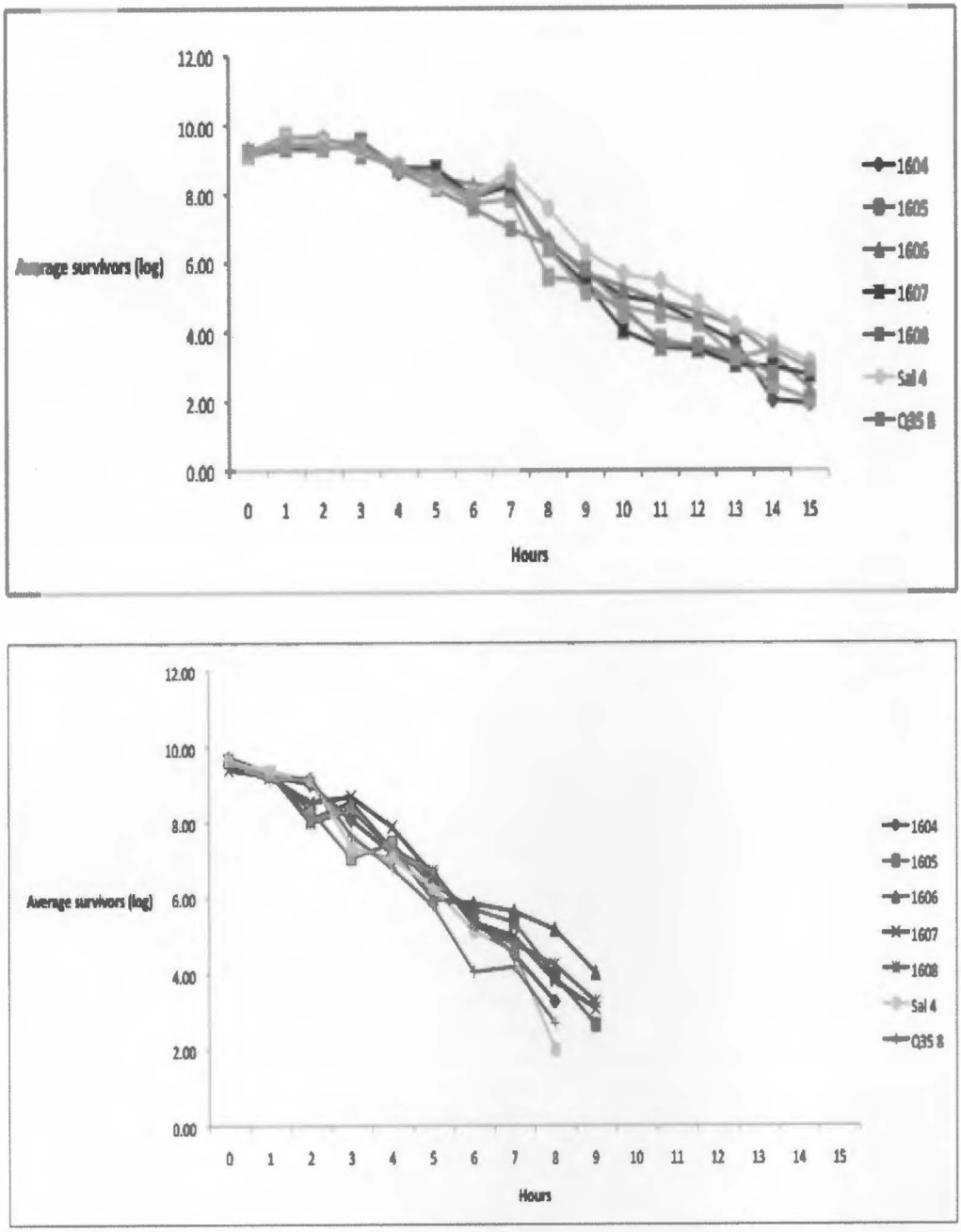


Figure 3.2. Survival curves of salami challenge and other *E. coli* strains exposed to acid stress (pH 3.5 adjusted using L-lactic acid) that had been prior acid adapted in a glucose amended medium (upper pannel) and non-adapted (lower panel).

Comparison of the responses of different salami challenge strains and other K12 variant *E. coli* strains to acid challenge involving BHI broth adjusted to pH 3.5 with

L-lactic acid. The inactivation responses are shown for cultures grown in (A) TSB+1% glucose and in (B) TSB alone, demonstrating the greater degree of ATR in strains exposed to acid end products deriving from glucose.

3.4.3. Experiment 3

A negative relationship between survival and salt concentration was observed (Fig. 3.3). For all 3 strains, challenge with L-lactic acid, regardless of the salt concentration, resulted in lesser inactivation when compared to the DL-lactic acid counterparts. Under experimental conditions the type of lactic acid used, demonstrated a clear effect on *E. coli* inactivation in a strain dependent manner. DL-lactic acid overall had a more deleterious effect on survival.

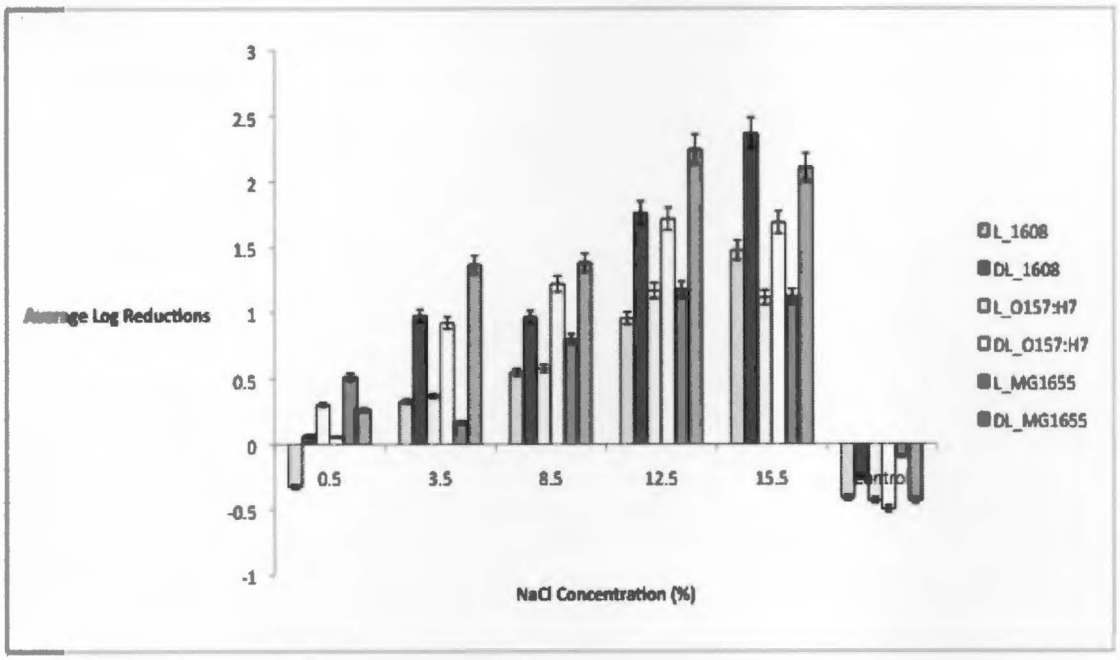


Figure 3.3. Inactivation effects of L- and DL- lactic acid (each added to reach pH 4.5, ~35 mM) on different *E. coli* strains in the presence of different NaCl levels.

3.4.4. Experiment 4

An inverse relationship between survival and salt concentration was observed. Significant survival differences were found when the NaCl levels were above 3.5%. No significant differences in survival were found for cultures challenged with 0.5% and 3.5% at their respective temperatures. However, the magnitude of this pattern was exacerbated by the increasing temperature. When comparing the survival rates at 25˚C with 37˚C, there was greater than three-fold increase in inactivation rates across all salt concentrations. Under experimental conditions increasing temperature demonstrated an amplifying effect on *E. coli* inactivation under combined acid-salt stress.

3.4.5. Experiment 5

The number of colonies on the agar plates decreased throughout the acid challenge with no colonies observed 16h following the challenge. The number of culturable cells was below 10-20 CFU ml-1, the approximate detection limits of standard agar plate counting. Thus, no colony forming units were observed using standard plate count techniques. However, epifluorecscent studies revealed that approximately 35% of the observed cells within a particular microscopy grid (done in tripliate) showed to fluoresce green, suggesting viability.

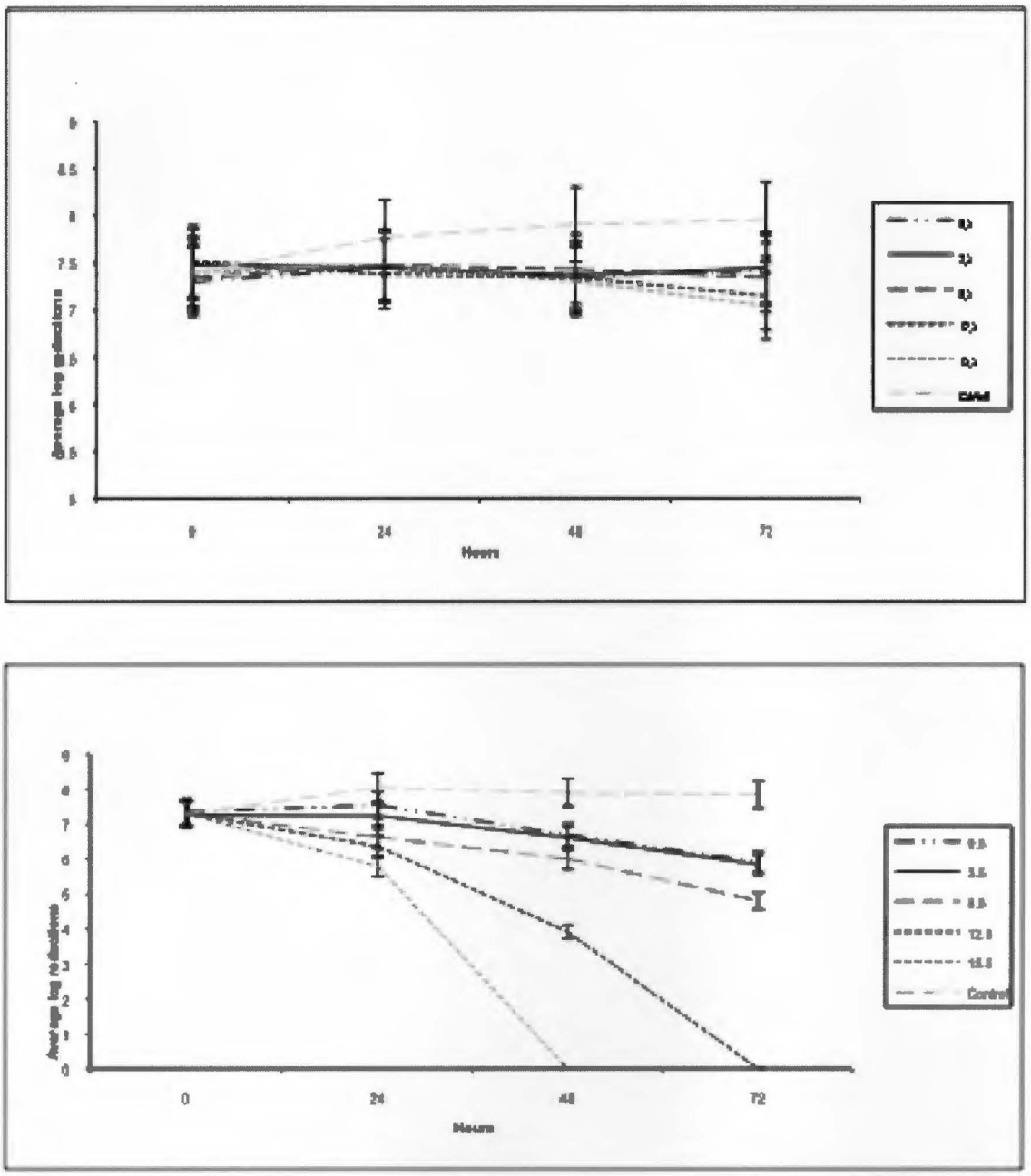


Figure 3.4. Inactivation rate comparisons for acid challenge studies under 5 different NaCl concentrations challenged at 25°C (upper panel) and 37°C (lower panel).

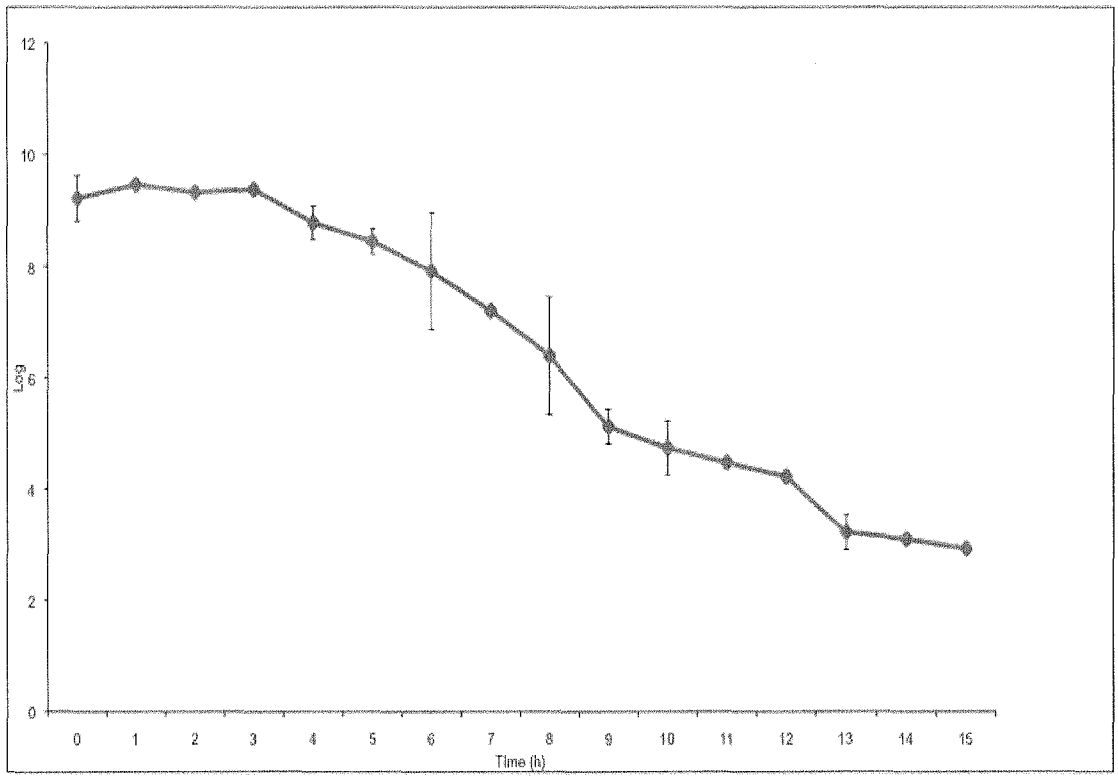


Figure 3.5. Survival curve of an *E. coli* K-12 variant strain following acid challenge at pH 3.5.

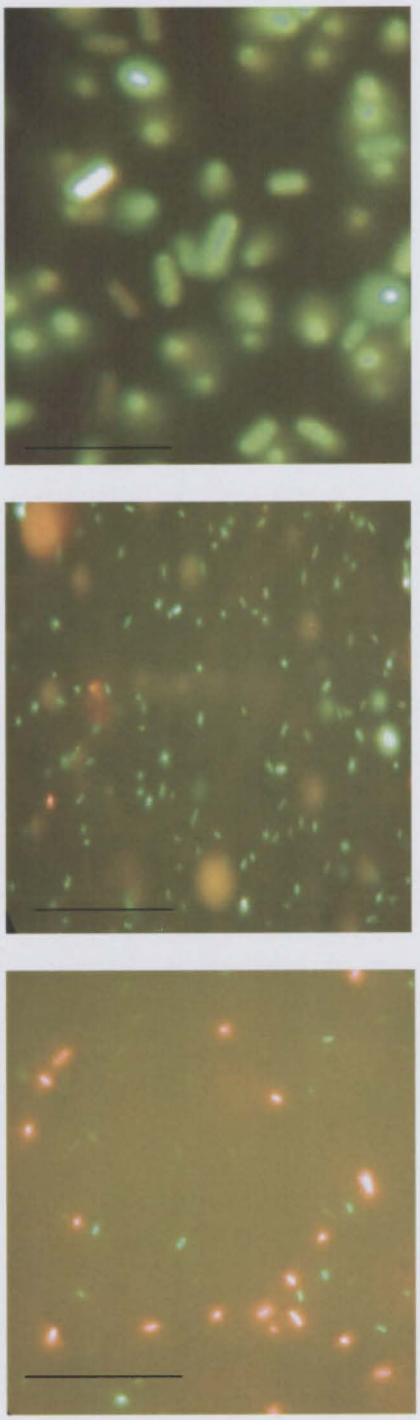


Figure 3.6. Fluorescent microscopic image of bacterial cells stained with Syto-9 and propidium iodide prior to (top panel) and 4h (middle panel) and 24h (lower panel) following acid challenge. Scale bar = 5 μm (top photo), 25 μm (middle, bottom photos).

3.5. Discussion

In the first experiment, we wanted to observe whether the 'working cultures' (cultures kept in -80°C storage with a high turnover ratio) from the CSIRO – Food Science Australia Division in Cannon Hill, behaved differently to the same lyophilised cultures held at CSIRO – Food Science Australia Division Cannon Hill, Queensland and those also stored at CSIRO – Food Science Australia Divisions in North Ryde, NSW and Werribee, Victoria (see experiment 3.1). The experiment revealed that in general, the working cultures possessed a higher ability to resist the given acid challenge compared to the other stored "clones". Perhaps an explanation for this phenomenon could be due to the recurrent growth and storage of the working cultures, as opposed to those held in storage continually for 4 to 5 years. Another explanation might be the type of media in which they were grown and kept in during storage. The work of Gawande & Griffiths (2005) provided some insights to the latter; in their work starved *E. coli* cultures subsequently grown in TSB, showed a 30% increase in their ability to survive frozen storage when compared to those grown in LB. The results for this experiment lead to the conclusion to utilize only the working cultures from CSIRO – Food Science Australia Division Cannon Hill in all further experiments. This conclusion was drawn with the aim to challenge the most robust strains available rather than strains exhibiting a less acid adapted phenotype.

An interesting observation from these experiments was the fact that the control strains, including a K-12 strain, did not show dramatic acid sensitivity when compared to the salami validation strains. In fact some validation strain clones shoed to be more acid sensitive than the controls. These preliminary experiments suggest

that certain conditions within the salami model demonstrated in Duffy *et al*, 2000 have a direct effect on the acid resistance phenotype of the validation strains. Similar results were found in the work of Large et al, 2005 were shiga toxin-producing clones did not show to be significantly more resistant to acid than commensal strains under certain challenge conditions; but demonstrated to be more acid resistant under other conditions. It was found that magnesium concentration played an important role in the variation of acid resistance amongst 30 strains (Large et al, 2005).

In the second experiment we wanted to confirm whether acid adaptation enhanced the ability of these strains to resist a given acid challenge (See experiment 3.2). In conjunction to previous literature the strains survived the given acid challenge for 3h longer than their non-acid adapted counterparts. One of the first authors to describe this phenomena were Buchanan & Edelson (1996), in their work the ability of 8 *E. coli* strains to resist acid challenge was evaluated when grown in TSB to stationary phase in the presence or absence of 1% glucose. All cultures grown in the presence of glucose demonstrated a significantly higher acid resistance than their glucose-absent counterparts. It was therefore concluded that further experimental research in our laboratory was to be performed using acid adapted *E. coli* cultures. This conclusion was drawn with the aim to perform *E. coli* inactivation studies using robust cultures rather than acid sensitive ones.

It must be noted however that, commercial TSB contains 0.25% glucose which could have trigger a small acid tolerance response. However, as observed in experiment 3.2, there was a substantial statistical difference in survival rates between the cultures challenge with TSB with those challenged with 1% glucose. In addition, pH measurements throughout the construction of growth curves (results not shown) did

not show dramatic pH changes during growth and upon entry into stationary phase. The pH values from the cultures grown with the added glucose was an average of 4.5 where those with the non-added glucose did not go below 6.5.

In the third experiment, we wanted to explore whether the lactic acid conformation had a significant effect in the ability to resist an acid challenge (See experiment 3.3). This was done as in some of the existent literature, the lactic acid conformation is often not specified (Abdulraouf *et al.*, 1993). For example in the work of Abdoul Rouf *et al.* (1993) and Leyer *et al.* (1995), two commonly cited publications; neither the brand nor the kind of isomer for the lactic acid used is mentioned (Abdulraouf *et al.*, 1993; Leyer *et al.*, 1995). In addition, the work of Duffy *et al.* (2000) which is the basis of this project, the lactic acid isomer conformation used was also not specified (but was assumed to be L-lactic acid). In this third experiment we confirmed that the lactic acid isomer conformation can influence the ability of three *E. coli* strains to resist the acid challenge. Lactic acid in the DL conformation appeared to have a more deleterious effect on *E. coli* survival. In other inactivation experiments it has been found that DL lactic acid has a greater inactivation effect than any of the isomers alone, this has been suggested to be due to a synergistic effect between both isomers (Goyal *et al.*, 2008). The work of William and Stuart (2002)it was demonstrated that L-lactate was more deleterious than D-lactate on E. coli O157:H7 and non-O157:H7 *E. coli* cultures. Since L-lactate is the most commonly used form of lactic acid in the current literature it was decided to utilize this isomer for all acid challenge experiments in this thesis.

In the fourth experiment we wanted to study the influence of temperature and salt concentration in *E. coli* inactivation (See experiment 3.4). Using one of the *E. coli* challenge strains as a model organism, we observed that reductions of the challenge temperature resulted in reductions of the magnitude of the given acid challenge. It was also observed that for NaCl levels above 3.5%, the higher the salt concentration the higher the inactivation effect. No significant differences were found for cultures challenged in acid supplemented with 0.5% NaCl and those challenged in acid supplemented with 3.5% NaCl. Similar observations regarding the effects of temperatures were made in the work of Garren *et al.* (1997) where *E. coli* survival to acid challenge was prolonged at 25°C when compared to 32°C. In regards to the supplementation of NaCl, literature sources suggest that in some instances the correlation between salt supplementation and *E. coli* inactivation was relatively linear and positive. That is, the more NaCl in the challenge media the higher the inactivation effect. However, some authors have observed that supplementation of NaCl to an acid challenge between the 1% and 4% can sometimes enhance *E. coli* acid survival. This is exemplified in the work of Jordan & Davies (2001), where they suggested that the presence of NaCl between 4% and 6% enhanced the recovery and survival of acid stress *E. coli* O157:H7 cultures. The fact that in this fourth experiment only one *E. coli* (non-O157:H7) strain was used, led to question whether other strains in our laboratory would behave in a similar manner. In addition, the fact that *E. coli* acid resistance has been proven to differ depending in the amino acid content of the media (Large *et al.*, 2005); also led to question if the sensitivity of the *E. coli* strains in our laboratory would be affected by the type of challenge media. Both of the latter queries led to the work presented in Chapter 5.

In the fifth experiment we wanted to study whether cultivability was a true representation of inactivation (See experiment 3.5). This was performed in order to confirm the accuracy of the results observed in chapter 2. In chapter 2, proteome analysis were taken 24h following the challenge, however under the same experimental conditions we found that for this experiment no culturable colonies occurred after 16h of given acid challenge. The use of viability stains revealed that viable but-non culturable cells (using standard plate count techniques) were present 24h following the acid challenge. We therefore concluded that the observed proteomic profiles for the strains used in chapter 2 were valid. The concept of viable but-non culturable was described in detail by Bloomfield *et al* (1998). Briefly, the term viable but non-culturable is generally used for cells that cannot be cultured in the laboratory but capable of growing and thus potentially able to cause infection. Bloomfield *et al* (1998), refer to this type of cells as active but non-culturable to avoid a possible oxymoron; they suggest that the current methods used in microbiology fail to provide the cells the adequate conditions to support growth following acid challenge (Bloomfield *et al.*, 1998). The work of Rigsbee *et al* (1996) also suggested that environmental stresses can often cause *E. coli* O157:H7 strains to enter a viable but non-culturable state (Rigsbee, 2007). The results from this experiment suggest that cells that entering a viable but non-culturable state may perhaps be the best candidates for molecular challenge studies. It can be hypothesized these cells have undergone a stress so major that they are unable to replicate under certain conditions (standard incubation techniques) but still manage to remain viable through the expression of certain mechanisms. Mechanisms that might be of interest for understanding the ability of cells to resist environmental stresses.

3.6. Conclusion

The results from the series of experiments assisted in comparing the behavior of the strains used in this project with some of the *E. coli* acid resistance knowledge reported in previous literature. It became clear that the acid resistance phenotypes of *E. coli* cultures in question was affected by certain experimental parameters; in this case: storage (Experiment 1), acid adaptation (Experiment 2), type of lactic acid used (Experiment 3), challenge temperature and salt concentration (Experiment 4). It became apparent that in order to reach a more accurate conclusion of the behavior of *E. coli* strains to acid challenge, it is necessary to take into account the independent effects of the experimental parameters. For example, in Experiment 3.2 no significant differences in survival rates were observed between the salami challenge strains and the controls when challenged under the same treatments. This was unexpected as the salami challenge strains appeared to be more robust than the controls in the work of Duffy *et al* (2000). It was concluded that expecting these strains to behave in the same manner was not accurate as the experimental parameters were not the same. In the work of Duffy *et al* (2000), the strains were challenged under a high NaCl concentration (12.5%) and at 15°C, a completely different set of experimental parameters to those from Experiment 2 (Figure 3.2). In experiments 3.4 we confirmed that the magnitude of the effects of acid challenge is altered with temperature and NaCl concentration.

The observed results in Duffy *et al* (2000) and the comparisons of the obtained results in these experiments led us to hypothesize whether the salami challenge strains were

only more robust to acid challenge, when compared to the controls, in the presence of NaCl.

4. Chapter 4. Development of an agar substitute for Escherichia coli acidic studies in semi-solid media

"A man should look for what is, and not for what he thinks should be"

Albert Einstein (1879-1955)

Chapter Preface:

The main aim of this chapter was to develop a suitable semi-solid media for acid challenge studies. Evidence suggests that *E. coli* cells in a planktonic state (free-floating) have an inherently lower capacity to resist acid than their sessile (attached or established at a surface) counterparts. By studying *E. coli* cells in a semi-solid media we hoped to facilitate the means for *E. coli* to exist in a sessile state and observe for differences in survival rates when compared to cultures challenged in broth. In addition, the development of a suitable semi-solid media for acidic challenge studies, creates an opportunity to study *E. coli* in a media that more resembles salami without the added complexity. We successfully developed a semi-solid media suitable for acidic studies and observed a greater capacity to resist acid for the cultures challenged in such when compared to their planktonic counterparts.

ABSTRACT

An agar substitute was developed for comparative *E. coli* acid survival studies between broth and semi-solid acidified media. Out of a series of five carrageenan mixtures with or without the addition of carboxylmethylcellulose (CMC), the blend combination of 1.2% PS911 and 0.3% GP379 was determined to be the most adequate. Using a selection criterion, this blend proved to be the best candidate for semisolid acid studies at pH levels of 4.5. The selected blend had better consistency, homogeneity and stability than the other blends. When compared to agar, the selected blend demonstrated the ability to remain rigid and have less syneresis when inoculated with bacteria and after acid addition. The acid resistance of two *E. coli* strains, an O157:H7 strain and an Australian salami validation strain were compared in acidified semi solid media and in acidified broths. Results showed no significant differences in survival between the broths and the blends at a neutral pH. Under the same lactic acid concentration, growth was observed for both strains challenged in the blend (pH 5.5), while inactivation occurred for the cells challenged in the broth (pH 4.5). When more lactic acid was added to adjust the pH of the blend to pH 4.5, inactivation occurred but at a lesser rate than with the planktonic counterparts challenged at the same pH. Significant differences were found for cells challenged in the semi-solid media when compared to those challenged in broths. The results from this study suggest that carrageenans can be used for modeling acidified semi-solid matrices. They also suggest that *E. coli* cells challenged in a semi-solid environment might possess a higher acid resistance than their counterparts challenged in broth.

4.1.Introduction

The understanding of the *E. coli* acid stress response is of relevance to food safety. It is believed that the ability of pathogenic *E. coli* to persist in the food chain is partly due to their intrinsic characteristic acid resistance (Conner & Kotrola, 1995; Lin *et al.*, 1996). Extensive research regarding the persistence of pathogenic *E. coli* in acidic environments has been generated due to its association with acidic food products. Information from acid challenge studies has and can be used for the implementation and regulation of safer food manufacturing practices (Cheng *et al.*, 2003; Lin *et al.*, 1996). Although many acid challenge *E. coli* assays have been performed utilizing actual food products (Abdulraouf *et al.*, 1993; Glass *et al.*, 1992a; Hsin-Yi & Chou, 2001; Lekkas *et al.*, 2006; Leyer *et al.*, 1995), most literature has been obtained from *in vitro* acid challenge assays using broth systems. Bacteria challenged in broths are considered to be in a planktonic state (free floating). However, it has been hypothesized that in nature, most bacteria exist in a sessile state and/or as biofilms (Costerton *et al.*, 1987b). It is believed that planktonic bacteria represent <0.1% of the bacterial population of quantitatively studied ecosystems (Costerton *et al.*, 1987b). In terms of food, with the exception of milk, sauces, juices and other liquid products, it can be said that bacteria are mostly present in semi-solid heterogeneous mixtures rather than in homogeneous fluids. It can also be assumed that within a semi-solid heterogenous mixture not all bacteria might be in a planktonic state. This is inferred based on the possibility that bacteria might attach to the solid structures within the semi-solid matrix. To date studies have demonstrated that there are extensive metabolic differences between planktonic and sessile cells of the same population

(Brown & Williams, 1985; Costerton *et al.*, 1987a; Lorian *et al.*, 1985; Walker *et al.*, 1998). For example bacterial cells in biofilms have been demonstrated to be more resistant to environmental stresses than their planktonic counterparts (Finlay & Falkow, 1997; Holmes & Evans, 1989; Hoyle & Costerton, 1991). It can be therefore hypothesized that some bacteria within a population in a semi-solid heterogenous mixture might exist in a non-planktonic state and differ phenotypically to those in a planktonic state.

It must be emphasized that most of the information regarding the *E. coli* 's acid stress response has been generated using planktonic cells. In this work we suggest that the current understandings need to be complemented using data from studies in acidified semi-solid heterogenous mixtures. To date the *E. coli* acid stress response in food has been investigated, however experimental parameters are often restricted due to the complexity in the media. Even when bacteria is successfully inoculated and distributed within an actual food matrix, its isolation and enumeration has been proven to present many challenges (Bolton *et al.*, 1996; Rossen *et al.*, 1992). For example, the isolation of pure cultures from a mixture of pork and beef mince would be very difficult, as there is no guarantee that the mince is free of other bacteria. In addition, separating pure cultures from complex mixtures involves some physical and chemical processes, which could act as an stressor affecting the proteome and transcriptomes. Thus, the use of less complex artificial heterogenous mixtures (*in vitro* food matrices) might therefore be a better approach. Previous laboratory studies using artificial semi-solid matrices for sessile studies have been documented (Mitchell & Wimpenny, 1997; Walker *et al.*, 1998). The work of Mitchell and Wimpenny (1997), assessed the differences in bacterial colony morphology dependent on the

microtopology of the matrix. The authors selected the use of agar for its common use in microbiological studies (Mitchell & Wimpenny, 1997). However due to the intrinsic characteristics of agar as well as some preliminary studies in our laboratory (not published), we believe that agar is not the most adequate candidate for acidic studies in heterogeneous semi-solid matrices. At pH 4.5 or below, we have observed that the structure of agar is somewhat affected in terms of rigidity and consistency.

In this study, an agar substitute for acidic *E. coli* studies in semi-solid heterogeneous mixtures was developed using a blend of carragennans. Carragennans are naturally occurring polysaccharides extracted from red seaweed that are widely used in food ingredients, including those with low pH (Stephen *et al.*, 2006). Once the agar substitute was developed, the survival in the carrageenan blend of two *E. coli* strains an O157:H7 and an Australian salami validation strain was studied and compared to that of broths. Comparative survival studies between the blends and broths were performed under three main conditions, at neutral pH, under the same L-lactic acid concentration and under the same pH value (4.5).

4.2. Materials and Methods

4.2.1. Agar and carrageenan blend development studies

Standard laboratory agar (Oxoid, LP0011) was prepared according to manufacturer specifications and used as a control for the carrageenan mixtures. The agar substitute was developed using a blend of two carrageenans with or without the addition of

CMC (carboxymethylcellulose). All blends were supplemented with brain heart infusion (BHI) solids at a concentration of 37g/L. The carrageenans used in this study were Gelcarin – GP-379 (Swift and Company Limited, 4522211) and Gelcarin – PS-9111 (Swift and Company Limited, 4900704). A series of five mixtures using different amounts for each carrageenan including the addition of CMC were tested (Table 4.1). The most adequate combination was selected using a selection criterion based on setting temperature, hardness, homogeneity and stability after the addition of acid and/or bacteria (see below). To prepare the carrageenan blends, all solid ingredients were firstly dry mixed prior to the addition of water. In a separate container, approximately 80% of the total water (w/w) required for the mixture was added. The container was centered underneath the probe of a high sheer mixer (Silverston, L1RT). To ensure adequate mixing, the high sheer mixer probe was submerged well below the surface but a few centimeters from the bottom ensuring it did not touch the base. The high sheer mixer speed was set to 50,000 RPM and the dry-mix of ingredients was added to the water slowly to prevent lump formation. The mixing speed was increased as the solution became more viscous. Once all ingredients were added to the high sheer mixer, mixing was performed for an extra minute at a maximum speed of 80,000 RPM to ensure thorough mixing. The remaining left over water was then added and mixed using a plastic spatula. Blends were either immediately sterilized or stored in the chiller for a maximum of two days. Samples were subjected to four sterilization treatments in order to achieve better homogenization upon setting. The 4 different sterilization treatments were as follows: 121 °C for 25 °C min, 111 °C for 25min, 111 °C for 100min, and 100 °C for100min. To further improve homogeneity, blend aliquots for experimental use were brought to boil using a magnetic stirrer and a hot plate prior to autoclaving. Immediately after

boiling, aliquots were autoclaved for 90 min at 110 °C. Once autoclaved, aliquots were gently shaken and transferred to a 45 °C water bath and allowed for the temperature to equilibrate. Temperatures were monitored using an external thermometer (Fluke, G2 –IR Thermometer).

4.2.2. Selection criteria studies

Blend homogeneity and stability

All the selection criteria experiments were performed using triplicate 50ml aliquots, placed in 70 mL sterile jars (Sarstedt, 75.9922.730). Immediately after dispensing, the temperature for each blend was monitored until setting was achieved. Samples were categorized as set, once no dripping fluids were apparent when turning the jar upside down. Once set, homogeneity was studied by observing the samples against a light source. Adequate homogeneity was categorized by the absence of layering and lumps. Samples with different color layers or lumps were classified as not homogenous.

Hardness of samples was assessed using two parameters: their ability to stand and maintain the jar-shape once they were dislodged from the jars and by their ease to be dissolved using a mechanical stomacher. Samples were dislodged from the jars by gentle tapping and squeezing of the jar. In some instances if the samples were not strong enough, squeezing of the jar will cause the samples to become brittle and lose their shape. Such samples were classified as very weak. To assess the effects of a five day incubation period at 37 °C, duplicates of all samples were tested using the same parameters. In addition, the presence of syneresis following incubation was also assessed. Syneresis was categorized by the presence of free water in the jars and around the dislodged samples.

4.2.3. Acidic studies

L-lactic acid (80% solution, Sigma 27715) was added to each of the 50ml blend aliquots at various concentrations prior to and following sterilization at 60°C, 50°C and 45 °C. Thereafter the stability and homogeneity of the series of blends were assessed exactly as mentioned above. The pH of the samples was monitored using a probe suitable for semi-solid media. However, since pH is dependent on temperature, pH measurements were performed at room temperature once the blends were set. When the lactic acid was added prior to autoclaving, pH was monitored using a standard pH probe. Monitoring of pH values were also performed every 24 h during incubation.

4.2.4. Blend-bacterial studies

Strains and inoculums preparations

Two *E. coli* strains were used throughout this study, genome-sequenced pathogenic O157:H7 strain (Sakai, ATCC BAA-460) and a non-pathogenic (EC1606) strain previously demonstrated to have high organic acid resistance (Duffy *et al.*, 2000). The strains were recovered from -80C° storage by placing one Protect Bead® into 10 ml of nutrient broth (NB, Sigma – 70122) and incubated overnight (24 h). For each strain a loopfuls of the overnight cultures were streaked on nutrient agar plates (NA, Sigma N4019) and incubated for 24 h to verify purity and isolate individual colonies. Single

colonies were then used for each replicate. Prior to any experiment, strains were grown in NB for 24 h at 37°C.

Bacterial inoculations

Bacterial strains were inoculated to the samples at 42°C. However, bacteria inoculation studies were only performed on those blend combinations that met the abovementioned selection criteria. A range of bacterial inoculums was tested until the starting bacterial concentration after setting was around 10^4 CFU ml-1. Stability following bacterial inoculation was assessed every 24 h after incubation at 37°C using the previously described parameters.

Bacterial enumeration

The 50ml blend aliquots were aseptically dispensed into sterile stomacher bags, to which 50 ml of 0.85% saline solution was added. The gel and saline solutions were then stomached for 90 s using a mechanical stomacher (Interscience, Bag Mixer). Due to occasional frothing, the stomached mixtures were left to sit at room temperature for 2 min. Thereafter, 100 μL of each sample was aseptically extracted using wide-bore pipette-tips and pippetted into 900 μL of saline. Dilutions were subsequently performed five times until a 10^4 CFU ml-1dilution was achieved. Following dilutions, 100 μL of each were plated accordingly onto tryptic-soy agar (TSA, Sigma -22092) plates supplemented with 0.2% glucose and 0.2% yeast extract (TYSG). Plates were incubated for 24 h and colony-forming units were enumerated.

4.2.5. Bacterial-broth studies

Broth studies were performed using BHI broths that were prepared using de-ionised water at a concentration of 37g/l. For acidified broths, filter sterilized lactic acid was added at 45°C following autoclaving at different concentrations. Bacterial inoculums were then added at different concentrations at exactly 42°C in order to achieve $>10^5$ CFU ml-1. Upon inoculation, samples were shaken for 10 s and immediately dispensed in 50 mL aliquots into 70 ml jars, which were then placed into an iced water bath for 30 s. The aim of this exercise was to mimic the rapid heat loss observed in the carrageenan blend where the temperature of each aliquot averaged 39°C after 2 min. Bacterial enumerations were performed as described for the blend studies.

Table 4.1. Summary for the five combinations trials for the development of an agar substitute for sessile bacteria acidic studies

Blend	GP 379 %	PS 911 %	CMC %	BHI %	Distilled water %
1	0.3	1.2	0	3.7	94.8
2	0.3	1.2	0.25	3.7	94.55
3	1.2	0.3	0	3.7	94.8
4	1.2	0.3	0.25	3.7	94.55
5	0.75	0.75	0.25	3.7	94.55

4.3. Results

Of the five mixtures, the most adequate was Blend 1, consisting of the carragenans 1.2% PS-9111 and 0.3% GP-379 and BHI solids (Table 1). All other blends did not meet selection criteria requirements (See figure 3.1). Blend 5 did not appear to be completely homogeneous after setting. Blends 3 and 4 began to harden at around 45°C. The addition of CMC (0.25%) to Blend 2 resulted in a more stable blend, however the increased hardness decreased its ability to be dissolved. Some semi-solid lumps were still present after 2 minutes of stomaching. Agar appeared to meet most of the points within selection criterion. However, the addition of acid affected the blend consistency by weakening it and making it less stable. Similarly, without the addition of acid, the inclusion of bacteria to the agar resulted in brittle and weak semi-solid structure.

Sterilization studies for a homogeneous Blend 1, was achieved at 110 C° for 100 min. Preliminary studies, revealed that the standard sterilization method (121°C, 25 min) resulted in a non-homogenous mixtures upon setting (regardless of the carrageenan combination). At standard sterilization conditions, upon setting, the blend appeared in multiple layers, with the carrageenans appearing to settle at the bottom of the Schott bottles. Such a phenomenon was also observed for all other blends (results not shown). The lack of homogeneity appeared to affect the setting temperatures, as not all triplicate samples set at the same temperature. Multi-layering was resolved when the carrageenan mixture was boiled for 2 min prior to autoclaving and gently-stirred prior to setting. However, some lumps were still visible once the sample had set. A

successful homogenous and sterile mixture was achieved at sterilization settings of 110°C for 90 min.

The chosen carrageenan combination was selected based on a setting temperature of 42°C, as well as the consistency and stability after setting. The same parameters were then tested with the addition of acid and a 96-hour incubation period at 37°C. Finally, blend stability was tested against bacterial inoculation. The findings were then compared to those of standard agar.

Lactic acid-addition studies were only performed for Blend 1 and agar. When lactic acid was added prior to sterilization, it was found that neither Blend 1 nor the agar set after autoclaving. Both the agar and Blend 1 appeared to remain in a gelatinous state regardless of the external temperature. It was also found that the addition of acid at temperatures higher than 50 C° appeared to weaken the structure of the matrices. The higher the temperature at which the acid was added following autoclaving the weaker the structures after setting. Lactic acid (80% solution) was added at a concentration of 6.2 mL/L to achieve a pH value of 4.5. When acid was added at a similar concentration as the broth studies (4.2 mL/L) the final pH after setting was 5.5. Interestingly at such concentration (4.2 mL/L) for Blend 1, the pH was also 4.5 when the lactic acid was added prior to autoclaving.

In the presence of bacteria, there was a significant difference between the stability of the standard agar and that of Blend 1. Syneresis after overnight bacterial growth at neutral pH (7) was more pronounced for the agar than for Blend 1. The blend also appeared to be less brittle in structure than the agar. Bacterial inocula were added at

concentrations of 100 μl/100 ml to 300 ml of the mixture at exactly 42°C. At these concentrations the initial bacterial counts averaged 3×10^5 CFU ml-1 for all bacterial studies. At neutral pH (pH 7) a 2 log increase was observed following 24 h incubation at 37°C. Bacterial numbers remained stable for up to 5 d after inoculation (see Fig. 3.2A). Similar results were observed when bacteria were challenged at pH 5.5, however, a slight decrease in the population was observed for the strain O157:H7 Sakai in the fifth day of incubation (see Fig. 3.2B). When bacteria were challenged at values of pH 4.5 no survivors were found three days after inoculation (see Fig. 3.2C). For broth studies, at neutral pH, there were no significant differences in bacterial survival when compared to Blend1. At pH 4.5 and 4.1, however, no survivors were found 2 days and 1 day after inoculation respectively (Fig. 3.2B and 3.2C)

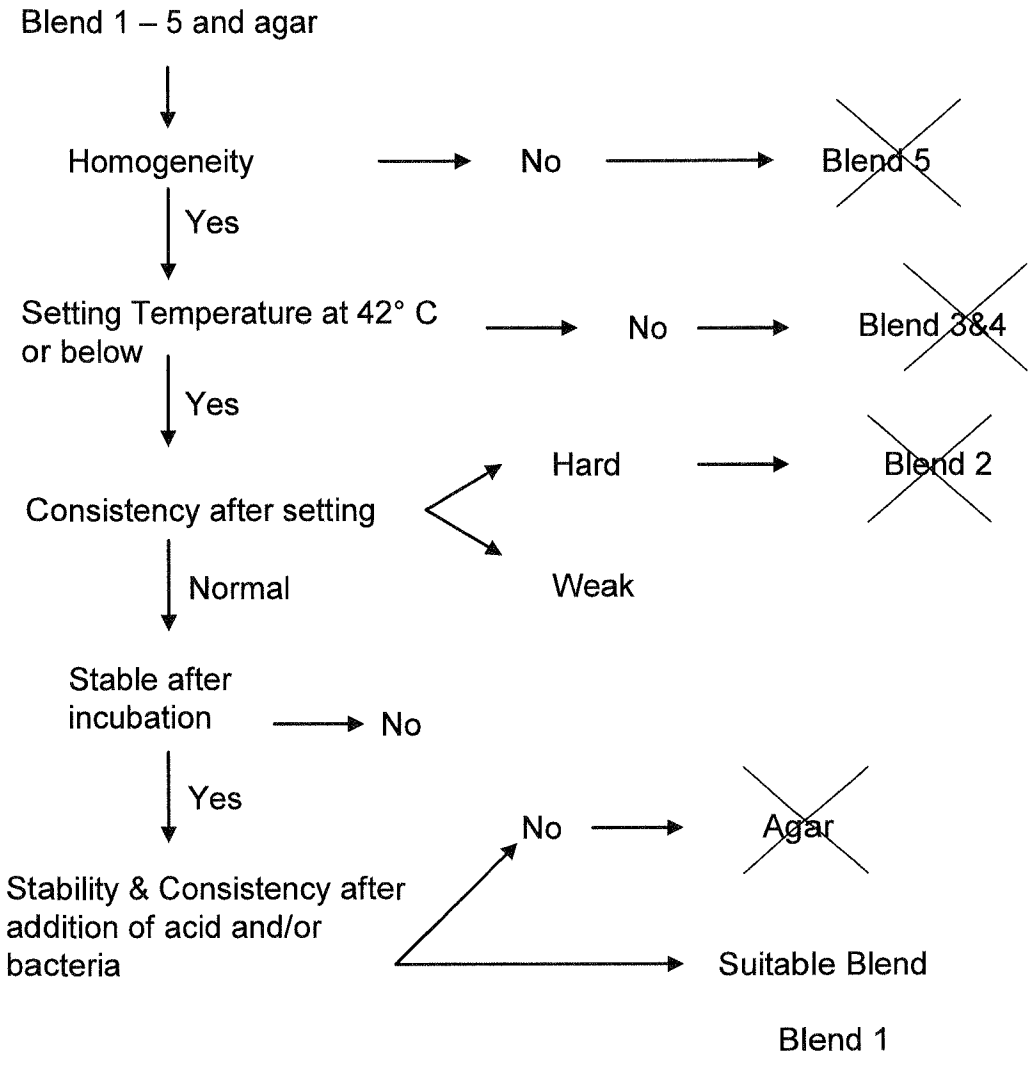
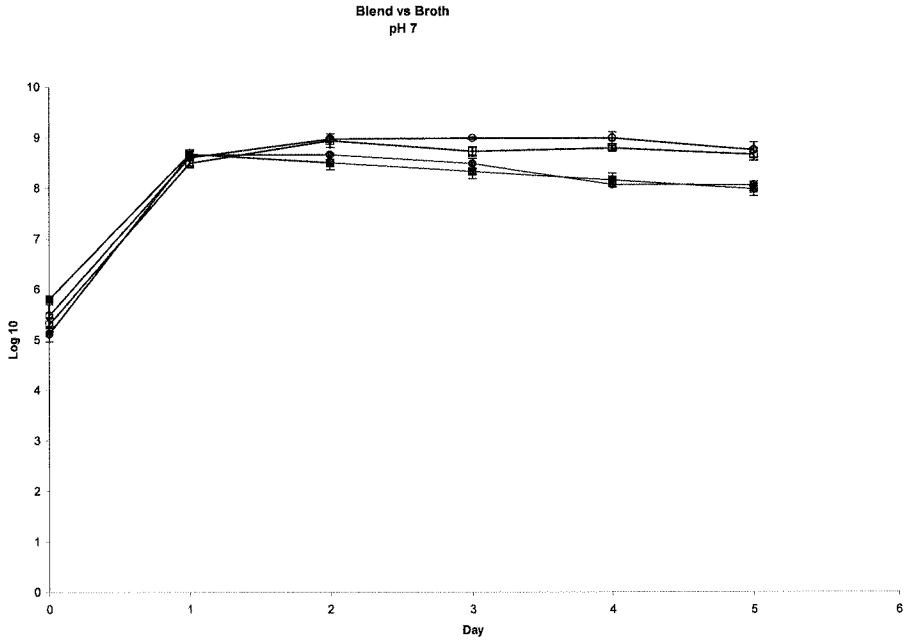
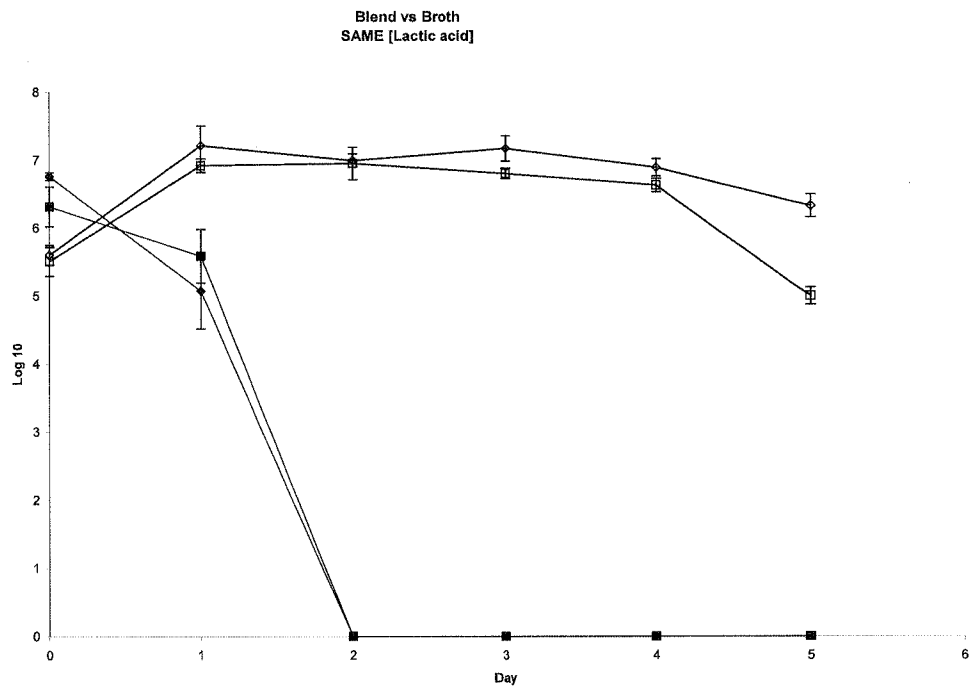


Figure 4.1. Schematic representation of the selection criterion used to select the most adequate carrageenan mixtures (with or without the addition of CMC), using agar as control.

A


Blend vs Broth
pH 7
Log 10
Day
0
1
2
3
4
5
6
7
8
9
10

B


Blend vs Broth
SAME [Lactic acid]
Log 10
Day
0
1
2
3
4
5
6
7
8

C

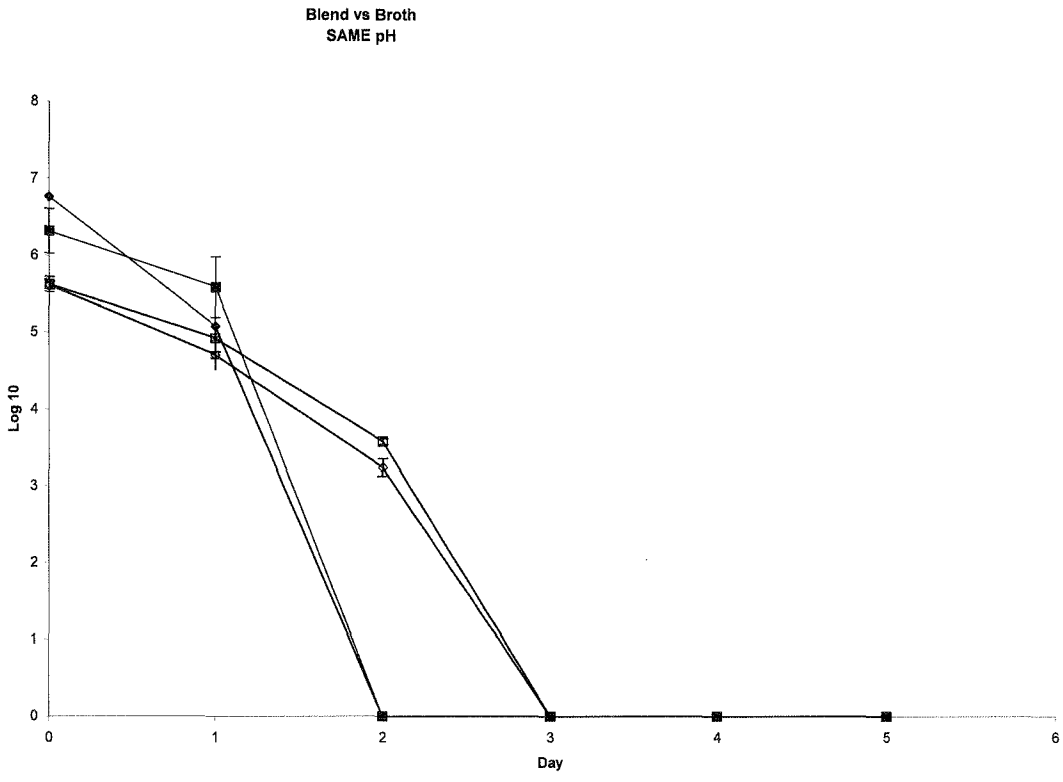


Figure. 4.2. Survival curves of 2 *Escherichia coli* strains, including an Australian salami validation strain (circles) and a pathogenic O157:H7 strain (squares), challenged in broths (closed symbols) and in Blend 1 (opened symbols) for a period of 5 days at 37 °C. (A) bacteria challenged at pH 7; (B) bacteria challenged under the same lactic acid concentration (4.5 ml/l) with varying pH values; (C) bacteria challenged at pH 4.5 with varying lactic acid concentrations between the broths (4.5 ml/l) and Blend 1 (6.2 ml/l).

4.4.Discussion

Agar is the most commonly used semi-solid media for current bacterial research (Mitchell & Wimpenny, 1997). However, in the laboratory agar does not appear to adequately set under acidic conditions (pH $\leq$4.5). Similar findings where the physicochemical properties of gels are affected by pH have previously been reported (Feng & Hultin, 2001; Motoki., 1994).In this study carrageenans were used to substitute for agar due to their use in acidic food products such as salad dressings, as well as for their structural similarities to agar (Stephen *et al.*, 2006). The carrageenan mixture (Blend 1), containing PS9111 (1.2%) and GP379 (0.3%), and supplemented with BHI solids proved to be the best candidate for acidic-sessile challenge studies. Results showed that the final concentration of carrageenans (1.5%) resembled the average concentration utilised in standard pre-made agar media. However, in contrast to standard pre-made agar media, it was found that sterilization at 121°C for 25 min, resulted in a non-homogenous mixture when set. The lack of homogeneity may be a result of irreversible structural damages caused by the temperature and pressure (~140 KPa). Improvements to setting-homogeneity were made when the carragenan blends were boiled prior to autoclaving. This process appeared to reduce the damages caused by standard sterilization settings. A homogenous mixture was obtained when the sterilization temperature was lowered to 110°C and the sterilization time was increased to 90min. Emphasis was placed on the homogeneity of the blends in order to achieve a consistent distribution of acid and/or bacteria throughout the samples. It was found that the addition of acid to samples that were not homogeneous resulted in variations in pH values within the sample. Different pH gradients within the sample

could have had an effect on the survival of bacteria. In a similar manner it was hypothesized that bacteria would not be evenly distributed in non- homogeneous samples, therefore their survival in acid could also be affected.

Bacterial inoculation studies revealed that Blend 1 appeared to remain stable following the addition of acid and/or bacteria. The absence of visible syneresis following inoculations, regardless of the pH, suggested the reduced chance excess of bacterial cells remaining in a planktonic state. When compared to agar, Blend 1 remained stable at lactic acid concentrations of 7mL/L suggesting its use for bacteria acidic assays at pH levels as low as 3.5.

The monitoring of pH revealed that the addition of 4.2 ml/l of lactic acid resulted in pH values of 4.5 and 5.5 for the broth and the carrageenan blend respectively. The addition of 6.2 ml/l of lactic acid to the broth and blend resulted in pH values of 4.1 and 4.5 respectively. These values suggest that upon setting the carrageenan blend somewhat buffers the added acid. Interestingly, such buffering is not observed when the carrageenan mixture is not autoclaved. Perhaps, the buffering phenomenon is due to the inclusion of some lactic acid molecules into the gel structure, indicating that less unbound lactic acid molecules are present in the mixture. Therefore, it can be assumed that bacteria are exposed to less lactic acid molecules once the gel had set. Survival studies agreed with the latter theory, under the same lactic acid concentration as the broths (pH 4.5) there was an approximate 2 log difference for both bacterial strains 24 h to 48 h after the sessile challenge. Cultures showed a 6.5 log decrease 24 h to 48 h after being challenged within broth.

The setting temperature for the carrageenan mixture (42 °C), proved to be suitable for bacterial challenge studies. For Blend 1, the estimated initial bacterial numbers following inoculation matched the actual numbers after stomaching and enumeration. However, for the cultures challenged in broths, an approximate 2 log reduction for the estimated number of cells was observed immediately after inoculation. Preliminary studies demonstrated that duplicate inoculations to broths at 37 °C did not display the 2 log reduction observed at 42 °C. At this temperature the initial counts matched those of Blend1 (results not shown). Such phenomena clearly indicate that heat is dissipated at a faster rate for the carrageenan mixture than for the broths, and that the observed log reductions are due to temperature. Temperature studies revealed that, if not placed on ice, the broth cultures remained at 42 C° for up to 90 sec, while the carrageenan mixture reached 39 C° in the same time frame (results not shown).

Bacterial challenge studies revealed that cells in broths displayed a lesser tolerance to both acid challenges; same pH and same lactic concentration. The substantial difference in survival rates for both strains challenged under the same lactic acid concentrations could be easily linked to the differences in pH; pH 5.5 for Blend 1 and pH 4.5 for the broths. However, when the pH for Blend 1 was adjusted to 4.5 by increasing the amount of lactic acid added, both strains displayed enhanced tolerance than what is observed in the broths at the same pH. These results, suggest that bacterial tolerance to an acid challenge could potentially be higher for cells challenged in a semi-solid heterogeneous mixture than when challenged in broths. An important observation from these studies is the fact that the pathogenic strain showed no significant difference to any of the acid challenge studies from this chapter. This is an important observation as it corroborates the fact that the validation strains (or at

least this particular one) have similar resistance to acid challenge as the pathogenic *E. coli* O157:H7 strain. This fact further supports their use as salami validation strains in the salami industry.

In terms of food safety research, the findings of this study might be of relevance, since most of the current knowledge regarding the acid stress response of *E. coli* cells has been obtained from cells challenged in broths. When challenged in a semi-solid environment, *E. coli* cells might exist in different physiological states such as planktonic, sessile and perhaps even as biofilms; having a direct influence in their acid resistance phenotypes (Costerton *et al.*, 1987b). For example, when challenged in broths, four *E. coli* acid resistance systems have been characterized, AR1, AR2, AR3 and AR4. The induction of these systems is influenced by the entry into stationary phase (AR1) and the presence of glutamine (AR2), arginine (AR3) and lysine (AR4) (Foster, 2004). Such knowledge has been obtained for cells challenged in broths; however it remains possible that when cells are challenged in a semi-solid environment other complimentary acid resistance systems might come into play. It is known that the attachment of bacterial cells to surfaces triggers many physiological changes in the cell (Costerton *et al.*, 1987b). It may be that the induction of the attachment-genes may trigger the induction of some of the genes involved in acid resistance. For example in a study it was found that the majority of associated acid resistance genes can be induced upon other environmental changes such as entry into stationary phase and osmotic shifts (Weber *et al.*, 2005). Perhaps the osmotic shift created by the transfer of broth cultures (initial inoculums) to the semi-solid matrix could have caused the expression of some acid resistance genes (Weber *et al.*, 2005)

In conclusion, the current work presented an agar alternative for the study of bacterial cells in an acidified matrix, using a blend of two carragenans. Comparative acidic studies between cells challenged in the carragenan blend and broth, demonstrated a reduced acid persistence in the latter. These results suggest that cells challenged within a semi-solid matrix may have a stronger persistence to an acid challenge than their planktonic counterparts. Complementing the acid resistance knowledge obtained from broth studies with studies in semi-solid media might assist in better hurdle design for pathogenic *E. coli*. In addition, it may assist in better ways of controlling spoilage, which is also a major contributor to economic losses in the food industry.

5. Chapter 5: Challenge medium composition a new factor on *Escherichia coli* acid challenge studies

"What gets us into trouble is not what we don't know

But what we know for sure that just ain't so"

Samuel Langhorne Clemens (1835-1910)

CHAPTER PREFACE

In this chapter the effects of the constituents of 4 standard media on the acid resistance phenotypes of 12 *E. coli* strains was investigated. In addition the effects of NaCl supplementation to an acid challenge were also investigated. The experimental set up was constructed with salami manufacturing in mind. The pH value of 4.5 was selected, as it is the average pH that is reached following the fermentation process. During the process of formulating the correct parameters for *E. coli* challenge at pH 4.5 we noted that each media had unique buffering capacities following the addition of lactic acid. It was therefore decided to challenge the panel of strains with 3 different parameters in mind; when lactic acid was added to reach pH 4.5, when lactic acid was added at a given concentration without adjusting the pH values, and when lactic acid was added at a given concentration adjusting the pH values to pH 4.5. The main finding of this work was the observation of different patterns of survival amongst the three main parameters. Some strains that proved robust for a particular treatment when compared to the other strains appeared to be acid sensitive in another parameter. Similar findings were observed for the acid challenge supplemented with NaCl at different concentrations.

ABSTRACT

The survival of 12 *Escherichia coli* strains (including four pathogenic O157:H7 serotype strains) in 4 different media: brain heart infusion (BHI), tryptic soy broth (TSB), nutrient broth (NB) and minimal media (MM), with various acid challenge conditions was examined to determine the influence of external nutrient availability and buffering capacity on acid resistance. The challenge conditions included 96 h exposure to 3 different lactic acid treatments: varying lactic acid concentration (37.2, 5.5, 32.1 and 4.4 mM) to pH 4.5; and constant lactic acid concentration (34.6 mM) with or without pH adjustments to 4.5. *E. coli* acid resistance phenotypes were also evaluated 96 h following acid challenge (pH 4.5) in four different media supplemented with 0.5%, 3.5%, 8.5% and 12.5% (w/v) NaCl. Acid challenge studies revealed substantial variation in survival amongst the 12 strains during challenge with the 3 different treatments and across the 4 challenge media. Survival also varied across the 4 media supplemented with the various NaCl concentrations. The current work demonstrated that the constituents of each media influenced the parameters, acid concentration and pH, of a given acid challenge directly influencing the degree of inactivation. It was also demonstrated that under standardized parameters, equal lactic acid concentration and pH, the constituents of each media had a direct influence on the acid resistance phenotypes of individual strains and of the population as a whole. Overall, BHI appeared to offer the most protection against acid challenge, including that with high NaCl concentration. In addition, some strains challenged in TSB supplemented with 3.5% NaCl had increased survival than those challenged in TSB supplemented with 0.5%, 8.5% and 12.5% NaCl.

The current work suggests that challenge media is a factor that should be considered when designing an acid challenge study as it can influence inactivation likely due to altered phenotypes of the *E. coli* strains. Moreover, our observations suggest that the constituents of the challenge media should be taken into account for the accurate determination of *E. coli* survival to acid challenge.

5.1. Introduction

Within foods, during food processing and within the gastrointestinal environment, pathogenic bacteria often are exposed to acidic environments including a range of organic acids. Some manufactured food products rely on low pH and/or the presence of organic acids for the inactivation of contaminating pathogens (Brul, 1999; Shelef, 1994; Sofos, 1993). The current implementation of hurdles is based not only on centuries of empirical knowledge but also on the outcomes of more recent scientific research.

To date many studies have focused on the characterization of various *E. coli* acid resistant phenotypes under different experimental conditions. Such research has included a variety of acidulants, temperatures, media, pH ranges as well as growth and adaptation stages (Cheville *et al.*, 1996; Jordan & Davies, 2001; Lekkas *et al.*, 2006; Yuk & Marshall, 2005). It has been found that different stages of growth affect acid resistance regardless of nutrient availability. Most acid resistance genes are induced in the later stages of stationary phase (Arnold & Kaspar, 1995). Consequently, cells are more sensitive to acid at lag and exponential phases than at stationary phase. In addition, it has been demonstrated that acid adapted *E. coli* cells possess a greater resistance to acid than their non-adapted counterparts (Buchanan & Edelson, 1999a; Leyer *et al.*, 1995). Acid adaptation is usually obtained by exposing *E. coli* cells to a mild acid stress or by growing the cells in the presence of fermentable carbohydrates. Buchanan and Edelson (1996) demonstrated that enterohemorrhagic *E. coli* cells grown in (TSB) supplemented with 1% glucose

showed a substantial increase in survival rates when compared to cells grown in the absence of glucose. The studies of Layer *et al* (Leyer *et al.*, 1995), showed that acid-adapted *E. coli* O157:H7 cells had enhanced resistance to a lower pH and persisted longer in dried fermented salami. It is also known that the presence of certain nutrients in the growth media and the challenge media have an effect on the acid resistant phenotypes of *E. coli* cells (Hersh *et al.*, 1996; Iyer *et al.*, 2003; Richard & Foster, 2004). To date at least 4 different *E. coli* acid resistance systems, AR1, AR2, AR3 and AR4, have been characterized. AR1 is stationary-phase-dependent and requires the presence of glucose but not of amino acids in the media (Castanie-Cornet & Foster, 2001a; Gong *et al.*, 2003; Iyer *et al.*, 2003; Lin *et al.*, 1996). The 3 remaining systems however require the presence of glutamate (AR2), arginine (AR3) and lysine (AR4) and are induced during growth (Gong *et al.*, 2003; Iyer *et al.*, 2003; Lin *et al.*, 1995).

Recent studies have now confirmed that *E. coli* survival in acidic environments can be positively influenced by the presence of sodium ions (Na+) (Casey & Condon, 2002; Chapman *et al.*, 2006). In contrast to previous reports, these studies have shown that the presence of Na+ in concentrations between 2% and 4% can enhance *E. coli* acid resistance (Casey & Condon, 2002; Chapman *et al.*, 2006; Jordan & Davies, 2001). In another study it was The work of demonstrated that reduced levels of extracellular Na+ in the growth media suppressed the expression of the gadE, gadA, gadBC ; genes involved in the glutamate-dependant acid resistance system, AR2 (Gong *et al.*, 2003; Richard & Foster, 2007).

To the best of our knowledge, the type of challenge media used in acid challenge studies is not considered to be an important factor. Although gross differences such as nutrient and no-nutrient are taken in consideration, the effect of minor differences such as buffer content or nutrient type is often ignored. In this study, 4 of the most commonly used acid challenge media were investigated. Each medium was selected to ensure that the study included a range of media with variation in specific nutrient composition and concentration as well as buffering components. Three treatments (A, B and C) were selected to cover the variety of ways in which previously reported acid challenge systems have been designed. Treatment A represented an assay where uniform pH values in different media were compared. For this assay each challenge media required different concentrations of lactic acid. On the other hand, for treatment B, acid molarity was kept uniform, resulting in each medium having a unique pH. Treatment C represented an *in vitro* challenge assay where molarity and pH were uniform in all media. Uniformity in pH values was obtained by adjusting the media using NaOH or HCl. As salt is frequently used in combination with acid in food processing and preservation regimes, this study also investigated the effect of varying concentrations of NaCl (0.5, 3.5, 8.5 and 12.5% w/v) in media acidified with lactic acid to pH4.5 on the survival of each of the test strains.

5.2. Materials and Methods

5.2.1. Strains and inoculum preparation

Twelve *E. coli* strains comprised of 4 pathogenic O157:H7 strains, 5 non-pathogenic Australian salami validation strains (Duffy *et al.*, 2000), 1 uncharacterized non-pathogenic *E. coli* isolated from a salami plant and 2 *E. coli* K12 control strains were used throughout this study (Table 5.1).

All strains were recovered from storage at -80C° by placing one Protect™ bead (TSC-Technical Service Consultants) into 10 ml of TSB (Oxoid – CM0876) and incubated at 37 C° for 24 h with shaking. Cultures were streaked on to Tryptic Soy Agar (TSA, Oxoid – CM0131) supplemented with 0.2% glucose (Sigma – G7528) and 0.2% yeast extract (Oxoid – LP0021) (TYSG) and incubated at 37 C° for 24 h. Strains were subsequently grown under the same conditions and re-streaked prior to storage at 4 C° (working cultures). Working cultures were kept for the length of the experiment but were not stored for longer than six weeks.

Prior to acid adaptation strains were grown in TSB to stationary phase (24 h±1 h) at 37°C with shaking at 200 RPM. Acid adaptation was performed for all strains prior to any challenge. Strains were acid adapted by inoculating 20 µl of the overnight TSB cultures in 200 ml of TSB+1% glucose and incubating them for 24 h±0.2h at 37°C with shaking at 200 RPM. Two milliliters of each culture were then centrifuged at 3500 RPM for 10 min at 37°C and the supernatant was discarded. Pellets were then resuspended in 2ml of each challenge media at 37°C.

5.2.2. Broth preparations, acid and acid- salt challenge studies

The broths used in this study were; Brain Heart Infusion (BHI, Oxoid – CM1032), TSB, Nutrient Broth (NB, Oxoid – CM0501) and Davis Minimal Media (DMM, Bacto Laboratories – 275610). All media were prepared as specified by the supplier. NaCl (Sigma- S 3014) was prepared as a sterile 5 M solution and L-lactic acid (80% solution, Sigma - 27715) were added as appropriate after sterilization (120°C, 140kPa for 15min) to prevent variations caused by autoclaving.

Three treatments (A, B, C) were designed to study the effects of pH and lactic acid concentration on bacterial survival in each of the four media. For treatment A, lactic acid was added at different concentrations until each media reached a pH of 4.5 ± 0.2 (WP80 pH meter, TPS, Australia). For treatment B, lactic acid was added to each media to a final concentration of 34.7 mM and the pH values were recorded. For treatment C, lactic acid was also added to a each media to a final concentration of 34.7 mM, however the pH of each media was adjusted using either HCl or NaOH until each media reached pH 4.5±0.2.

To study the effects of different salt concentrations in addition to pH, lactic acid was added to reach pH 4.5 ± 0.2 as for treatment A. NaCl was then added at four different concentrations; 0.5 %, 3.5 %, 8.5% and 12.5% (w/v). The pH was monitored following the addition of NaCl. Changes in pH due to the addition of NaCl Were counteracted by the addition of HCl or KOH until the target pH was achieved.

5.2.3. Serial dilutions and enumeration

Enumerations were performed by counting the number of colony forming units on TYSG plates. Serial dilutions were made in 0.085% (w/v) saline solution using 96-deep well plates (Nunc T969799901) and an automatic 12-channel multi-channel Pippettor (Eppendorf). One hundred microliters of each dilution were then plated on solidified semi-dried TYSG agar. All agar plates were stored for a maximum period of 72 h and surface-dried for a maximum period of 25 min prior to inoculation. Inoculums were spread using sterile-disposable spreaders (Sarstedt - 86.1569.005). The plates were incubated for approximately 24 h at 37°C and a further 24 h at room temperature if the colony size had not developed sufficiently for accurate enumeration.

5.2.4 Statistical analysis

Data was analyzed using Minitab® 14 statistical software (Minitab Inc. Mineapolis, MN, USA). Significant differences of the means of average survivors were determined using one-way analysis of variance. A p value of < 0.05 was regarded as statistically significant. All further references to significant difference indicate $p < 0.05$.

Table 5.1. Panel of 12 *E. coli* strains used in this study

Strain	CSIRO FSA Designation	Serotype	Source
1	EC1591	O157:H7	Industry isolate, US, Doyle
2	EC1592	O157:H7	Human Isolate, US, Doyle
3	EC1596	O157:H7	ATCC 43895
4	EC1608	Unknown	Salami challenge strain (Duffy *et al.*, 2000)
5	EC2935	Unknown	Salami challenge strain
6	EC2936	Unknown	Salami challenge strain
7	EC2937	Unknown	Salami challenge strain
8	EC2938	Unknown	Salami challenge strain
9	EC2940	OR:H48K-12	MG1655
10	EC2941	O157:H7	ATCC BAA-460, Carlton Gyles
11	EC2971	K-12	M534, Tom Ferenci
12	Sal4	Unknown	Persistent isolate from an Australian salami factory

Table 5.2. Lactic acid concentrations and pH values for treatments A, B and C for the panel of 12 *E. coli* strains challenged in four different media.

Medium	**pH (L-lactic acid concentration) Treatment conditions:**		
	A	B	C
Brain heart infusion broth	pH 4.5 (35.5 mM)	pH 4.7 (34.6 mM)	pH 4.5 (34.6 mM)
Trypticase soy broth	pH 4.5 (32.1 mM)	pH 4.2 (34.6 mM)	pH 4.5 (34.6 mM)
Nutrient broth	pH 4.5 (4.4 mM)	pH 2.9 (34.6 mM)	pH 4.5 (34.6 mM)
Minimal medium	pH 4.5 (34.6 mM)	pH 4.8 (34.6 mM)	pH 4.5 (34.6 mM)

5.3. Results

5.3.1. Effects of acid concentration and pH on cell survival

For treatment A, when lactic acid was added to each media to reach pH 4.5, variations in the lactic acid concentrations were observed (Table 5.2). The lowest value was for NB (4.4 mM) and the highest for MM (37.22 mM). The average survival for each strain varied depending on the challenge medium (Fig 5.1A). When challenged in NB, 7 strains [2, 3, 4, 5, 9, 10 and 11, Table 5.1] showed significantly higher survival rates than when challenged in BHI and TSB. There were no significant differences in survival rates between strains 7 and 12 when challenged in BHI and NB. In addition, there were no significant differences in survival rates for strains 1, 6 and 7 when challenged in TSB and NB. Comparisons between strains challenged in BHI and TSB, revealed that 7 strains [2, 3, 4, 5, 9, 10 and 12] had significantly higher survival rates in BHI than those in TSB. Only 2 strains (1 and 6) showed significantly higher survival rates when challenged in TSB than in BHI. No survivors were found for 4 strains [2, 3, 9 and 11] challenged in TSB and 2 strains [2 and 11] for cells challenged in BHI. Under these conditions (pH 4.5) no survivors were found for any strain when cells where challenged in MM.

For treatment B, when lactic acid was added to each media at a final concentration of 34.6 mM a range of variations in pH value and survival rates were observed (Fig 5.1B). The highest pH value was found for MM at 4.76 and the lowest for NB at 2.9 (Table 5.2). In terms of survival, similarly to treatment A, there were no survivors

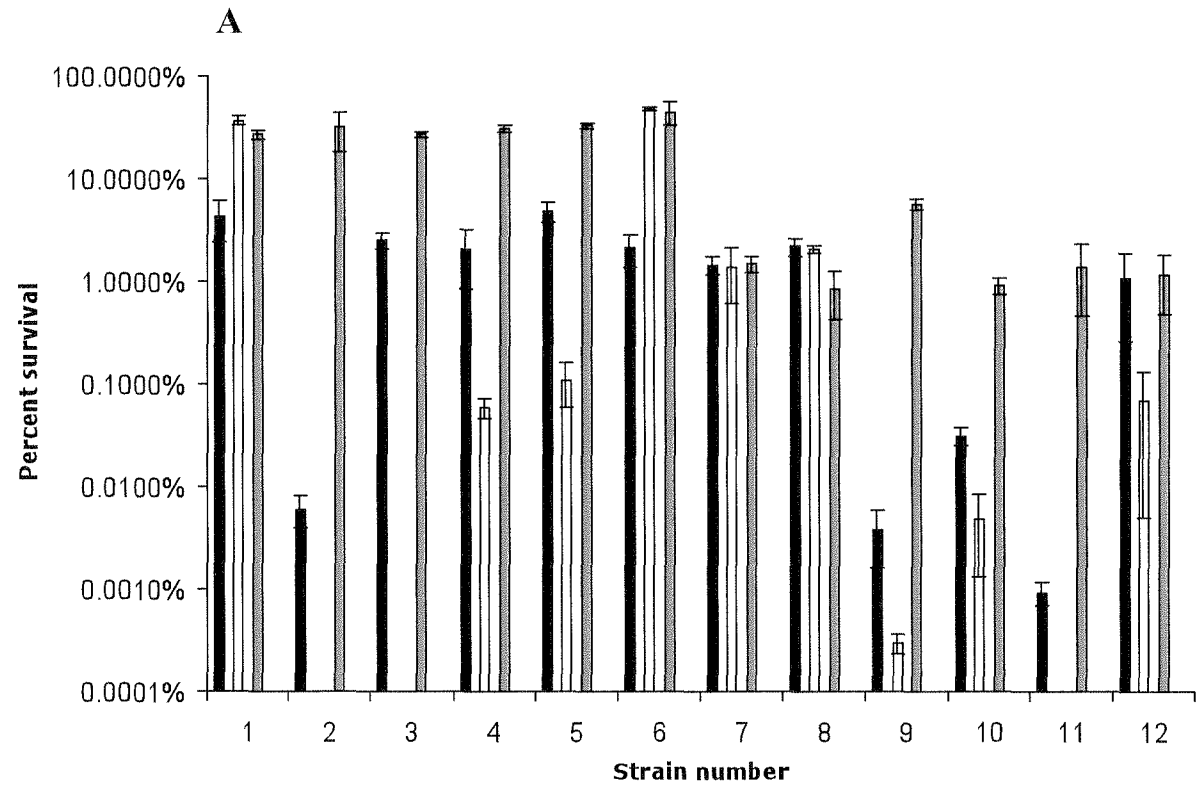
when cells were challenged in MM. However, there were also no survivors for any of the strains challenged in NB. For strains challenged in TSB, survivors were found for 7 strains [1, 4, 5, 6, 7, 8 and 12], all of which had significantly lower survival rates than the strains challenged in BHI. Survivors for all strains were found for cells challenged in BHI. Interestingly when compared to treatment A, less variation in the survival rates between strains challenged in BHI were observed. In this media only 3 strains [9, 10 and 11] appeared to have significantly lower survival rates than the rest of the population.

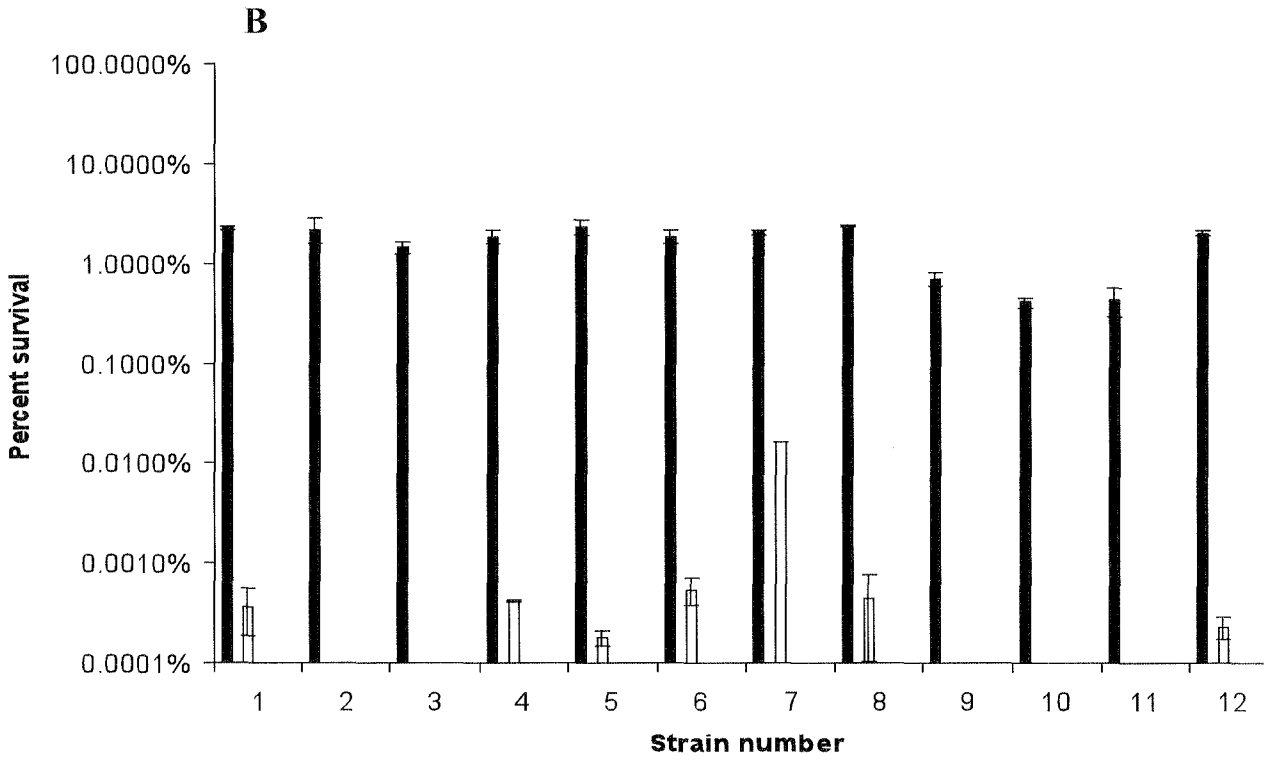
For treatment C, the lactic acid concentration of each media was kept constant at 34.64 mM and the pH was adjusted to 4.5±0.2 using either NaOH or HCl (Table 5.2). As observed in treatments A and B, there were no survivors for any of the strains challenged in MM (Fig 5.1C). For cells challenged in TSB, for most strains, the average percent survivors were higher and less variable than for those observed in treatments A and B. Comparisons between BHI and TSB revealed that 3 strains [4, 5 and 12] showed significantly higher average survival values for cells challenged in TSB. In addition, 6 strains [1, 2, 3, 6, 7 and 8] were found not to be significantly different between TSB and BHI. Strains 9, 10 and 11 showed significantly higher average survivors when challenged in BHI than in TSB. No survivors were found for strains 3, 9, 10 and 11 when challenged in NB. Most of the remaining strains challenged in NB showed higher survival rates than in treatment B and lower survival rates than in treatment A.

5.3.2. Effect of salt concentration at pH 4.5 with varied lactic acid concentrations

Results for the average percent survivors at 0.5% NaCl were the same as those for treatment A. However, for convenience, they are also summarized in Figure 5.2A. At 3.5% NaCl, similar to previous results (treatments A, B and C) there were no survivors for cells challenged in MM. At this NaCl concentration, 9 strains [1, 2, 3, 4, 5, 6, 9, 10 and 11] showed significantly higher percent survivors when challenged in NB than any other media. Strain 12 showed significantly lower average percent survivors when challenged in NB than in BHI. Four strains [1, 3, 5 and 11] showed significantly higher survival rates when challenged in BHI when compared to TSB. On the other hand only 1 strain [9] showed significantly higher survival rates when challenged in TSB than in BHI. No significant differences in survival rates were observed for strains 2, 4, 6, 7, 8 and 12 when challenged in BHI and TSB (Fig 5.2B). At 8.5% NaCl, no survivors were detected for any of the strains challenged in MM and TSB. When compared to the previous treatments, substantial decreases in percent survivors for strains challenged in BHI and NB were observed. No survivor cells were found for strains 3, 5, 8, 9 and 10 when challenged in BHI. Similarly, strains 3 and 10 failed to survive when challenged in NB. Strains 4, 5, 7, 8, 9, 11 and 12 showed significantly higher average percent surviving cells when challenged in NB than in BHI. Only strain 1 showed higher percent surviving cells when challenged in BHI than in NB (Fig 5.2C).

At 12.5% NaCl, survivor cells were found for only four strains [1, 2, 6 and 7] when challenged in BHI. No survivors were found for any other strains or in the 3 alternative challenge media (Fig 5.2D).


A
100.0000%
10.0000%
1.0000%
0.1000%
0.0100%
0.0010%
0.0001%
Percent survival
1
2
3
4
5
6
7
8
9
10
11
12
Strain number


B
100.0000%
10.0000%
1.0000%
0.1000%
0.0100%
0.0010%
0.0001%
Percent survival
1
2
3
4
5
6
7
8
9
10
11
12
Strain number

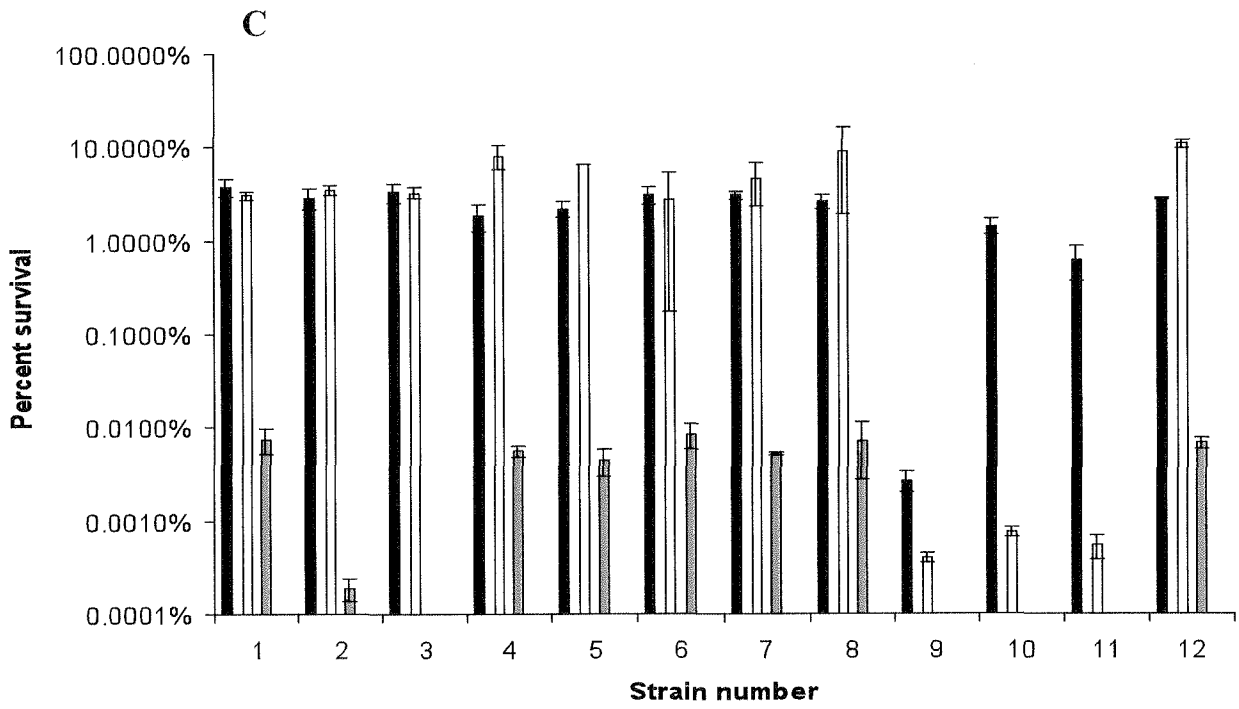
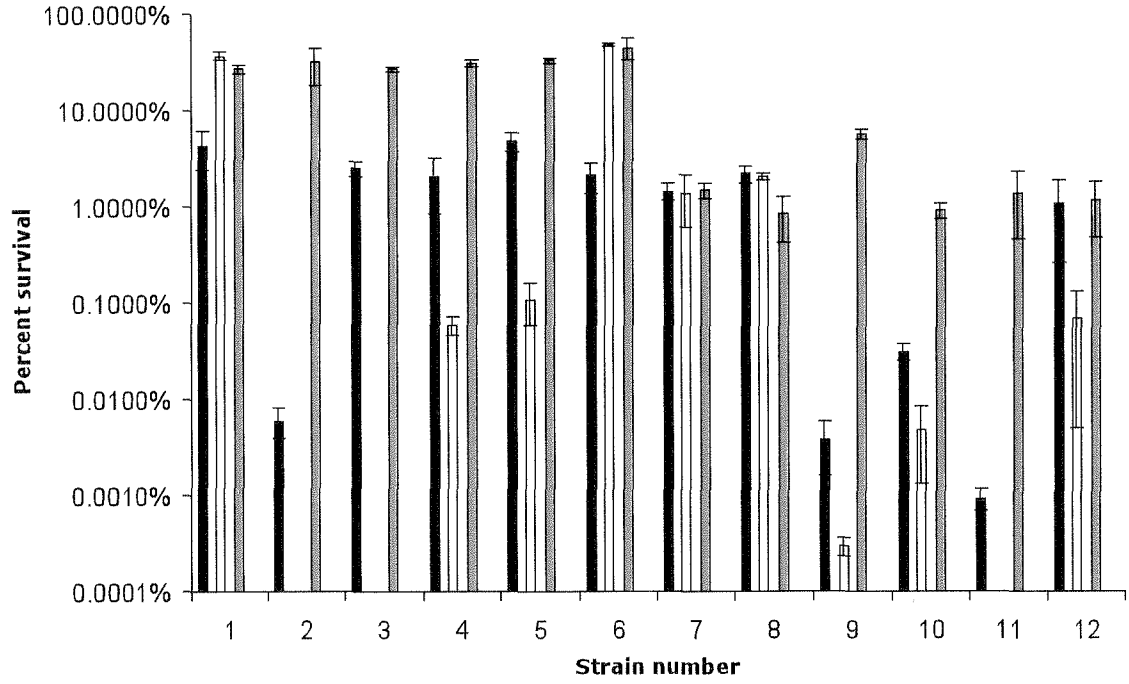


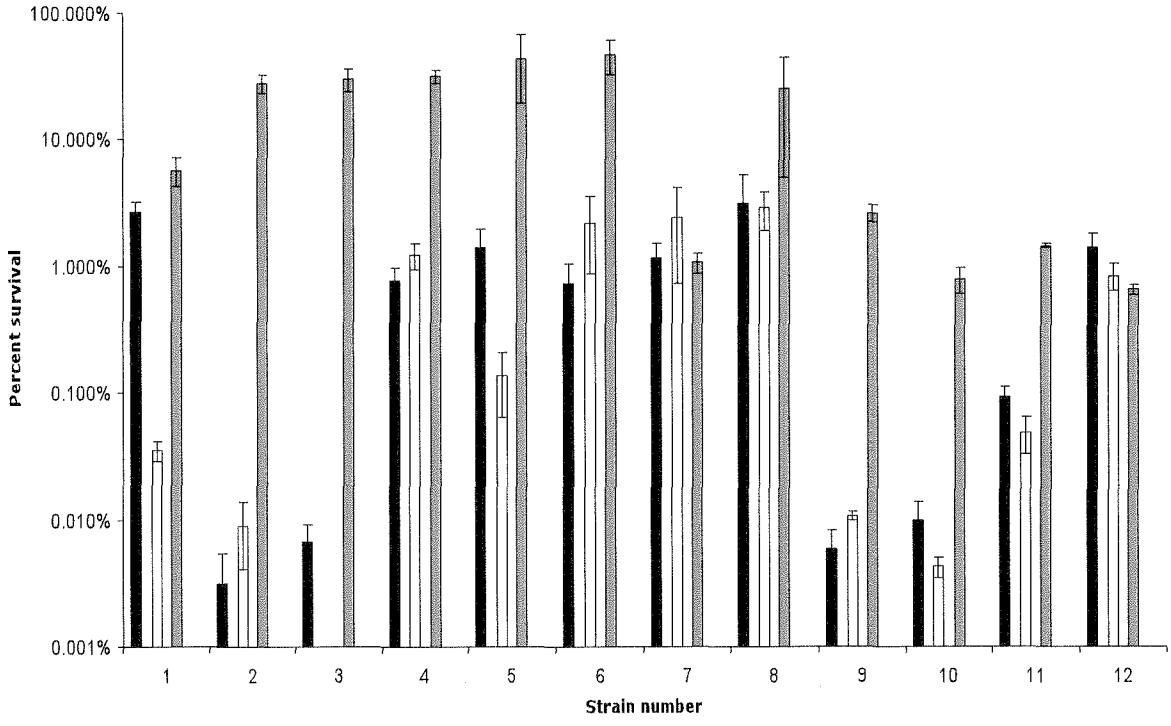
Figure 5.1. Media associated survival charts for different lactic acid concentrations

Percent survival charts for the panel of twelve *E. coli* strains challenged in four different media; BHI (■), TSB (□), NB (▒) and MM (■), under three different treatments. Graphs refer to treatment conditions A, B, C in which pH levels and lactic acid concentration vary, please refer to Table 5.2. For treatment A the acid challenge involved a L-lactic acid adjustment to pH 4.5. In Treatment B 34.6 mM lactic acid was added to each medium with the pH unadjusted. For treatment C the amount of lactic acid added was 34.6 mM and the pH was adjusted to pH 4.5 with HCl/NaOH as required.

A


100.0000%
10.0000%
1.0000%
0.1000%
0.0100%
0.0010%
0.0001%
Percent survival
1
2
3
4
5
6
7
8
9
10
11
12
Strain number

B


100.000%
10.000%
1.000%
0.100%
0.010%
0.001%
Percent survival
1
2
3
4
5
6
7
8
9
10
11
12
Strain number

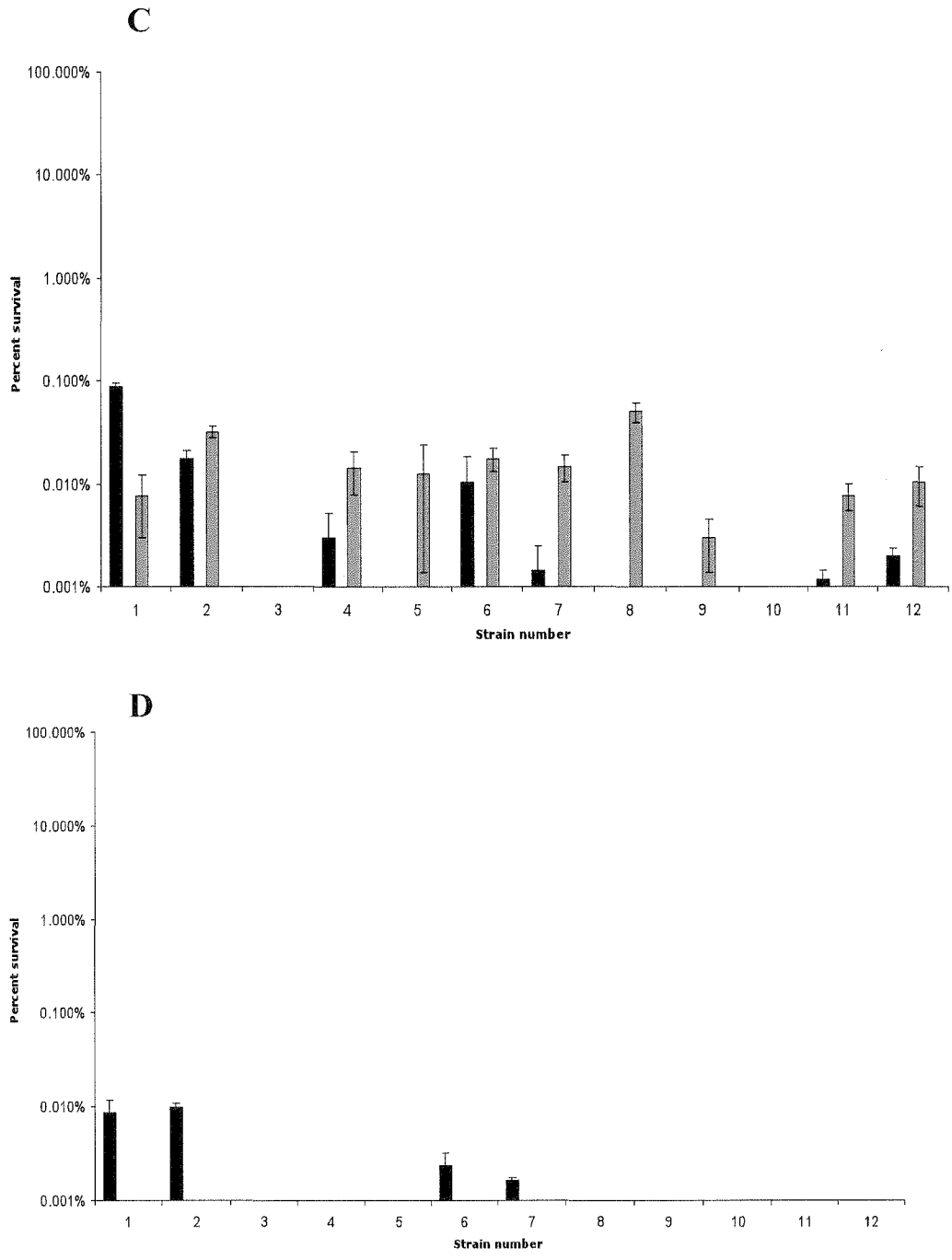


Figure 5.2. Percent survival charts for the panel of 12 *E. coli* strains challenged with treatment A supplemented with 0.5% (A), 3.5% (B), 8.5% (C), 12.5% (D) NaCl in four media; BHI (■), TSB (□), NB (▒) and MM (■).

5.4. Discussion

Studies revealed that each of the 4 media had a particular influence on the amount of lactic acid used to reach pH 4.5 and the pH values that resulted from using 34.6 mM of lactic acid (Table 5.2). Treatment A represented an acid challenge which determines the effects on *E. coli* survival using lactic acid to reach a given pH value of 4.5. Treatment B represented an acid challenge which determines the effects on *E. coli* survival using a given lactic acid concentration of 34.6 mM. The average percent survivors for the panel of 12 *E. coli* strains varied for each different media for both treatments. On the bases of pH values, treatment A, it can be concluded that NB is the more advantageous media for acid challenge for the panel of *E. coli* strains. This conclusion is equivocal as the lactic acid concentration of NB was lower than for any other media (Table 5). Other studies have previously demonstrated that the higher the concentration of organic acid in solution the stronger the inactivation efficacy of acid challenge (Ma *et al.*, 2002). Based on the results of treatment B (Figure 5, panel B), it can be concluded that BHI is more advantageous than any of the media tested as it offered the most protection for all the strains. This conclusion is also equivocal as BHI had higher pH values than those of NB and TSB (Table 5). Many studies have demonstrated that there is a correlation between the low pH values and *E. coli* inactivation efficacy of acid challenge (Buchanan & Edelson, 1999a; Buchanan & Edelson, 1999b). On a strain specific level, it was observed that the magnitude of each treatment varied depending on the challenge media. The magnitude of treatment A was very similar for strains 7 and 8 when challenged in NB, BHI and TSB. On the other hand, the magnitude of treatment A was different for strains 2, 3, 4, 5, 9, 10 and

11 across all media. These results suggest that the type of media had some influence in the acid resistance phenotypes of individual strains.

Average survivor values for treatment C, demonstrated that the constituents of each challenge media have a direct influence on the acid survival of the panel of 12 strains. It was observed that, there were no major differences in the magnitude of acid challenge for most strains (except strains 9, 10, 11) challenged in BHI and TSB. The magnitude of acid challenge was more pronounced for strains challenged in NB. These results suggest that nutrient content had a positive influence in acid survival as the listed nutrient composition more extensive for TSB and BHI than for NB and MM (no organic nutrients). It is known that the presence of glutamine or arginine, and to a lesser extent lysine, can influence the outcome of the acid challenge by inducing the acid resistant pathways AR2, AR3 and AR4 (Castanie-Cornet *et al.*, 1999; Lin *et al.*, 1995; Richard & Foster, 2007). Glutamate decarboxylase (AR2) is capable of raising the internal pH through the conversion of glutamate into GABA (/γ-aminobutyric acid) by consuming protons and releasing CO_2. The observed enhanced protection seen for cells challenged in BHI and TSB compared with the other media for treatment C in particular, may be due to a greater content of glutamate or other amino acids.

NB offers poor buffering capacity, whereas BHI and TSB contain 2.5 g/liter of disodium hydrogen phosphate and 2.5 g/liter of di-potassium hydrogen phosphate, respectively. MM contained 7 g/liter of dipotassium phosphate and 2 g/liter of monopotassium phosphate. A correlation between buffering composition, pH values and lactic acid concentration was observed. At similar lactic acid concentrations, both

the quantity and type of buffer influenced final pH values (as seen in treatment B; Table 5.2) thus resulting in different levels of unprotonated lactic acid that can diffuse into the cell and cause inhibition of growth due to cytoplasm acidification. Lactic acid is a considered a monoacid, a type of acid commonly referred as weak acid (Anderson, 1987). The tendency of monoacids to dissociate is directly related to the pH of the media (Anderson, 1987). The lower the pH, the higher the amount of unprotonated lactic acid in solution and the greater the bacteriocidal effect is (Anderson, 1987; Cardenas, 2008; Hammes & Tichaczek, 1994). Since the pKa of L-lactic acid is 3.87 unprotonated concentrations were mostly at about 7 mM.

In the case of NB, with no buffering capacity, for Treatment B and C, survival was abolished and severely reduced due to the combined low pH and the respective high proportion of unprotonated lactic acid (equal to >33 mM). In turn, the high buffering capacity of the medium in the case of MM offered no protection to the cells due to the lack of available nutrients, e.g. glutamate, arginine, lysine, compatible solutes.

The supplementation of salt to treatment A resulted in variation in the average survival pattern for each individual strain across each treatment and each challenge medium. The results observed in this study link the presence of Na^+ and Cl^- ions and its interactions with the challenge media to differences in acid resistance phenotypes across the panel of *E. coli* strains. In contrast to what was expected (increased acid sensitivity with increased NaCl concentrations) it was found that the supplementation of 3.5% NaCl to each of the media did not result in a general reduction of acid resistance for the panel of strains. Rather it was found that the addition of 3.5% NaCl to NB and TSB increased the acid resistance of some strains, while the survival for

cells challenged in BHI was not dramatically altered. At concentrations of 8.5% and 12.5% NaCl, BHI and NB appeared to be advantageous for some strains (Figure 5.2, panels C and D). Perhaps the high nutrient content played a protective role against osmotic stress, for example through provision of compatible solutes (Warnecke & Gill, 2005).

Other authors have observed similar findings, where the presence of NaCl at concentrations between 2 to 6% enabled enhanced survival, recovery and growth of *E. coli* when subjected to various acid challenges (Casey & Condon, 2002; Chapman *et al.*, 2006; Jordan & Davies, 2001). The work of Richard and Foster (Richard & Foster, 2007) revealed that cells challenged in media containing low Na+ and high K+ concentrations were 80% less resistant to acid than cells challenged in high Na+ and low K+. Their work suggests that high Na+ levels are involved in inducing the gadA/BC genes involved in the characterized acid resistance system (AR2). Of relevance to this study are the findings by Jordan and Davies (Jordan & Davies, 2001) and Casey and Condon (Casey & Condon, 2002) that pathogenic *E. coli* demonstrate enhanced growth and survival during acid treatment when challenged in tryptone based media. In the present study acid resistance in the presence of salt was observed for some strains challenged in TSB but not in BHI.

While we have observed that varying conditions of acid concentration, pH, buffer and nutrient composition influences the acid resistance profiles of cultures, the nature of our experimental design has not permitted for the accurate quantification of the influence of each individual parameter. This is because, each media varied in the amount of nutrient and buffer content, varying the individual effects on pH and *E. coli*

nutrient availability. An experimental approach that would determine the individual effects of nutrient availability and buffering capacity could be design by preparing a challenge media in the laboratory so that the concentration amounts and content of the challenge media could be adjusted on an individual basis. For example, mimicking the components of TSB and studying the effects on *E. coli* survival when changing one variable at time (i.e. buffer composition).

The results from this experimental work demonstrated that the presence of NaCl has a direct and pronounced effect on the *E. coli* acid resistance phenotype. In general it was observed that for strains challenged in NB and to a lesser extent in TSB, the presence of NaCl at 3.5% resulted advantageous for acid survival when compared to those challenged in media with 0.5% NaCl. The effects of the presence of NaCl at a genetic level on acid adapted *E. coli* cultures to acid challenge were studied in the next chapter.

6. Chapter 6 Responses of *E. coli* O157:H7 Sakai following lactic acid challenge with and without NaCl supplementation as determined using transcriptomic analysis

"It is never possible to predict a physical occurrence with unlimited precision"

Max Plank Ernst (1858-1947)

CHAPTER PREFACE

In this chapter microarrays were used to analyze the mRNA transcripts of stationary phase cultures prior to and following the challenge of two treatments; lactic acid challenge at pH 4.5 and lactic acid challenge at pH 4.5 supplemented with 3.5% NaCl. The experimental parameters for this study were based on the fermentation stage of salami manufacturing where the final average pH reaches 4.5 and the salt concentration is roughly 3.5%. Previous published data as well as data within this thesis (previous chapter in particular) had suggested that the presence of salt at 3.5% might be advantageous to acid challenge. We were therefore also interested in the particular genes that might be involved in contributing to this enhanced acid resistance. Interestingly we found that a considerable amount of the up-regulation of genes for the stationary phase cultures specifically occurs following challenge with lactic acid conditions and also under acidic conditions in which media were supplemented with 3.5% salt.

The concentration of lactic acid used for analyzing the acid stress response in the presence or absence of NaCl was 35mM. This concentration is based on the challenge studies from previous experiments. In addition, when added to TSB and BHI it results in a final pH concentration of approximately 4.5 reducing the need to use excessive amounts of HCL or NaOH to adjust the media to the target pH, 4.5. The use of a much lower concentration was considered as lactic acid at this concentration can result in very quick inactivation of a large population of the challenge cultures. However, based on the results from previous chapters it was inferred that an hour exposure at pH 4.5 did not eventuate in large reductions of the population for most of

the challenged cultures; yet it would be sufficient to evoke a significant response that could be studied using microarray techniques.

ABSTRACT

The magnitude of acid resistance of *E. coli* O157:H7 Sakai (ATCC BAA-460) cultures under laboratory conditions can be influenced by nutrient availability and stage of growth. In this study, transcriptomes of stationary phase *E. coli* O157:H7 Sakai cultures grown in TSB + 1% glucose were analysed following challenge with 35 mM lactic acid at pH 4.5 and also the same acidic conditions including 3.5% NaCl in TSB. From comparisons of the acid-adapted stationary phase cultures with log phase controls increased expression of key acid resistance genes such as *asr*, *gadB* and *gadC* was observed. Almost 80% of the total down-regulated genes were shared between the stationary phase cultures prior to and following both challenges (523 genes). A total of 5 and 30 genes were exclusively down- and up-regulated for cultures challenged with lactic acid alone, for cultures challenged with lactic acid supplemented with salt the numbers of genes with altered expression were 62 and 33, respectively. A total of 17 and 36 genes were observed to be down- and up-regulated for cultures challenged with both treatments. The vast majority of genes (60%) found to be up-regulated for the acid adapted and acid challenged cultures were of bacteriophage origin. The exposure to acid challenge in the presence and absence of NaCl essentially promulgates the expression of prophage genes overall, potentially suggesting induction of lysogeny. Since phage genes also include possible virulence factors the results also suggest that the ability of acid adapted stationary phase *E. coli* O157:H7 Sakai cultures virulence could be also being further enhanced when exposed to lactic acid challenges.

6.1. Introduction

Non-thermal inactivation of *Escherichia coli* (*E. coli*) O157:H7 strains is essential for the safe manufacture of dry fermented food products. Dry fermented food products such as salami, are considered ready to eat and are not cooked before consumption. Pathogen inactivation and preservation in fermented food products is achieved by fermentation and drying. These processes create an inhabitable environment for most pathogenic bacteria and other food spoilage microorganisms by reductions in pH and water activity, coupled with the effects of NaCl and other curing agents. The presence of lactic acid, the by-product of lactic acid bacteria, and that of NaCl in conjunction with the drying process; are believed to be the major stressors encountered by the unwanted microbes. The inactivation of *E. coli* O157:H7 is believed to mainly occur due the synergistic effect of acid and osmotic stresses (Metaxopoulos *et al.*, 1981; Pidcock *et al.*, 2002).

In recent decades, pathogenic *E. coli* has emerged as an important food pathogen. To date food associated disease involving *E. coli* O157:H7 in dry fermented food products has been documented (Duffy *et al.*, 2000; Leyer *et al.*, 1995; Nightingale *et al.*, 2006). It is believed that the particular characteristics of *E. coli* O157:H7 strains to resist environmental stresses, together with the manufacturing methods of fermented food products makes them suitable vehicles of infection (Tilden *et al.*, 1996). Information regarding the ability of *E. coli* O157:H7 to resist an acid challenge in the presence of NaCl is of relevance to dry fermented food products manufacturing.

Research has identified the highly efficient mechanisms used by *E. coli* O157:H7 to resist environmental stresses (Lin *et al.*, 1996). Of particular importance is its intrinsic ability to resists acid stress. At least four acid resistance mechanisms have been characterized (Castanie-Cornet *et al.*, 1999; Castanie-Cornet & Foster, 2001b; Price *et al.*, 2004). In recent years the presence of NaCl in the immediate environment has been found to play a role in the ability of *E. coli* to resist acid challenge (Chapman *et al.*, 2006; Jordan & Davies, 2001). In the work of Jordan and Davies, the presence of 4 to 6% NaCl proved advantageous for the growth and recovery of acid-stressed *E. coli* O157:H7 cultures. In the work of Chapman *et al* (2006), increased NaCl concentrations were associated with increased survival of *E. coli* 0157H:7 cultures in model acidic sauces. Transcriptome analysis demonstrated genetic expression overlap between osmotic and acid stress in *E. coli* cultures (Weber *et al.*, 2005); suggesting that the presence of NaCl might play a protective role in acid resistance. In the work of H. Richard and J. W. Foster, the presence of sodium was found to play an important role in the expression of gadA/BC, genes involved in *E. coli* acid resistance (Castanie-Cornet & Foster, 2001a; Richard & Foster, 2007).

In the present study, mRNA transcripts of acid-adapted stationary phase *E. coli* O157:H7 Sakai cultures challenged with lactic acid alone (pH 4.5) and acid supplemented with 3.5% NaCl (pH 4.5) analyzed. It was hypothesized that the presence of NaCl during acid challenge of O157:H7 Sakai would evoke a larger and/or different gene expression than when challenged with acid alone. It was further hypothesized that some of the genes expressed under the NaCl and acid treatment would be associated to those expressed under the treatment of acid alone. Analysis revealed altered expression of over 1900 genes following acid adaptation and entry

into stationary phase. The challenge of stationary phase cultures with lactic acid (pH 4.5) revealed the increased expression of over 45 genes. Supplementation with 3.5%NaCl to the lactic acid challenge (pH 4.5) revealed the increased expression of over 300 genes. The findings herein suggest that the presence of NaCl in an acid challenge might be advantageous for the survival of *E. coli* cultures.

6.2. Materials and Methods

6.2.1. *E. coli* Sakai strain

The *E. coli* strain used in this study wasO157:H7 Sakai. This strain was first isolated from the Sakai outbreak in Japan (Michino *et al.*, 1999) and was obtained from the American Type Culture Collection (ATCC BAA-460). Prior to each experiment the strain was recovered in triplicate from −80°C storage by aseptically placing one Protect Bead (brand) in 10 ml of Tryptone Soy Broth (TSB). The cultures were then incubated in a 37°C water bath with shaking for 22 hrs. A loop full of the cultures was then streaked onto Tryptone Soy Agar (TSA) and incubated for 22 h at 37°C. Following incubation, a single colony was inoculated into 10ml of TSB and incubated in a 37°C water bath for a further 22 h. These cultures were then categorized as working cultures.

6.3. Growth and acid challenge conditions

Two types of growing media were used throughout the experiment, TSB and TSB supplemented with 1% Glucose (TSB + G). TSB was used for growing the non-acid adapted cultures and for subsequent acid treatments. TSB + G was used for growing the acid adapted cultures. The presence of glucose in the growing media has been demonstrated to offer greater resistance to acid challenge when compared with cells grown without it (Buchanan & Edelson, 1999a; Duffy *et al.*, 2000). Two acid treatments were used in this experiment to challenge the stationary phase cultures. Treatment A consisted of TSB supplemented with 35mM of lactic acid. Treatment B

consisted of TSB supplemented with 35 mM lactic acid and 3.5% NaCl. To counteract the effects of NaCl on pH, KOH was added until pH 4.5 was reached.

For non-acid adapted cultures, 20 µl of the working cultures were inoculated into 50 ml of TSB. For acid adapted cultures, 20 µl of the working cultures were inoculated into 50 ml of TSB + 1% glucose. Cultures where incubated in a 37°C water bath with shaking for a period of 24 h. Absorbance readings were taken using (Pharmacia LKB, Novaspec II) every 30 min for the first 8 h, every 1 h for the following 4 h, and every 2 h for the remaining period. Absorbance measurements were recorded in triplicate and the different stages of growth were graphed using Microsoft Excel (results not shown). Once the growth stages were determined two time points were selected, mid-log phase and late stationary phase. Cultures were considered to reach mid-log phase at 3.5 h and was well into the stationary phase after 17 h after inoculation.

Once cultures reached the stationary growth phase, 45 ml aliquots of each culture were transferred into sterile 50 ml centrifuge tubes and spun for 10 min at 5000 rpm at 37°C. The supernatants were decanted and the bacterial pellets were resuspended in 1ml pre-warmed solutions (37°C) of either treatment A or treatment B. Cultures were challenged for 1 hr in a 37°C water bath with shaking. Following the challenges 45 ml aliquots were transferred into 50ml sterile-centrifuge tubes for RNA extractions.
Note that length of the challenge was chosen using the data collected in Chapter 5. Briefly, it was found that an hour into the challenge was sufficient to expose the cultures to the media without having a significant inactivation state. For the most

sensitive strains, the recovery rate (in the majority of the challenges) begun to be affected between 2 and 3 hours into the challenge.

6.3.1. RNA extractions

RNA preparations were obtained in triplicate for cultures at mid-log phase and for the stationary-phase cells prior to and following challenge with treatments A and B. Immediately at time of harvest cultures were placed on ice-water and transferred into disposable-sterile 50 ml tubes (Sigma-Aldrich, CLS430290) and centrifuged at 4°C for 10 min. Supernatants were then discarded and the pellets were resuspended in 1ml of a chilled solution containing 50% TSB/50% RNA Protect bacterial reagent (QIAGEN Handbook 74524). Cultures were incubated on ice water in this solution for no longer than 15 min. Thereafter the pre-mixed cultures were centrifuged at 4°C for 5 min at 10000 rpm and the supernatant was decanted. The bacterial pellets were stored at -20°C until further processing. RNA for each sample was extracted using an RNeasy Mini kit (74104) and according to the RNeasy Handbook (74104). Briefly, samples were lysed using lysozyme (Biochemika, Sigma-Aldrich, 62970) for 30 min at 25 C, RNA was trapped in specialised columns while all other cellular components were discarded using a mixture of reagents and enzymatic reactions. Once RNA was isolated samples were then sent to the Australian Genome Research Facility (AGRF) in Melbourne Australia, for analysis and quantification. Once quantified RNA profiling was obtained using the Affymetrix GeneChip ® Instrument System with the *E. coli* 2 genome array. The arraying method has been previously described by Shabala *et al.* (2009).

6.3.2. Array Analysis

Data form AGRF was analysed using GeneSpring GX Software Ver.10 (Agilent Technologies, USA). Quality Control (QC) and downstream analysis of the raw data was conducted using GeneSpring GX 10.0 software. QC of the samples was conducted by assessing the: (i) distribution of normalized intensity values, (ii) 3'/5' ratios of internal controls, (iii) normalized signal values of hybridisation controls and (iv) principal Component Analysis (PCA) scores of replicates. Raw data was normalized using the RMA summarization method and samples below the 20th percentile of normalized intensity values were removed. Significance analysis was conducted using one-way analysis of variance (ANOVA) using the multiple corrections testing method of Benjamin-Hochberg with a p-value cut-off of <0.01. Elements were considered to be differentially expressed if the fold change was >2 as compared to the control (exponential phase) cultures. Expression Analysis based on GO Terms was performed using GeneSpring GX 10.0 with a p-value cut-off of <0.05 and <0.5. To supplement our analysis, other web interface databases such as the Encyclopedia of *Escherichia coli* K-12 Genes and Metabolism (EcoCyc) and EcoliHub (www.ecolicommunity.org) were used.

6.4. Results & Discussion

Extensive studies have investigated the effects of acid challenge with or without the supplementation of salt on *Escherichia coli* (Chapman *et al.*, 2006; Richard & Foster, 2007). In this study the primary objective was to investigate at a transcriptomic level the effects of acid challenge alone and acid supplemented with salt, on acid adapted *Escherichia coli* cells in late stationary phase. The aim of our experimental set up was to challenge cells with maximized expression of previously identified acid resistance genes with the aim of finding genes not previously identified to have involvement to the *E. coli* acid resistance response. Challenge was applied to late stationary phase cultures as it is known that many of the genes that offer protection against acid challenge are growth phase dependent and are expressed upon entry into stationary phase (Arnold & Kaspar, 1995; Berry *et al.*, 2004). Adaptation to acid challenge was achieved with the supplementation of 1% glucose to the growing media, as it has been proven to provide a protective effect on *E. coli* cells to acid challenge (Buchanan & Edelson, 1999a). This phenomenon was also checked in our laboratory prior to commencing the experiment (results not shown). The use of cells at a mid-exponential phase (log cells) as negative controls was due to their high acid sensitivity. Several publications have documented remarkable differences in acid survival between log and stationary phase cells (Arnold & Kaspar, 1995; Berry *et al.*, 2004). The supplementation of salt to the acid challenge was of interest as in certain food products the presence of salt is believed to be a complimentary hurdle for bacterial growth and inactivation (Glass *et al.*, 1992b). The selection of a mild acid challenge at

pH 4.5 was also utilized to mimic the pH of uncooked manufactured food products such as salami, which their safety does not rely on the use of thermal inactivation methods.

Transcriptomes for the stationary phase cultures prior to and following challenge with treatments A (acid only) and B (combined acid and NaCl) were all compared using the log phase cultures as the base control. Analysis revealed that, 799 significantly (twofold up- or down- regulated) expressed genes were observed upon entry into stationary phase. Following challenge with treatments A and B, 799 and 768 significantly expressed genes were respectively observed (Fig. 6.1). Of the total expressed genes for each treatment, the percentage ratio of significantly up- or down-regulated genes was very similar for cultures in stationary phase and those challenged with treatment A, with approximately 28% to 72% and respectively. The percentage ratio of down- or up- regulated genes for the stationary phase cultures challenged with treatment B was somewhat different with 20% up-regulated genes and 80% down-regulated.

Comparisons for the down-regulated genes using the log phase cultures as base controls, revealed that close to 80% of the down-regulated genes were found to be commonly expressed for the stationary phase cultures prior to and following challenge with both treatments. Moreover, the expression ratio (M) for all the commonly expressed genes did not vary substantially across all three conditions (Table 6.1). Only 13% of all down-regulated genes for all treatments were exclusively expressed following challenge with treatments A and B, with the majority corresponding to treatment B, ~9.4% (Figure 6.1). Only about 1.2% of the down-

regulated genes were exclusive to the acid challenge alone, suggesting that such challenge did not evoke a significant down-regulating response on the stationary phase cultures. In contrast the fact that most of the exclusively down-regulated genes were observed following challenge of treatment B, suggest that the addition of salt to the acid challenge evoked a somewhat stronger response. Similar findings were observed for up-regulated genes in the work of Weber *et al* (2004), where a higher number of *E. coli* genes were found to be significantly up-regulated following the osmotic challenge of cultures as compared with the acid challenge alone. This was not unexpected, as the presence of salt at 3.5% in an acid challenge has been proven to have a direct effect on *E. coli* survival (Casey & Condon, 2002; Chapman *et al.*, 2006; Jordan & Davies, 2001).

In contrast to what was observed for the down-regulated genes, there was a greater variation in expression of the up-regulated genes. Comparisons revealed that approximately 23% of the total up-regulated genes were shared for the stationary phase cultures prior to and following challenge with treatments A and B. About 23% of the genes were shared between the stationary phase cultures and those challenge with treatment A. About 9% and 10% of the up-regulated genes were exclusively up regulated for the stationary phase cultures challenged with treatments A and B respectively (Figure 6.1).

Specifically acid induced genes

Out of the 30 significantly identified genes that were found to be exclusively up-regulated for cultures challenged with treatment 58% were from prophage origin. Of the latter only 4 homologous genes were identified (*exoD*, *betW*, *kilW* and *ssbW*).

Only 8 genes were homologous to genes in other *E. coli* strains *csgB*, *prrA*, *rutB*, *ybcK*, *ybeQ*, *ycdI*, *ydeS* and *ydjE*. GO Term Analysis constructed using the GO Analysis tool of Gene Spring and web-interfaced databases such as EcoCyc, revealed that most of the specifically induced genes were involved in binding, transporter, hydrolase and catalytic activity. The genes *prrA* and *cgsB* are known to play a role in the *E. coli* outer membrane. In the work of Guyer *et al* (1998) *prrA* was found to be present in pathogenicity islands of uropathogenic *E. coli* strains and play a role in iron transport (Guyer *et al.*, 1998). Overexpression of the *csgB* gene in *E. coli* leads to the formation of *CgsB* polymers that assemble curli on the cell surface (Bian & Normark, 1997). In the work of Loh *et al* (2006), *rutB* was identified as part of a larger operon involved in a previously underscribed pyrimidine degradation pathway (Loh *et al.*, 2006).

Specific acid 3.5% NaCl induced genes

Out of the 33 significantly identified genes that were exclusively up-regulated for cultures challenged with treatment B, 63% were prophage genes (Figure 6.1). Only the name for the *ybcK* gene of prophage origin was identified. The remaining genes included homologs of *chaB*, *ptrB*, *sufA*, *ybjL*, *yeaI*, *yebB*, *yebE*, *yfiE*, and *yfiC*. Similarly to those expressed following the acid challenge alone (treatment B) GO Term Analysis revealed that most of the specifically induced genes were involved in binding, transporter, hydrolase and catalytic activity. In the work of Zheng *et al* (2001), *sufA* was within the 30 most significantly expressed genes of an *E. coli* wild type strain following challenge with hydrogen peroxide (Zheng *et al.*, 2001). The protein SufA (IscA) plays an important role in iron-sulfur cluster biosynthesis. SufA with other Fe-S cluster assembly proteins and assemble the Fe-S cluster of biotin

synthase BioB (Zheng *et al.*, 2001). In this study the gene *bioD* encoding debiotin synthase was up-regulated in all treatments, perhaps suggesting an important role of biotin against a variety of different stresses. The gene *ptrB*, encoding for a serine protease that hydrolyzes peptide bonds following arginine and lysine residues (Pacaud & Richaud, 1975). Other studies have demonstrated the involvement of arginine and lysine on the *E. coli* acid resistance mechanism (Castanie-Cornet *et al.*, 1999; Foster, 2004). The membrane associated genes *yebB* and *yeaI* could suggest changes in the cell envelope as a response to the acid-salt challenge.

Acid and acid +3.5 NaCl induced genes

Out of the identified up-regulated genes expressed for cultures challenges with treatments A and B but not stationary growth phase (Table 6.1) about 91% were prophage genes with only one homolog identified, *ybcO*, which represents a hypothetical protein

Stationary-phase and acid induced genes

There were a total of 75 shared genes which were up-regulated upon entry to stationary phase and following challenge with treatment A. Of those about 56% were of prophage origin. Only 8 homolog gene names were identified; *tfaX*, *intR*, *ydeQ*, *ydaC*, *recT*, *recE*, *kilR*, and *sieB*. In the set of identified homolog genes, not of prophage origin, the most significant to acid resistance is the *ydeO* gene. Masuda *et al* demonstrated that acid resistance of *E. coli* cells is partially abolished with the deletion of *ydeO*. In their work, it is suggested that *ydeO* regulates the expression of other acid resistance genes (Masuda & Church, 2003). Perhaps some of the up-

regulated genes observed in this gene set are under the regulation of *ydeO* though the DNA-binding sites for this activator are yet to be defined.

Stationary-phase and acid and acid-salt induced genes

There were 75 up-regulated genes that were shared for cultures in stationary phase and those challenged with treatments A and B most of which (67%) were identified to be of prophage origin; 12 genes common to most *E. coli* strains were included in the group of upregulated prophage genes. These genes were: *lomU*, *stfR*, *xisW*, *gamW*, *antP*, *rzpD*, *intN*, *ninE*, *nohA*, *ybcY*, *ydfD* and *dicB*. Go Term analysis of the total genes revealed that the majority of genes were involved in transporter, oxidoreductase, hydrolase and binding activities. Within this gene set, several genes previously identified with acid resistance were also observed to be upregulated. These included those of major importance for acid resistance including *asr*, *gadB* and *gadC*. The acid shock precursor gene, *asr*, has been a subject of many studies and its expression has been demonstrated to enhance resistance to acid by *E. coli* (Seputiene *et al.*, 2006). Genes *gadB* and *gadC* are part of the well-characterized *E. coli* acid resistance system 2 (AR2) with *gadB* encoding a glutamante decarboxylase and *gadC* the associated glutamate:γ aminobutyric acid antiporter (Castanie-Cornet & Foster, 2001a). The presence of the abovementioned genes for the stationary phase cultures, suggest that the acid pre-adaptation with the supplementation of 1% glucose and the allowance to reach late stationary growth phase proved advantageous for acid challenge. Some well known other acid resistance associated genes such as *hdeA*, were found to be down regulated for stationary phase cultures prior to and following challenge with treatments A and B. The gene *hdeA* is known to be a putative chaperone associated with protective responses against severe acid shock usually

generated by mineral acid (e.g. exposure to pH 2.5, equivalent to gastric acid) (Mates *et al.*, 2007). The physiological state of the cultures grown here under conditions of milder acidic pH generated by lactic acid are quite different and thus leading to different responses to what has been observed under other scenarios e.g. sudden exposure to high levels of protons.

Genes of prophage origin

A high proportion of the total up-regulated genes from entry into stationary phase and following challenge with treatments A and B, were prophage genes. In contrast only 2% of the down-regulated genes were of prophage origin. Of the total up-regulated genes exclusively found upon entry into the stationary phase approximately 38% were from prophage origin (Fig 6.1). About 56% and 68% of the total up-regulated genes exclusively found following treatment A and B were from prophage origin respectively. As previously mentioned almost every single shared gene (92%) exclusively up-regulated following the challenge with treatment A and B was of prophage origin (Fig. 6.1).

Analysis of these prophage genes showed that they included many genes involved in phage replication mechanisms and structural proteins. Studies have demonstrated that the presence of certain antimicrobials can trigger genetic expression of pro-phage genes and the replication of lambdoid phages (Grif *et al.*, 1998; Leenanon *et al.*, 2003; Mellies *et al.*, 2007). Perhaps, as observed in this Chapter, the presence of lactic acid, an antimicrobial, also triggers the replication of genes from phage origin.

In the work of Leenanon *et al.* (2003) where stress conditions such as acid adaptation and starvation triggered the expression of *stx-II*, a gene contained within an integrated bacteriophage genomic island designated BP-933W. This might explain the reason why the up-regulation of prophage genes was observed upon entry into the stationary phase, even prior to the exposure of the acid present in treatments A and B. Leenanon *et al.* (2003) also demonstrated that while mRNA transcripts of genes of phage origin including that of the *stx* gene, were being detected no *Stx* toxin was actually produced. It would be interesting to observe if this case also applies to the challenge conditions presented in this work.

Table 6.1. *E. coli* Sakai genes showing up-regulation under different conditions of acidic stress.

Gene symbol	*E. coli* Sakai ORF no.*	Known or predicted function	stat vs log	acid vs log	acid/NaCl vs log
			Fold change*:		
yhhI	ECs0241	H repeat-associated protein of Rhs element, predicted transposase	2.13	2.36	2.18
yhhI	ECs0241, ECs0602, ECs0731	H repeat-associated protein of Rhs element, predicted transposase	2.64	2.92	2.56
hokE	ECs0620, ECs0621	small toxic polypeptide	2.04	-	-
ybeQ	ECs0682	hypothetical conserved protein	-	2.03	-
bioD	ECs0856	dethiobiotin synthetase	2.50	2.36	4.78
ybjL	ECs0927	predicted transporter	-	-	3.19
exoU	ECs1057, ECs2771	exodeoxyribonuclease VIII of prophage	2.03	-	-
	ECs1081, ECs1521	hypothetical protein	2.32	2.01	-
	ECs1088, ECs1781, ECs1961, ECs2189, ECs2262, ECs2746, ECs3498	hypothetical phage-associated protein	3.10	2.88	2.13
	ECs1088, ECs1781, ECs1961, ECs2189, ECs2262, ECs2746, ECs3498	hypothetical phage-associated protein	2.63	2.59	2.21
	ECs1089, ECs1209, ECs1381, ECs1665, ECs1690, ECs1919, ECs2220, ECs2477, ECs2637, ECs2744, ECs2794, ECs2933, ECs2958, ECs3132, ECs3490, ECs3863, ECs4024, ECs5243	putative transposase of prophage	2.18	-	-
	ECs1098	hypothetical phage-associated protein	-	2.21	-
	ECs1100	Prophage holin protein	-	2.01	-
	ECs1102	putative IS-associated protein of prophage	2.01	-	-
	ECs1103	putative IS-associated protein of prophage	2.07	-	-
	ECs1110, ECs2730	major head protein of prophage	2.19	2.22	2.02
	ECs1112, ECs2728	tail component of prophage	-	-	2.07
	ECs1116, ECs1555, ECs1805, ECs1985, ECs2164, ECs2238, ECs2723, ECs2947	minor tail component of prophage	2.42	-	-
	ECs1117, ECs1558, ECs2163, ECs2237, ECs2722, ECs2946	tail assembly protein of prophage	2.03	-	-
	ECs1119, ECs1988	hypothetical phage-associated protein	2.13	2.15	2.01
lomU	ECs1122, ECs1649, ECs1991, ECs2160, ECs2232, ECs2718, ECs2942	putative outer membrane protein Lom precursor of prophage	2.24	2.21	2.08

	ECs1123, ECs1228, ECs1808, ECs1992, ECs2717, ECs2941	putative tail fiber protein of prophage	2.20	2.07	2.09
stfR	ECs1123, ECs1650, ECs2159, ECs2231, ECs2717, ECs2941	putative tail fiber protein of prophage	2.98	3.00	2.57
xisW	ECs1161	putative phage-associated excisionase	2.08	2.52	2.21
	ECs1162	hypothetical phage-associated protein	-	2.33	2.35
	ECs1164	hypothetical phage-associated protein	-	2.12	2.18
	ECs1165	hypothetical phage-associated protein	-	-	2.14
	ECs1166	hypothetical phage-associated protein	-	2.11	2.24
	ECs1167	hypothetical phage-associated protein	2.05	2.42	2.59
	ECs1168	hypothetical phage-associated protein	-	-	2.32
	ECs1169, ECs3007	hypothetical phage-associated protein	2.36	2.71	2.62
	ECs1173	hypothetical phage-associated protein	-	-	2.53
exoD	ECs1174	exonuclease of prophage	-	-	2.13
betW	ECs1175, ECs3001, ECs5398	recombination protein Bet of prophage	-	-	2.15
gamW	ECs1176	hypothetical phage-associated protein	2.16	2.49	2.90
	ECs1176 (c1538)	hypothetical phage-associated protein	-	-	2.05
kilW	ECs1177, ECs1178, ECs2997, ECs2998	killing associated prophage protein	-	-	2.80
ssbW	ECs1179, ECs2996	single-stranded DNA binding protein of prophage	-	-	2.39
	ECs1183	hypothetical phage-associated protein	-	2.28	-
	ECs1186	hypothetical phage associated antirepressor CRO	-	2.01	2.88
	ECs1186 (c1547)	hypothetical phage associated antirepressor CRO	-	2.05	-
	ECs1187, ECs2988	putative regulatory protein CII of bacteriophage	-	2.15	2.85
	ECs1188	hypothetical phage-associated protein	-	2.24	2.74
	ECs1189	phage replication protein O	-	-	2.54
	ECs1190	phage associated replication protein P	-	2.27	2.89
	ECs1191	hypothetical phage-associated protein	-	2.36	2.79
	ECs1192, ECs2984	hypothetical phage-associated protein	2.07	2.42	2.26
	ECs1193, ECs2983	hypothetical phage-associated protein	2.05	2.35	2.16
	ECs1194, ECs2983	hypothetical phage-associated protein	2.99	3.33	2.76
	ECs1195, ECs2982	hypothetical phage-associated protein	2.20	2.54	2.45
	ECs1196, ECs2981	putative DNA N-6-adenine-methyltransferase of bacteriophage	2.13	2.60	2.46
ninE	ECs1197	conserved phage associated protein	2.10	2.16	-
	ECs1200	phage associated DNA-binding protein	-	2.45	2.34
	ECs1201, ECs2977	putative phage associated hypothetical protein	-	2.05	2.10
	ECs1203	antitermination protein Q of prophage	-	2.24	2.24
	ECs1210, ECs2971	hypothetical protein, phage associated	-	2.17	2.28
antPUV	ECs1214, ECs1533, ECs1785, ECs1965, ECs2185, ECs2258, ECs2967	putative prophage antirepressor	2.52	2.80	2.55

rzpD	ECs1215, ECs1623	phage associated hypothetical protein	2.23	2.65	2.59
	ECs1218	phage associated hypothetical protein	-	-	2.05
	ECs1219	terminase small subunit of prophage	-	2.23	2.10
	ECs1220	terminase large subunit of prophage	-	2.19	2.19
	ECs1221	portal protein of prophage	-	2.23	2.21
	ECs1222	hypothetical protein, bacteriophage	-	2.09	-
	ECs1224	phage associated hypothetical protein	-	2.10	2.10
	ECs1226	hypothetical phage-associated protein	-	2.16	2.26
	ECs1227	phage associated hypothetical protein	-	2.07	2.10
	ECs1228, ECs1992, ECs2231, ECs2941	tail fiber protein of prophage	-	2.37	3.06
	ECs1232	phage associated hypothetical protein	-	2.24	2.45
	ECs1233	phage associated hypothetical protein	-	2.49	2.29
	ECs1234	phage associated hypothetical protein	-	2.36	2.13
	ECs1235	phage associated hypothetical protein	2.06	2.45	2.28
	ECs1238	phage associated hypothetical protein	-	-	2.23
	ECs1239	phage associated hypothetical protein	-	-	2.01
	ECs1240	phage associated hypothetical protein	-	2.24	2.19
	ECs1241	phage associated protein with RNA processing domain	-	2.26	2.25
	ECs1242	phage associated hypothetical protein	-	-	2.12
	ECs1243	phage associated hypothetical protein	-	-	2.11
	ECs1246	phage associated hypothetical protein	-	2.29	2.09
	ECs1248	hypothetical phage-associated protein	2.36	2.71	2.62
	ECs1250	phage associated C4-type zinc finger protein (TraR family)	-	2.05	-
	ECs1251	phage associated anti-repressor protein	-	2.30	-
rutB	ECs1257	hypothetical isochorismatase family protein	-	2.04	-
	ECs1283	putative hemolysin activator/transporter protein ShlA/HecA/FhaA exoprotein family	-	2.04	-
	ECs1384	putative phage-associated helicase	-	2.02	-
	ECs1392	hypothetical protein	2.10	2.15	2.10
	ECs1397, ECs2796	hypothetical protein	-	-	2.30
	ECs1408 (Z1663)	hypothetical protein	-	2.03	-
csgE	ECs1416	curli assembly protein CsgE, predicted transport protein	2.13	2.06	-
csgB	ECs1419	curlin nucleator protein, minor subunit in curli complex	-	2.07	-
csgC	ECs1421	putative curli production protein	2.04	2.11	-
yceE	ECs1431	hypothetical protein	2.03	-	-
yceO	ECs1436	hypothetical protein	2.05	-	-
intN	ECs1501	putative integrase of prophage	2.02	2.01	2.00
exoOP	ECs1503, ECs1759, ECs2286	exodeoxyribonuclease of prophage	2.03	-	-
	ECs1505	phage associated hypothetical protein	-	2.00	2.39
	ECs1507	phage associated hypothetical protein	2.01	-	2.11
	ECs1508	phage associated hypothetical protein	2.26	2.54	2.69

	ECs1530, ECs2743	holin protein of prophage	-	2.25	2.14
	ECs1532, ECs1784, ECs1964, ECs2186, ECs2259	endolysin of prophage	2.15	2.21	-
	ECs1532, ECs2186	endolysin of prophage	2.28	2.33	-
	ECs1534, ECs1535	putative lipoprotein Rz1 precursor	-	-	2.12
	ECs1540, ECs1789, ECs1968, ECs2254	putative DNAse of prophage	2.45	2.41	2.12
	ECs1541, ECs1791, ECs1970, ECs2252	terminase small subunit of prophage	2.07	-	-
	ECs1542, ECs1792, ECs1971, ECs2251	terminase large subunit of prophage	3.70	3.26	2.60
	ECs1543, ECs1793, ECs1972, ECs2250	major head protein/prohead protease of prophage	2.12	-	-
	ECs1545, ECs1796, ECs1976, ECs2247	phage associated hypothetical protein	2.24	-	-
	ECs1546, ECs1797, ECs1977, ECs2246	head-tail adaptor protein of prophage	2.15	2.07	-
	ECs1547, ECs1798, ECs1978, ECs2245	putative structural component of prophage	2.81	2.42	2.15
	ECs1547, ECs1798, ECs1978, ECs2245, Z1368, Z1809, Z1905, Z3325, Z6039	putative structural component of prophage	2.78	2.29	-
	ECs1549, ECs1800	major tail subunit of prophage	2.03	2.08	2.03
	ECs1550, ECs1801, ECs1981, ECs2242	tail assembly chaperone of prophage	2.57	2.43	2.08
	ECs1550, ECs1801, ECs1981, ECs2242, Z1371, Z1812, Z3320, Z6036	tail assembly chaperone of prophage	2.20	-	2.05
	ECs1551, ECs1802, ECs1982, ECs2241	tail assembly protein of prophage	2.39	2.27	-
	ECs1554, ECs1804, ECs1984, ECs2239	minor tail protein of prophage	2.30	-	-
	ECs1559	tail assembly protein of prophage	2.11	2.09	-
	ECs1560	putative phage associated secreted effector protein	2.16	2.02	-
	ECs1578	phage associated hypothetical protein	2.24	2.33	2.02
	ECs1580	phage associated hypothetical protein	2.16	2.32	2.21
	ECs1584 (Z1843)	primase of prophage	-		2.02
	ECs1586	phage associated hypothetical protein	2.01	2.30	2.06
	ECs1595	holin of prophage	-	2.02	-
	ECs1610	integrase of prophage	-	2.05	-
ybcK	ECs1615	phage recombinase of prophage	-	2.03	-
ninE	ECs1617	phage associated hypothetical protein	2.18	2.16	2.07
ybcO	ECs1618	phage associated hypothetical protein	-	2.11	2.32
	ECs1620	antiterminator of prophage	-	2.07	2.06
	ECs1621	holin of prophage	2.19	2.12	-
	ECs1622	endolysin of prophage	2.06	2.08	2.00
nohAB	ECs1629	terminase small subunit, DNA packaging protein of prophage	2.27	2.37	2.04

	ECs1637, ECs2172	head-tail joining protein of prophage	2.13	-	-
	ECs1642	minor tail component of prophage	2.12	2.05	-
	ECs1654	phage associated hypothetical protein	2.13	2.19	-
ybcY	ECs1656	phage associated hypothetical protein	2.29	2.32	2.05
tfaX	ECs1657	phage associated hypothetical protein	2.15	2.40	-
	ECs1658	IS-associated protein of prophage	2.01	-	-
	ECs1659	IS-associated protein of prophage	2.07	-	-
hlyE	ECs1677	hemolysin E	-	2.43	2.03
prrA	ECs1693	iron complex TonB-dependent outer membrane receptor	-	2.03	-
	ECs1695	hypothetical protein	2.06	2.01	-
	ECs1699	ABC transporter, ATP-binding protein	2.10	2.01	-
	ECs1700	hypothetical protein	2.25	2.48	2.12
chaB	ECs1722	cation transport regulator	-	-	2.27
ychS	ECs1733	hypothetical protein	2.35	-	-
	ECs1758	phage associated excisionase	2.28	2.35	-
ydfD	ECs1760, ECs2285	phage associated hypothetical protein	2.97	3.19	2.90
dicB	ECs1761, ECs2284	prophage cell division repressor	2.19	2.46	2.27
	ECs1762	phage associated hypothetical protein	-	2.03	
	ECs1763	phage associated hypothetical protein	2.05	2.02	2.20
	ECs1764	phage associated hypothetical protein	-	-	2.08
dicC	ECs1766	phage associated DNA-binding transcriptional regulator	2.03	-	-
	ECs1767	phage associated hypothetical protein	2.26	2.11	-
	ECs1768	phage associated hypothetical protein	2.12	2.09	-
	ECs1769	phage replication protein	2.06	-	-
	ECs1772	intestinal colonization factor encoded by prophage	2.07	3.97	2.02
	ECs1774	phage associated hypothetical protein	2.32	2.42	2.02
	ECs1776	phage associated hypothetical protein	2.07	-	-
	ECs1778	phage associated hypothetical protein	2.27	2.21	2.08
	ECs1780	putative DNA adenine methyltransferase of prophage	2.22	2.15	-
	ECs1781, ECs2189, ECs2262, ECs3498	phage associated hypothetical protein	-	2.13	2.69
	ECs1782, ECs2188, ECs2261, ECs3497	holin protein of prophage	2.37	2.31	2.05
	ECs1795, ECs1975, ECs2248	portal protein of prophage	2.22	2.32	2.32
	ECs1797, ECs1977, ECs2246	head-tail adaptor of prophage	3.07	2.75	2.47
	ECs1803, ECs1983, ECs2240	phage tail length tape measure protein	2.14	-	-
	ECs1806 (Z1916, Z6029)	tail component of prophage	-	2.05	2.24
	ECs1806 (Z6030)	tail component of prophage	2.25	2.16	2.28
	ECs1808	tail fiber protein of prophage	2.26	2.19	-
	ECs1812	phage associated hypothetical protein	2.05	2.12	2.10
	ECs1813	putative phage associated integrase	2.24	2.39	2.24

	ECs1819	IS-associated protein of prophage	2.07	-	-
	ECs1820	IS-associated protein of prophage	2.01	-	-
yciE	ECs1829	hypothetical conserved protein	2.05	2.13	-
yciG	ECs1831	hypothetical protein	-	2.03	2.07
trpE	ECs1836	anthranilate synthase component I	2.29	-	-
trpL	ECs1837	trp operon leader peptide	-	2.09	2.40
	ECs1862	putative transcription regulatory protein	2.01	-	-
	ECs1863	multidrug-efflux transport protein	2.22	2.13	-
	ECs1864	multidrug-efflux transport protein	2.25	2.16	2.14
	ECs1865	putative outer membrane channel protein	-	2.03	-
	ECs1866	partial putative membrane transport protein	2.05		
ycjJ	ECs1873	putative amino acid/amine transport protein	2.15	2.18	
aldH	ECs1877	gamma-glutamyl-gamma-aminobutyraldehyde dehydrogenase	2.31	2.14	-
ordL	ECs1878	putative oxidoreductase	2.02	-	-
goaG	ECs1879	GABA aminotransferase	2.01	-	-
ycjQ	ECs1892	putative oxidoreductase, Zn-dependent and NAD(P)-binding	2.20	2.11	-
ycjS	ECs1894	putative dehydrogenase/oxidoreductase, NADH-binding	2.12	-	-
ycjT	ECs1895	predicted hydrolase	2.27	2.14	2.01
ycjU	ECs1896	putative beta-phosphoglucomutase	2.06	-	-
ycjV	ECs1897	putative ABC-type transporter, ATP-binding protein	2.02	-	-
ompG	ECs1898	outer membrane porin	2.18	-	-
abgT	ECs1917	predicted cryptic aminobenzoyl-glutamate transporter	2.01	-	-
abgB	ECs1921	predicted peptidase, aminobenzoyl-glutamate utilization protein	2.01	-	-
abgA	ECs1922	predicted peptidase, aminobenzoyl-glutamate utilization protein	2.04	2.01	-
intR	ECs1929	putative integrase of prophage	2.07	2.15	-
ydaQ	ECs1930	phage associated hypothetical protein	2.14	2.09	-
ydaC	ECs1931	phage associated hypothetical protein	2.22	2.08	-
recT	ECs1933	recombination and repair protein, phage-associated	2.13	2.07	-
recE	ECs1934	prophage-associated exonuclease VIII, 5' -> 3' specific dsDNA exonuclease	2.19	2.06	-
kilR	ECs1936	phage-associated killing protein (inhibitor of ftsZ)	2.10	2.13	-
sieB	ECs1937	phage superinfection exclusion protein	2.04	2.11	-
	ECs1942	putative regulatory protein Cro of prophage	2.05	2.03	-
	ECs1943	phage associated hypothetical protein	2.29	2.15	-
	ECs1944	phage associated hypothetical protein	2.18	2.05	-
	ECs1946 (Z2051)	phage associated hypothetical protein	2.07		-
	ECs1949	phage associated hypothetical protein	2.00	2.14	-

	ECs1950	phage associated hypothetical protein	2.25	2.08	-
	ECs1951	phage associated hypothetical protein	2.08	2.25	2.13
	ECs1952	phage associated hypothetical protein	2.36	2.31	-
	ECs1956	phage associated hypothetical protein	2.05	2.03	2.06
	ECs1957	phage associated hypothetical protein	2.06	2.20	2.34
	ECs1958	anti-terminator protein of prophage	2.55	2.69	2.25
	ECs1959	phage associated hypothetical protein	2.15	2.09	-
	ECs1960 (Z0958, Z3929)	phage associated hypothetical protein	-	2.09	-
	ECs1960, ECs2264, ECs2748	phage associated hypothetical protein	-	-	2.07
	ECs1963	phage associated hypothetical protein	2.06	2.03	2.13
	ECs1973, ECs2249, ECs5418, ECs5438, ECs5451	phage associated hypothetical protein	3.35	3.24	2.65
	ECs1974	portal protein of prophage	2.26	2.12	2.30
	ECs1983	tail length tape measure protein of prophage	-	2.06	-
	ECs1990	host specificity protein, tail fiber protein of prophage	2.34	2.26	-
	ECs2006	putative BigA-like protein	2.06	2.21	-
	ECs2007	putative outer membrane protein	2.07	2.06	2.12
ynbA	ECs2010	putative enzyme, predicted inner membrane protein	2.17	2.07	-
ynbB	ECs2011	putative CDP-diglyceride synthase	2.10	2.13	-
ynbC	ECs2012	predicted hydrolase	2.29	-	-
ynbD	ECs2013	predicted phosphatase, inner membrane protein	2.03	2.08	2.10
	ECs2016	hypothetical protein	2.09	-	-
	ECs2017	hypothetical protein	2.22	2.20	-
	ECs2018	hypothetical protein	2.03	-	-
	ECs2019	hypothetical protein	2.00	2.06	-
ycdI	ECs2027	putative transcriptional regulator, LysR family	-	2.06	-
ydcJ	ECs2028	hypothetical conserved protein	2.02	-	-
ydcK	ECs2033	predicted enzyme	2.13	-	-
	ECs2041	hypothetical protein	2.35	2.08	-
ydcQ	ECs2042	predicted DNA-binding transcriptional regulator	2.23	2.02	-
ydcS	ECs2044	predicted spermidine/putrescine transporter subunit	2.18	2.07	-
ydcT	ECs2045	putative spermidine/putrescine transporter ATP-binding subunit	2.30	2.05	-
ydcU	ECs2046	putative spermidine/putrescine transporter permease subunit	2.08	2.02	-
ydcV	ECs2047	putative spermidine/putrescine transporter permease subunit	2.07	-	-
yncB	ECs2053	putative oxidoreductase, Zn-dependent and NAD(P)-binding	2.21	2.01	-
yncC	ECs2054	putative DNA-binding transcriptional regulator	2.13	2.08	-
yncH	ECs2059	hypothetical protein	2.24	2.41	2.12

vgrE	ECs2060	hypothetical protein associated with Rhs element	2.27	2.17	2.13
yddH	ECs2065	hypothetical conserved protein	2.02	-	-
narV	ECs2068	cryptic nitrate reductase 2 gamma subunit	2.19	2.04	-
narW	ECs2069	nitrate reductase 2 (NRZ), delta subunit (assembly subunit)	2.03	-	-
narU	ECs2072	nitrite extrusion protein 2	2.15	2.11	2.19
yddJ	ECs2073	hypothetical protein	2.20	2.16	-
	ECs2075	IpaH-like protein	2.22	2.15	-
yddL	ECs2076	putative outer membrane porin protein	2.23	2.13	-
bdm	ECs2085	biofilm-dependent modulation protein	2.20	2.22	2.12
ddpB	ECs2090	D-ala-D-ala transporter subunit, permease	2.02	-	-
ddpA	ECs2091	D-ala-D-a la transporter subunit	2.16	2.03	-
ddpX	ECs2092	D-alanyl-D-alanine dipeptidase	2.13	-	-
yddV	ECs2095	predicted diguanylate cyclase	2.25	-	-
gadC	ECs2097	glutamate:gamma-aminobutyric acid antiporter	2.07	2.07	2.21
gadB	ECs2098	glutamate decarboxylase B	2.05	2.24	2.36
yddB	ECs2100	hypothetical protein	2.16	-	-
yddA	ECs2101	fused multidrug transporter, membrane component/ATP-binding component	2.22	2.03	-
ydeM	ECs2102	putative enzyme	2.12	2.09	2.03
ydeO	ECs2104	putative DNA-binding transcriptional activator	2.37	2.22	-
	ECs2105	hypothetical protein	2.46	2.48	2.37
ydeS	ECs2109	putative fimbrial-like adhesin	-	2.03	-
ydeK	ECs2117	predicted lipoprotein	2.17	2.09	2.11
lsrA	ECs2120	fused AI2 ABC-typetransporter, ATP-binding component	2.04	-	-
lsrC	ECs2121	AI2 ABC-type transporter, permease protein	2.08	-	-
lsrD	ECs2122	AI2 ABC-type transporter, permease protein	2.11	-	-
lsrB	ECs2123	LacI-type transcriptional regulator of AI2 transporter	2.16	-	-
lsrF	ECs2124	autoinducer-2 (AI-2) aldolase	2.02	-	-
lsrG	ECs2125	autoinducer-2 (AI-2) modifying protein	2.29	2.27	2.11
tam	ECs2126	trans-aconitate 2-methyltransferase	2.13	-	-
yneK	ECs2134	hypothetical protein	2.23	2.20	2.02
ydeF	ECs2141	putative MFS-type transporter YdeE	2.20	2.16	2.08
	ECs2142	hypothetical protein	2.35	2.06	-
ydeI	ECs2145	hypothetical conserved protein	2.26	2.04	-
ydeJ	ECs2146	competence damage-inducible protein A	2.31	2.18	2.04
ydfI	ECs2151	putative mannonate dehydrogenase	2.08	-	2.06
ydfJ	ECs2152	putative metabolite transport protein	2.11	2.05	2.19
	ECs2153, ECs2218	damage-inducible protein encoded within prophage	2.17	2.10	-

	ECs2163, ECs2722, ECs2946	tail fiber component of prophage	2.60	2.42	2.11
	ECs2181	phage associated hypothetical protein	-	2.08	2.08
	ECs2190 (Z1346)	phage associated hypothetical protein	2.14	2.03	-
	ECs2222	IS-associated protein of prophage	2.01	-	-
	ECs2223	IS-associated protein of prophage	2.07	-	-
	ECs2243	major tail subunit of prophage	2.22	2.08	-
renP	ECs2273	exclusion protein Ren of prophage	2.03	-	2.01
	ECs2277	phage associated hypothetical protein	2.13	2.22	2.17
	ECs2278	phage associated hypothetical protein	2.11	2.06	-
	ECs2282, ECs2283	phage associated hypothetical protein	-	-	2.14
	ECs2283, ECs5428	phage associated hypothetical protein	2.19	2.09	2.04
ynfA	ECs2288	conserved inner membrane protein	2.05	-	-
asr	ECs2303	acid shock-inducible periplasmic protein	2.05	2.32	2.25
uidB	ECs2323	glucuronide transporter	2.04	-	-
ydhS	ECs2375	conserved protein with FAD/NAD(P)-binding domain	2.04	-	-
sufA	ECs2391	iron-sulfur cluster assembly scaffold protein	-	-	2.07
ydiQ	ECs2404	putative electron transfer flavoprotein	2.00	-	-
ynjI	ECs2468	predicted inner membrane protein	2.00	-	-
ydjE	ECs2476	putative transport protein	-	2.10	-
yeaI	ECs2494	predicted diguanylate cyclase	-	-	2.29
ptrB	ECs2555	protease II	-	-	2.04
yebE	ECs2556	hypothetical conserved protein	-	-	2.84
yebB	ECs2572	hypothetical protein	-	-	2.28
	ECs2625	phage associated hypothetical protein	-	2.00	2.06
	ECs2716	phage associated hypothetical protein	2.22	2.04	-
	ECs2768	putative inhibitor of cell division encoded within prophage	2.03	2.05	-
	ECs2978	phage associated hypothetical protein	-	2.02	-
	ECs2979	phage associated hypothetical protein	-	2.06	-
	ECs2985	exclusion protein Ren of prophage	-	-	2.11
	ECs2988	regulatory protein CII of prophage	-	2.09	-
trmJ	ECs3411	tRNA/rRNA methyltransferase	2.20	2.19	-
yfiC	ECs3441	putative enzyme	-	-	2.02
yfiC	ECs3443	putative enzyme	-	-	2.36
	ECs3859 (Z2562)	putative transposase	-	-	2.05
treA	ECs4399	trehalase, periplasmic	2.05	-	-
ffs	ECs5378	4.5S RNA; component of ribonucleoprotein	2.40	4.04	4.24
	ECs5417, ECs5431, ECs5437	phage associated hypothetical protein	2.23	2.06	-
	ECs5421	phage associated hypothetical protein	2.27	2.31	2.66
	ECs5422	phage associated hypothetical protein	-	2.04	-
	ECs5423	phage associated hypothetical protein	2.26	2.46	2.33
ydaE	ECs5433	phage associated hypothetical protein	2.01	-	-

hokB	ECs5440	small toxic polypeptide, phage encoded	2.01	-	-
	ECs5444	phage associated hypothetical protein	2.26	2.09	-
rzpQ	ECs5458	putative murein endopeptidase of prophage	2.07	2.12	-
dicF	ECs5459	putative RNA encoded within prophage	2.01	-	-
	ECs5470	phage associated hypothetical protein	-	2.09	-
ssrS	ECs5516	6S RNA	2.08	2.23	2.24
	ECs5565 (Z2255)	unknown protein associated with Rhs element	-	-	2.05

*Prophage genes detected with probes on the Affymetric *E. coli* v. 2 chip. Include many with identical or near indentical paralogs that occur within different integrated prophage genomic islands on the Sakai geneome thus they cannot be distinguished from the array data.

**Only genes that show significant upregulation (>2 fold) are shown.

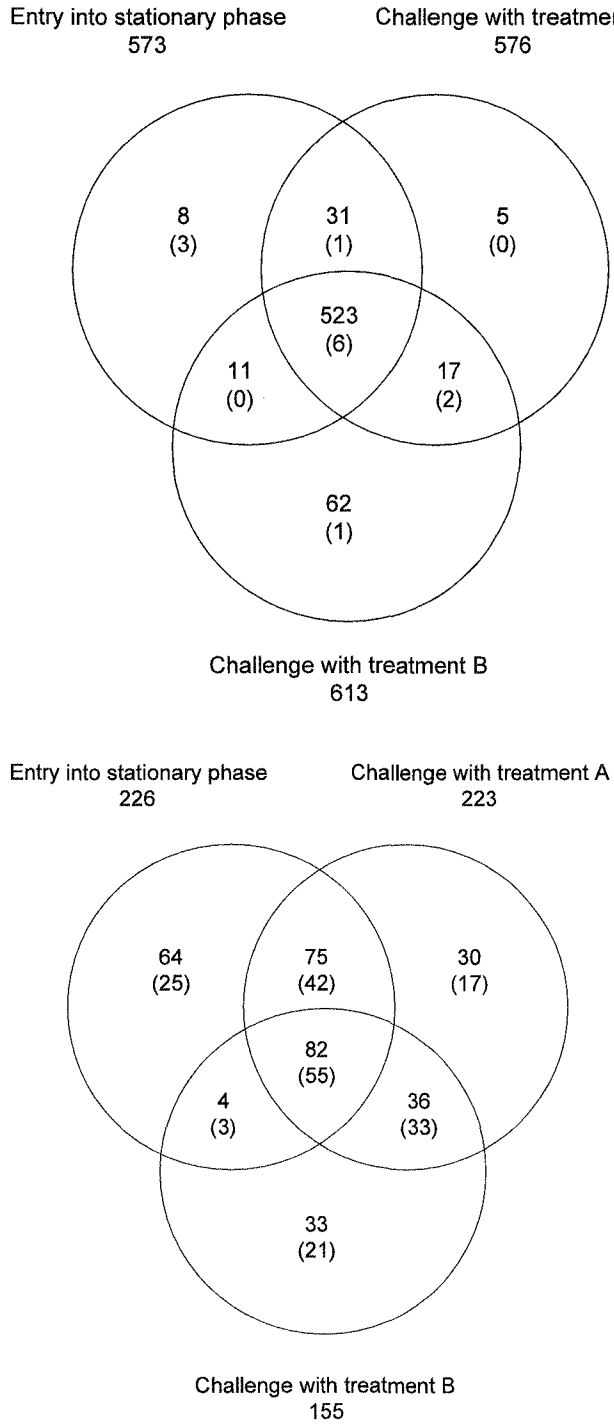


Figure 6.1. Venn Diagrams for down-regulated (upper diagram) and up-regulated genes (lower diagram) for stationary phase cultures prior to and following challenge with treatments A and B using log phase cultures as negative controls. Represented in parenthesis is the number of genes from prophage origin.

7. Concluding Chapter

"Enlightenment can be reached moments prior to total despair"

Santiago Ramirez (PhD Candidate 2005-2009)

7.1. CONCLUSION

The preliminary aim for this thesis was to characterize the acid response of a panel of *E. coli* salami validation strains. More specifically we wanted to gain insights on the mechanisms that allowed the validation strains to distinctively survive the challenge described in the work of Duffy *et al* (2000). In the first experimental work we attempted achieve this by using a proteomic gel-based approach. It was observed that each of the strains had similar but not identical, protein expression patterns prior to challenge. As opposed to what was hypothesized, there were no distinctive similarities in protein expression profiles exclusive to the validation strains, that distinguished them from the controls, following acid challenge. Similarities in protein expression following challenge would have given an indicator on the mechanism that provide the validation strains their unique ability to withstand the acid challenge.

Perhaps another approach could have been to perform 2D-gel electorphoresis of a pool culture from all the validation strains and compare it with a pool of non-sensitive strains. An example of this technique is described by Page *et al*, (1999), where the proteomes of normal human luminal and myopethelial breasts cells where compared using 2D-gel electorphoresis. In their work, 10-pooled samples of luminal and samples of breast cells from different patients were used to compare protein expression profiles for each cell type. Proteome comparisons revealed 1,738 unique proteins for each cell type out of a total of 43,302 observed proteins.

Other researchers have been successful in identifying differences in protein expression profiles in *E. coli* cultures under different treatments. In the work of Yohannes *et al* (2005) proteomes of *E. coli* K-12 cultures were compared under the presence or absence of two polyamines, spermine and spermidine. 2D-gels revealed

31 uniquely expressed proteins in the presence of the polyamines. On another study, 33 proteins were found to have significant expression profiles when comparing proteomes of *E. coli slyA* mutants and non-mutants (Spory et al, 2002).

An option to further understand the mechanisms of *E. coli* acid resistant strains using 2D techniques, would have been to perform survival studies under different experimental conditions (as described in Chapter 5) until significant differences between a group of acid sensitive and acid resistant ones. Once identified, pooled proteomes from each group of cells can be studied and protein expression profiles can be compared under a series of different treatments. For example, acid challenge under nutrient rich media, the presence or absence of glucose, nutrient less media, different acidulants, and different salt concentrations.

As demonstrated throughout this thesis there were some instances where the validation strains did not show higher resistance to acid challenge than the acid sensitive strains. In chapters 3 and 5, some experiments (Fig 3.2 upper panel and Figure 5.1 and 5.2) demonstrated that the validation strains had similar acid sensitivity than the acid sensitive strains. Perhaps the challenge conditions for the proteomic experiments, with no NaCl added, did not trigger a response sufficient to differentiate the acid sensitive strains from the non-sensitive ones. Using 2D-gel separation techniques provides a cheaper and quicker alternative than microarray studies.

Nevertheless, a critical analysis of the work performed in Chapter 2, revealed that the gel-based proteomic approach used was not sufficient for comparing the different proteomic expressions that might distinguish the salami validation strains from the acid sensitive controls. The proteomic approached taken in Chapter 2 was perhaps somewhat basic and could have been expanded with a more focused separation,

expanding the pI range and the length of the IPG strip. It would be very interesting to compliment the Microarray results with a 2D proteomic separation approach. Perhaps, the observed trasncriptome differences observed in Chapter 6 can be observed at a proteomic level using a more focused proteomic gel-based approach.

From the lessons learnt throughout the preparation of this manuscript, to characterize the mechanisms which allowed the validation strains to survive the salami model described in Duffy *et al* 2000, both transcriptomes and proteomes from the cultures need to be extracted under the exact same conditions. This is said because as shown in Chapter 5, the ability to resist a given challenge, whether is acid alone or acid supplemented by salt, can vary dramatically upon the challenge conditions. Transcriptomic and proteomic extractions from a replication a salami model using actual cure meats could have been extremely beneficial for this project and would have revealed interesting insights to better characterize the strains in question. If these extractions proved difficult to achieve, the semi-solid *in vitro* model developed in this thesis could have proved to be a great alternative. In fact preliminary studies (results not shown) demonstrated that the *in vitro* semi solid matrix was not affected by the addition of NaCl up to 8.5%.

Unexpectedly, certain acid challenge experiments within this thesis did not demonstrate that the validation strains were more acid sensitive than the controls. As previously discussed the different challenge conditions (i.e. the presence of NaCl) might have contributed to this phenomenon. Temperature is perhaps another crucial factor that might have contributed in the selection of the salami validation strains. Unfortunately, this factor was not really taken into consideration in a serious manner throughout this project. Experiment 3.4 (Chapter 3) revealed that the higher the

temperature the more pronounced the response to the challenge is. The results from this experiment suggested that the low temperature in the salami model in the work of Duffy *et al* (2000) contributed to reducing the magnitude of the acid-salt challenge.

These results are in accordance with those of Glass *et al* (1992) in that survival of *E. coli* O157:H7 cultures was not affected when challenged at pH 4.7 for 24 hrs at 5°C. Perhaps temperature, together with salt is a crucial factors in the survival of the challenge strains and the differentiation the validation strains from the acid sensitive strains and perhaps other pathogenic O157:H7 strains. Phenomenon not observed in all acid challenge studies in this thesis (i.e. chapter 3 and 4), where there was no difference in survival between the acid sensitive strains and the validation strains. It could have been interesting to replicate the studies from chapter five at different temperatures and observe the patterns of survival amongst the twelve strains.

One fact that became clear from this manuscript is that the ability of the salami validation strains to withstand acid challenge is enhanced in the presence of glucose (Chapter 3). It also became apparent that when TSB is used as a challenge media the validation strains show a considerably higher resistance to challenge with acid alone and supplemented by NaCl. In addition, it can be hypothesized that the validation strains might posses a higher tolerance to acid-salt challenge when compared to other strains (Chapter 5).

We began the characterization process for the salami validation strains by comparing their phenotypic responses to acid challenge with that described in literature. We were interested in observing the individual effects of parameters described in Duffy *et al* (2000) on acid resistance phenotypes (e.g. higher acid resistance) of the salami

validation strains that might assist in distinguishing them from other *E. coli* strains. In the work of Duffy *et al* (2000) all strains were acid adapted by the introduction of glucose at 1% during exponential growth. This was performed in order to introduce the acid challenge to a robust strain population rather than to a sensitive one (Personal communication with Paul Vanderlinde author in the work presented in Duffy *et al* (2000). The effect of the presence of glucose in acid adaptation has previously been described in the work of Buchannan and Edelson (1996). It was possible that the particular ability to resist the challenge in the work of Duffy *et al* (2000) was a direct consequence of the acid adaptation process. This is, the presence of 1% glucose in the growth media, evoked a phenotypic response, not found in the sensitive strains, that assisted the validation strains in surviving the challenge for longer. However, the work of Chapter 3 (Experiment 2) eliminated this possibility as there was no significant difference in the average survivor rates following the acid challenge between the acid adapted validation strains and the acid sensitive controls. This again points towards theorizing that it was synergistic effects of temperature, high NaCl levels and the acid present in the challenge media that differentiate the validation strains from others.

Critical analysis of this experimental work together with the variations in the experimental parameters across the literature (Table 3.1) raised two main questions; if the physical state of the media could also significantly influenced the acid resistance phenotypes of *E. coli*; and whether the type of challenge media used could significantly influenced the acid resistance phenotypes in *E. coli*. Both questions were studied in greater detail and gave rise to Chapters 4 and 5 respectively.

In Chapter 4 it was questioned whether *in vitro* acid challenge studies of *E. coli* cultures challenged in broth was accurate enough to represent the phenotypic behavior of *E. coli* stress response in actual salami. Due to the excess complexity of the salami batter and the challenges involved in organism enumeration and isolation, an *in vitro* semi-solid matrix was developed. The findings of this work demonstrated that cultures challenged in a semi-solid matrix show an enhanced resistant to acid challenge when compared to their counterpart challenged in broth. This is an important finding as a vast majority of challenge studies done *in vitro*, used to obtain information relating to food products, utilize broth as opposed to semi-solid matrices. These results might explain discrepancies observed in comparative studies between laboratory and food models; with the cultures challenged in food models.

It would have been very interesting to perform comparative survival studies between the semi-solid matrix and actual salami. The semi-solid matrix was found to be able to withstand most of the conditions of salami manufacturing allowing for comparative studies including, NaCl, lactic acid and temperature challenges.

The study of the phenotypic behavior of a larger culture population could bring some insights regarding the behavior of acid resistant *E. coli* cultures in semi-solid matrices as opposed to broth cultures. Perhaps elucidating the that arise from cultures existing or transiting in semi-solid matrix interfaces.

In Chapter 5, it was questioned whether the type of challenge media used has a direct influence on the acid resistance phenotypes of *E. coli*. In conjunction, it also questioned whether the addition of NaCl (an extra variable) at various concentrations to an acid challenge would also be affected by the type of challenge media. The findings from this experiment demonstrated that the type of challenge media had a

direct influence on the acid resistance phenotypes of *E. coli* cultures. To a certain extent these findings suggested that a challenge media with a higher buffer and nutrient content provided a protective effect for *E. coli* against acid challenge alone and supplemented with NaCl. However, the individual effects of the buffer concentration and composition together with the different nutrient content and composition found in each media could not be accurately assessed. It would be interesting to perform a large experiment where the ability of a large population of *E. coli* to resist an acid challenge is assessed based on the differences of individual variables in the challenge media; being nutrient or buffer content and composition. For example, it would be interesting to assess the independent influence on acid resistance between the disodium hydrogen phosphate and the K_2HPO_4 present in BHI and TSB respectively. These findings are of relevance to the scientific community, as more attention should be paid to the effects that the different commercial buffers have on the dissociation levels of the acidulants of choice for acid challenge experiments. Levels that have been proven to have a direct influence in bacterial survival, seen in Chapter 5 and in the work of Buchanan and Edelson (1999) and Cardenas *et al* (2008).

In the final experimental Chapter, the effects of NaCl supplementation in acid challenge were analyzed at a genetic level using an acid adapted *E. coli* O157:H7 strain as a model organism. The results demonstrated that the entry into stationary phase and the subsequent acid and acid salt challenge on the acid-adapted cultures resulted in a large number of up-regulated genes from pro-phage origin. This phenomena is of relevance as it appears that the presence of a stressor, acid alone or acid supplemented with 3.5%NaCl, evokes the possible sequestration of the *E. coli*

metabolism for bacteriophage replication; in turn decreasing the ability of *E. coli* to survive the given challenge (Grif *et al*, 1998). This phenomenon might explain the observed differences in the ability of certain strains to resist acid challenge under various experimental conditions (Chapter 5). Perhaps the presence of certain nutrients or other compounds in the immediate environment could act as a trigger or as an inhibitor for phage replication. It would be interesting to repeat the work of Chapter 6 using an *E. coli* salami validation strain as a model organism. This might provide some insight to the underlying mechanisms that might provide certain *E. coli* strains with the remarkable ability to withstand an acid challenge under high NaCl concentrations. The gained knowledge might compliment some of the findings from this manuscript and ultimately allow for the provision of knowledge to the food industry so that current food safety methods for salami manufacture and other fermented meats can be developed.

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