Chapter 1: INTRODUCTION

1.1 Introduction

Goat milk production has increased immensely over the last 20 years with a 58 % increase in goat milk production as compared to cattle (14 %), buffaloes (36 %) and sheep (2 %) milk (FAO, 2001). According to Klinger and Rosenthal (1997), goat milk ranks third in global milk production after cow and buffalo milk. Goat milk is an important source of human nutrition in developing countries. It used as a supplement in the treatment of allergic reactions of infants to cow milk and soy based infant formulas and in the processing of connoisseur cheeses (Chadan, Attaie and Shahani, 1992; Haenlein, 2004). Goat milk is therefore of growing importance to the dairy industry.

1.2 Problem statement

In South Africa, goat milk is mostly produced by small-scale farmers (Kyozaire, 2005) who lack dairy technology training and food hygiene education. Poor milk handling and limited cooling facilities that result in high microbiological load of pathogens (Giesecke, Du Preez and Petzer, 1994) such as *Escherichia coli* O157:H7 during milk collection and transport to dairy production and retail centers are thus not uncommon. *E. coli* O157:H7 is of particular concern because it is a natural inhabitant in the gut and on the skin and hides of goats. It has also been isolated from raw and pasteurized milk as well as cheeses made from unpasteurized milk (Wang, Zhao and Doyle, 1997). It causes haemorrhagic colitis and haemolytic uremic syndrome (Riley, Remis, Helgerson, McGee, Wells, Davis, Herbert, Olcott, Johnson, Hargrett, Blake and Cohen, 1983; Padhye and Doyle, 1992). According to the Communicable Disease Surveillance Centre (CDSC, 2000), 11 % of the total *E. coli* O157:H7 infections in England and Wales in 1999 were due to consumption of *E. coli* O157:H7 infected dairy products. Prolonged survival of *E. coli* in fermented dairy has been attributed to its ability to adapt to acidic pH (Jordan, Oxford and O'Byrne,

1999) by activating acid resistance genes that maintain internal pH close to neutral (Masuda and Church, 2003). Acid-adapted *E. coli* O157:H7 will not only persist in fresh and fermented dairy products but will also be better conditioned to survive the acidic environment in the human gut to cause infection.

The lactoperoxidase (LP) system has been recommended as a valid alternative to refrigeration preservation of fresh milk when appropriate cooling facilities are lacking (Björck, 1987). The LP system is an antimicrobial system consisting of the lactoperoxidase enzyme, thiocyanate and hydrogen peroxide. The LP enzyme is naturally found in milk, however the thiocyanate and hydrogen peroxide components need to be added from exogenous sources to activate the LP system (FAO, 1999).

The activation of LP system post-pasteurization of milk has been found to improve the keeping quality of milk (Marks, Grandison and Lewis, 2001). However, the effect of the LP system in combination with low pH and heat treatment on acid-adapted *E. coli* in goat milk has not been studied. Cross-protection of acid-adapted *Salmonella* Typhimurium against activated LP has been reported in Brain Heart Infusion (BHI) broth (Leyer and Johnson, 1993). Cross-protection of acid-adapted *E. coli* O157:H7 in food is however poorly understood. The potential hazards associated with the levels of acid-adapted microbial pathogens recovered from food can be underestimated. Lin, Smith, Chapin, Baik, Bennett and Foster (1996) reported that once acid tolerance response is induced, *E. coli* remains acid resistant in foods during prolonged storage. These aspects therefore need particular attention when dairy products are being produced for the commercial market, especially when milk is supplied by small-scale milk farmers that do not practice optimal hygiene milking and processing.

Though the LP system has been recommended as a safe and efficient method to use alone or alongside other preservation treatments to control spoilage and pathogenic microbes especially in milk supplied on a small-scale, its use on a broader scale is limited. Since publication of “Guidelines for the preservation of raw milk by use of lactoperoxidase

system" by the Codex Alimentarius Commission (1991) which emphasized that "the LP system should not be used for milk intended for international trade", there have been debates concerning safety of activated LP milk and how developing countries can benefit from its usage. There have also been concerns that the LP system inhibits acid production of lactic acid bacteria used in fermentation of milk and that the reduced acidity of activated LP fermented milk products could enhance survival of acid-adapted pathogens (FAO/WHO, 2006). In order to fully understand the impact of LP system on milk safety and on the broader economic status of milk production, the stress response of lactic acid bacteria and acid-adapted pathogens subjected to the activated LP system needs to be investigated. This study will provide valuable information to standardize the use and application of LP system.

Although stress response of acid-adapted *E. coli* O157:H7 has been studied in laboratory systems, the mechanism of cross-protection of acid-adapted *E. coli* O157:H7 in dairy systems has not been well characterized. LP in combination with low pH and heat treatment on acid-adapted *E. coli* in goat milk is novel. Also, the survival of acid-adapted *E. coli* O157:H7 during fermentation of activated LP milk has not been studied. To effectively control stress adapted pathogens in food, it is necessary to determine their mechanism of resistance using molecular and physiological studies in stressful environments (Chung, Bang and Drake, 2006). Their changes in gene expression and cell membrane profiles in stressful environments (Chung *et al.,* 2006) are also important in designing methods to effectively control their presence in food systems.

Chapter 2: LITERATURE REVIEW

2.1 Goat milk production

In Africa, goats are reared first for their meat but can be a significant source of milk production (Jaitner, Njie, Corr and Dempfle, 2006). Goats contribute 15 % to the total milk supply compared to 69 % from cattle and 11 % from sheep in Southern and Eastern Africa (Degen, 2007). In South Africa, about 60 % of rural households of former homelands own goats while less than 30 % own cattle (Statistics South Africa, 1999; Shackleton, Shackleton, Netshiluvhi, Mathabela and Phiri, 1999). Countries like Somalia, Sudan, Kenya, Mali, Ethiopia, Namibia and Botswana have bigger pastoral communities that rely significantly on goat milk in the dry season (Degen, 2007).

Quantitative data for goat milk production in pastoral communities are scant because they are produced on a small-scale mainly for home consumption, and relatively small amounts of goat milk products enter the formal market (Shackleton, Shakleton and Cousins, 2001). Small-scale goat milk production in rural centers makes use of minimal infrastructure. Milk is harvested mainly by hand milking or via use of semi-intensive systems (Degen, 2007). Here, goat milk is consumed raw or processed into artisanal soured milk products. This demand for goat milk for home consumption is increasing due to increase in human population (Haenlein, 2004). Rural small-scale goat milk production has a promising potential to contribute significantly to global milk production and is thus an avenue that needs further development. A less popular type of goat milk production is practiced on a commercial scale in urban areas. Intensive systems are used to produce good quality goat milk under hygienic conditions to be used for dietetic purposes or for processing into connoisseur cheeses (Degen, 2007).

Goat milk has been compared to cow milk and considered superior in terms of its digestibility, medical advantage as a substitute for cow milk (Haelein, 2004), and its potential economic role in rural development (FAO, 2001).

2.1.1 Anti-allergenic properties of goat milk

The health benefits of goat milk are related to allergic reactions to cow milk proteins prevalent among children less than 4 years (El-Agamy, 2007). Several clinical studies have shown that the common cow milk proteins that give positive skin reactions in infants are α-lactalbumin, α-s-1-casein and β-casein (Haelein, 2004; El-Agamy, 2007). Although goat milk proteins are similar to cow milk proteins in their general classifications, they differ in their frequencies and genetic polymorphisms (Grosclaude, 1995). The α-s-1-casein is the major α-s-casein protein in cow milk and it is also the major cause of allergic reactions to cow milk. In goat milk, the α-s-2-casein variant is the dominant α-s-casein (Ambrosoli, De Stasio and Mazzoco, 1988). The α-s-2-casein does not give positive skin reactions and is more digestible compared to α-s-1-casein (Ambrosoli *et al.,* 1988).

2.1.2 Nutritional properties of goat milk

Nutritional studies with Spanish rats that had malabsorption syndrome showed that these rats had significantly improved digestibility and improved copper and iron absorption when fed with goat milk compared to cow milk (Barrionuevo, Alferez, Lopez Aliaga, Sanz Sampelayo and Campos, 2002). In a similar study, Alferez, Barionuevo, Lopez Aliaga, Sanz Sampelayo, Lisbona, Robles and Campos (2001) showed that goat milk reduces total cholesterol levels due to the higher levels (36 % in goat milk versus 21 % in cow milk) of medium chain triglycerides (MCT). It is believed that the improved absorption of minerals in the digestive tract may be partly due to higher levels of essential amino acids in goat milk compared to cow milk. For example, the improved copper absorption is due to high cystine content in goat milk (Haelein, 2004). Table 1 shows differences in essential amino acids and the fatty acid contents of goat and cow milk. Goat milk contains higher MCT, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), all known to have beneficial health properties

particularly for cardiovascular conditions and for treatment of gastrointestinal disorders (Haelein, 2004). Also, Le Jaouen (1981) reported that the higher content of small fat globules in goat milk compared to cow milk makes goat milk more digestible which gives it a nutritional advantage.

Table 1: Average essential amino acid and fatty acid composition (g/10g milk) in proteins and lipids of goat and cow milk

Nutrients	Goat milk	Cow milk	Difference (%) for goat milk
Essential amino acids			
Threonine	0.163	0.149	+9
Isoleucine	0.207	0.199	+4
Lysine	0.290	0.261	+11
Cystine	0.046	0.030	+53
Tyrosine	0.179	0.159	+13
Valine	0.240	0.220	+9
Fatty acid composition			
C6-14 total MCT	0.89	0.61	+46
C4-18 total SFA	2.67	2.08	+28
C16:1-22:1 total MUFA	1.11	0.96	+16
C18:2-18:3 total PUFA	0.15	0.12	+25

MCT: Medium chain triglycerides; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids (Posati and Orr, 1976)

2.1.3 Goat milk products

Most dairy goat breeds reared on a small scale are dual purpose goats kept for their milk and meat. Goat milk is consumed raw, pasteurized or sterilized. It is also used in the production of connoisseur goat cheeses such as blue veined cheese, Feta and Manchego,

which are highly patronized in developed countries (Harding, 1995; Haenlein, 2004). Other goat milk products include goat milk powder, yoghurt, butter oil and cream (Pandya and Ghodke, 2007). In other societies, left over milk is allowed to sour naturally in clay pots, calabashes, or any suitable container into several indigenous dairy products such as Madila (Ohiokpehai, 2003), peculiar to Botswana and Amasi (Gran, Gadaga and Narvhus, 2003), which has a wider consumer base in the Southern African region.

2.1.4 General bacterial quality of goat milk

Although goat milk has several benefits, the major factor limiting its production is high losses of raw milk due to souring at ambient temperatures. There have been thorough studies of the microbiological quality of cow milk. However, information on the assessment of the microbiological quality of raw and processed goat milk is limited. Spoilage of raw goat milk by bacterial fermenters naturally present in milk, in the surrounding atmosphere or through fecal contamination hours after milk collection is an economic problem to goat milk production at rural centers. Microorganisms that occur in milk are usually due to unhygienic milk handling rather than transmission from the goat (Thompson and Thompson, 1990). In their study, Thompson and Thompson (1990) observed that hand milking as well as the cleanliness of the milker and the milking parlour present opportunities for contamination of raw milk.

Foschino, Invernizzi, Barucco and Stradiotto (2002) studied the general bacterial quality of raw goat milk in Bermago, Italy. In their study, they isolated several pathogens including *Escherichia coli, Listeria monocytogenes, Salmonella* spp., *Staphylococcus aureus* and *Staphylococcus caprae*. Lactic acid bacteria dominated the natural microflora of raw milk. These were composed mainly of lactobacilli and lactococci. Other bacteria such as enterococci, *Micrococcus*, coliforms and yeasts were also isolated (Foshino *et al.,* 2002). In another study conducted on bulk tank goat and ewe milk from 403 different farms in Switzerland, *Enterobacteriaceae* was isolated from 61.6 % of goat milk samples, *S. aureus* was detected in 31.7 % of goat milk, 23.0 % of the goat milk samples

were positive for *Mycobacterium avium* subsp. *paratuberculosis,* and 16.3 % were positive for Shiga toxin-producing *E. coli* (Muehlherr, Zweifel, Corti, Blanco and Stephan, 2003).

Several lactic cultures and yeast have also been isolated from artisanal goat cheeses from around the world. These comprise mainly of the genus groups *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Micrococcus, Leuconostoc*, and *Candida* (Tornadijo, Ferenso, Bernardo, Sarmiento and Carbello, 1995; Sablé, Portrait, Gautier, Letellier and Cottenceau, 1997). The origin of milk (i.e. the dairy farm) has been identified as a major factor affecting the variability of bacterial composition of milk (Foschino *et al.,* 2002; Oliver, Jayarao and Almeida, 2005). The interplay of several elements including composition of feed and contamination during milk collection determines the bacterial quality of raw milk (Foschino *et al*., 2002).

2.2 Significance of *Escherichia coli* O157:H7 as a foodborne pathogen

The occurrence of bacterial pathogens in milk and milk products is of significant public health concern. Prevalence of pathogens such as *S. aureus, L. monocytogenes, Campylobacter jejuni* and *E. coli* in milk has been well established over the years; however, little is known about the occurrence of shiga toxin-producing *E. coli* (STEC) in milk (Oliver *et al.,* 2005). Jayarao and Henning (2001) isolated several pathogens from bulk tank milk including STEC. Enteroheamorrhagic *E. coli* (EHEC), which is a subtype of STEC, is of particular importance due to the severity of disease, with most EHEC infections caused by *E. coli* O157:H7 (Oliver *et al.,* 2005; Chung *et al.,* 2006).

According to Perna, Mayhew, Posati and Blattner (2001), more than 75,000 cases of *E. coli* O157:H7 foodborne infections occur annually. *E. coli* O157:H7 is of critical public health significance because it has a low infectious dose of up to 100 cells and therefore is highly pathogenic. It causes acute illnesses including diarrhea associated haemorrhagic colitis (HC) characterized by severe abdominal cramps, watery diarrhea and subsequently

bloody diarrhea with little or no fever (Riley *et al*., 1983). Karmali, Petric, Lim, Fleming and Steele (1983) also reported that *E. coli* O157:H7 causes haemolytic uremic syndrome (HUS) manifested by acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia which follow bloody diarrhea. Non-bloody diarrhea, with long term sequelae is also typical of *E. coli* O157:H7 (Karmali, 1989; Paton and Paton, 1998).

Many ruminants including healthy cattle, sheep and goats naturally harbour *E. coli* O157:H7 in their intestinal tract (Oliver *et al.,* 2005). These ruminants shed *E. coli* O157:H7 suggesting that they provide a specific niche for them (Griffin and Tauxe, 1991; Hancock, Besser, Rice, Ebel, Herriott and Carenter, 1998). Transmission of *E. coli* O157:H7 occurs mainly by food and water, but also occurs by person-to-person contact and occupational exposure (Mead and Griffin, 1998). Foods such as unpasteurized milk and dairy products, undercooked hamburgers, apple juice and vegetables have been implicated in *E. coli* O157:H7 outbreaks (Steele, Murphy and Rance, 1982; Doyle, 1991; Oliver *et al.,* 2005). A list of milk, fermented milk and milk contact surfaces from which EHEC has been isolated is presented in Table 2.

Table 2: EHEC isolated from milk, milk products or contaminated milk contact surfaces

Location	Source	Reference
Argentina	Unpasteurized milk	Perez *et al*., 1994
Canada	Milk/milk filters	Borczyk *et al.,* 1987; Clarke *et al.,* 1989
Egypt	Unpasteurized milk	Abdul-Raouf *et al.,* 1996
France	Cheese from unpasteurized milk	Dechênes *et al.,* 1996
Italy	Unpasteurized milk	Foschino *et al.,* 2002
Scotland	Contaminated milk pipes/yoghurt	Morgan *et al*., 1988; Upton and Coia, 1994
Switzerland	Bulk tank milk	Muehlherr *et al.,* 2003

2.2.1 Virulence factors and pathogenesis

The major defining virulence factor of *E. coli* O157:H7 is the shiga toxin found on a bacteriophage gene inserted in the EHEC chromosome (Fig. 2.1). All *E. coli* O157:H7 cells carry the shiga toxin as well as other potential virulence factors in their chromosome and on a 60 MDa plasmid (Fig. 2.1) (Nataro and Kaper, 1998). Virulence genes such as *espA* or *espB* and genes that encode intimin and a type III secretion pathway are present in a 35 kDa LEE pathogenicity island in the chromosome of *E. coli* O157:H7 cells (McDaniel, Jarvis, Donnenberg and Kaper, 1995; Jarvis and Kaper, 1996). The genes found on the pathogenicity island encode attachment and effasive phenotype that is not always apparent in *E. coli* O157:H7 infections (Nataro and Kaper, 1998).

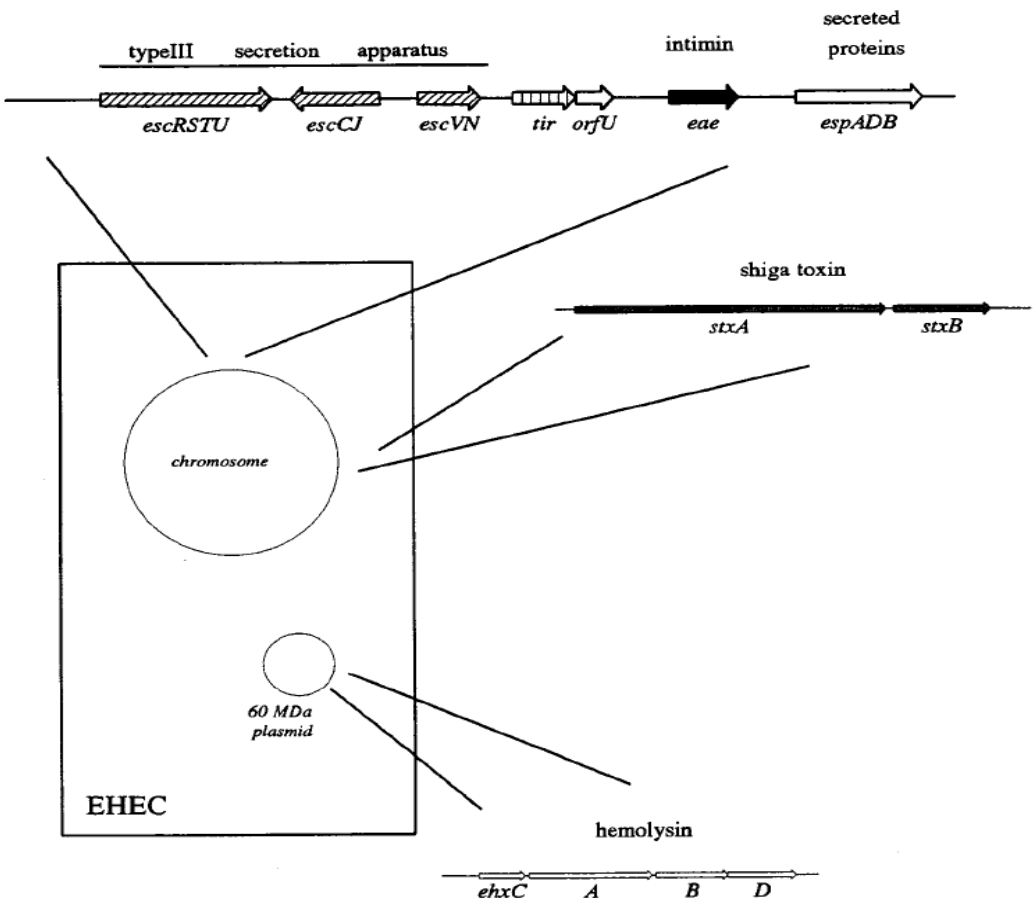

Figure 2.1: Genes involved in pathogenicity of Enterohaemorrhagic *Escherichia coli* (Nataro and Kaper, 1998)

Once ingested *E. coli* O157:H7 cells are able to survive the stressful conditions in the gut to cause disease in the intestine. *E. coli* O157:H7 produce one or two shiga toxins indistinguishable from the *Shigella dysenteriae* type 1 toxin (Nataro and Kaper, 1998). These are shiga toxin 1 encoded by *stx*1 and shiga toxin 2 encoded by *stx*2 (Nataro and Kaper, 1998). Most *E. coli* O157:H7 strains produce shiga toxin 2. Both shiga toxins are composed of an A subunit and five B subunits (Mead and Griffin, 1998). The B subunits bind to a glycolipid called globotriaosylceramide (Gb_3), located in eukaryotic cell membranes, for endocytosis of the toxin. Once in the cell, the A subunit inactivates the 60S ribosomal unit, blocking protein synthesis in the eukaryotic cell (Mead and Griffin,

1998). In addition to the shiga toxins, *E. coli* O157:H7 contains a plasmid that encodes a haemolysin which enables *E. coli* O157:H7 to use blood released into the intestine as a source of iron (Law and Kelly, 1995). The *E. coli* chromosome also contains a locus for erythrocyte effacement genes that encodes the adhesion molecule, intimin. Intimin enables *E. coli* adherence to mucosal cells in the large intestine, disrupting solute transport to cause non-bloody diarrhoea (Mead and Griffin, 1998). Post diarrhoeal haemolysis and renal failure can occur when the shiga toxins enter the blood and bind to Gb_3 rich endothelial cells of the kidney. Apart from the kidney, other organs such as the brain may be affected leading to a wide range of complications (Mead and Griffin, 1998). These are shown in Fig. 2.2A & B.

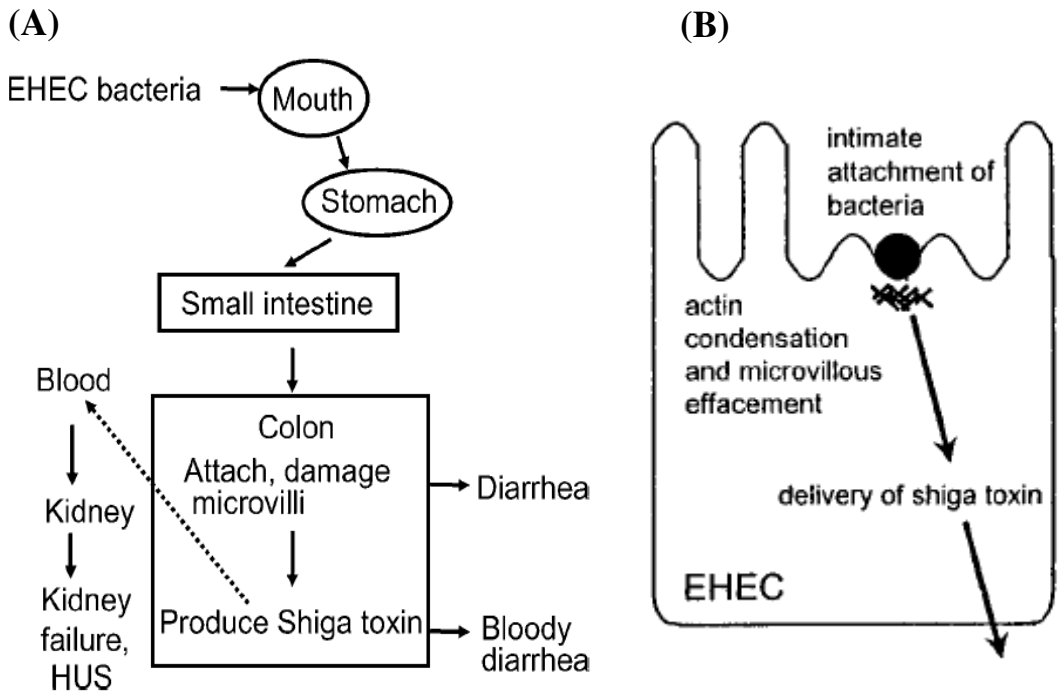

Figure 2.2: (A) The invasion pathway and diseases caused by Enterohaemorrhagic *Escherichia coli* (EHEC) (Gyles, 2007);
(B) Interaction of EHEC with the mucosal cells in the large intestine (Nataro and Kaper, 1998)

2.3 Preservation technologies applied in dairy processing

Due to the high occurrence of *E. coli* pathogens in dairy ruminants and severity of disease associated with *E. coli* O157:H7 contaminated dairy products, processing and preservation methods employed in processing of milk and milk products have to be designed to prevent their survival and growth. Preservation technologies commonly applied in dairy systems include chilling, pasteurization, acidification and recently, LP system to control bacterial numbers in milk and milk products. These technologies have been effective in destroying pathogenic bacteria or preventing their growth in dairy systems. However, recent studies have shown that stationary phase *E. coli* have the ability to adapt to multiple stresses, making it more virulent and difficult to eliminate in dairy products (Beales, 2004; Chung *et al.,* 2006).

2.3.1 Pasteurization of milk

Pasteurization has long been established as a heat treatment that is aimed at eliminating pathogenic vegetative microflora that are most resistant to thermal treatment, which is currently *Coxiella burnetii,* and significantly reducing non-pathogenic bacteria that may cause spoilage of raw milk (Hayes and Boor, 2001). Pasteurization temperatures applied to raw milk are listed in Table 3. Of all the pasteurization temperatures, the most commonly used temperature time combination is the high temperature short time (HTST) pasteurization where raw milk heat treated at 72 °C for 15 s (Hayes and Boor, 2001). Though less common, some small scale milk producers make use of the low temperature long time (LTLT) combination to pasteurize their raw milk at 63 °C for 30 min because of the ease in measuring the time temperature combination with their available infrastructure.

Table 3: The minimum pasteurization temperature and time combinations

Pasteurization temperature (°C)	Time
63[a]	30 min
72[a]	15 s
89	1 s
90	0.5 s
94	0.1 s
96	0.05 s
100	0.01 s

[a]Pasteurization temperatures commonly applied in dairy processing. The pasteurization temperature of milk containing ≥ 10 % fat or added sweeteners should be increased by 3 °C (Hayes and Boor, 2001)

Unlike sterilization, pasteurization is considered cheaper and more effective in eliminating vegetative bacteria without significantly altering the nutrients in milk (Ohiokpehai, 2003). Nonetheless, pasteurization preservation has to be used in conjunction with cooling to prevent growth of bacteria that survive the pasteurization treatment (Ohiokpehai, 2003).

Pasteurization is not always practiced by small-scale dairies due to lack of adequate equipment and fuel for pasteurization, and also to avoid destruction of native starter cultures that play a key role in natural fermentation and development of unique flavours of artisanal dairy products (Gran *et al.,* 2003).

2.3.2 Fermentation

Spontaneous fermentation of raw milk has been the traditional alternative to pasteurization and chilling preservation for decades. Fermentation preservation involves the production of organic acids from oxidation of carbohydrates by microbial fermenters, mainly lactic acid bacteria (LAB) which results in a product with desirable sensory

properties and enhanced shelf-life (Caplice and Fitzgerald, 1999). LAB mediate the process of fermentation via intricate metabolic and biochemical pathways that produce several end products and by products such as organic acids, alcohol, carbon dioxide, hydrogen peroxide and broad spectrum antimicrobial compounds that inhibit spoilage and pathogenic microorganisms present in the substrate (Caplice and Fitzgerald, 1999). All LAB ferment hexoses, mainly lactose, into lactic acid. Lactic acid serves multiple purposes in fermented milk. These include reduction of the pH of the product to preserve it, it enhances syneresis in cheese production, and it improves coagulation of caseins in the manufacture of yoghurt and sour cream (Hutkins, 2001).

Lactic acid is produced via two principal pathways that characterize LAB into homolactic fermenters and heterolactic fermenters. The homofermentative LAB produce lactic acid as the sole or major end product of fermentation via the Embden-Meyerhoff-Parnas pathway yielding 2 mol of adenosine triphosphate (ATP) and 2 mol Pyruvic acid per mole of hexose metabolized (Hutkins, 2001). Pyruvate is subsequently reduced to lactic acid resulting in ≥ 90 % conversion of glucose to lactic acid. This is shown in Fig. 2.3. The LAB that fall into this category include *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Pediococcus* (Caplice and Fitzgerald, 1999; Hutkins, 2001).

Alternatively, the heterofermentative LAB use the phosphor-ketolase pathway to generate 1 mol of ATP per mole of hexose and equimolar concentrations of lactic acid, acetic acid, ethanol and carbon dioxide. Most *Lactobacillus* spp. and *Leuconostoc* spp. are heterofermentative (Fig. 2.3) (Hutkins, 2001). The pathway used by LAB will have significant effect on texture, flavour, and quality of the fermented product. Also, product yield from both pathways may vary depending on conditions such as available substrate and incubation temperature (Hutkins, 2001).

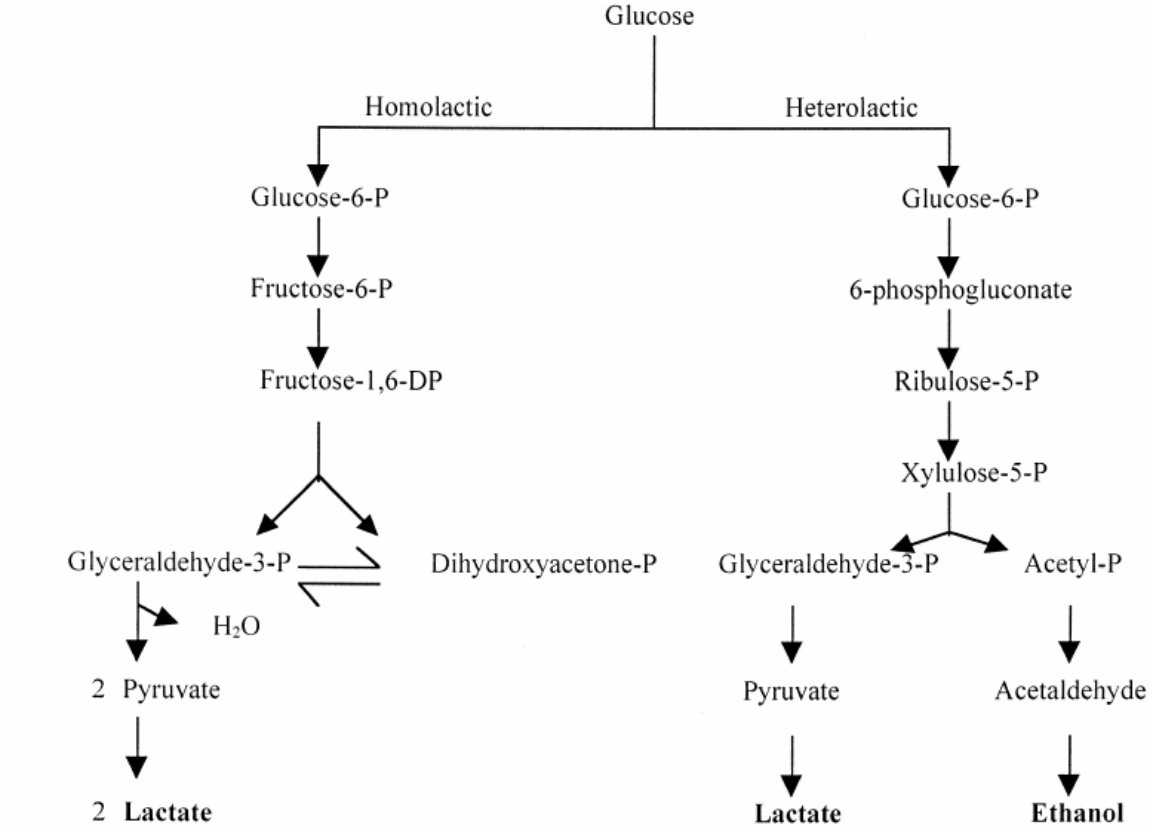

Figure 2.3: Glucose fermentation in homofermentative and heterfermentative lactic acid bacteria (Caplice and Fitzgerald, 1999)

The transport of lactose into the cell is also important because it can dictate the catabolism pathway for glucose metabolism. Also, since active transport is an energy consuming activity, much of the energy generated during catabolism is consumed in nutrient transport (Hutkins, 2001). Lactose enters the cell via a lactose permease (lactose carrier) into the cell followed by cleavage of lactose into glucose and galactose (Caplice and Fitzgerald, 1999). They can alternatively be transported into the cell via a phosphopyruvate dependent phosphotransferase system (PTS) followed by cleavage into glucose and galactose-6-phosphate. Most *L. lactis* used in dairy fermentations make use of the lactose PTS for transport of lactose into the cell (Caplice and Fitzgerald, 1999).

In addition to preservation of the fermented products by the end products of LAB

fermentation, LAB also produce organoleptic compounds and enzymes that influence the aroma, texture and taste of fermented dairy products (Hutkins, 2001).

2.3.2.1 Traditional fermented milk

Amasi is a traditional soured raw milk product made by allowing raw milk to ferment at ambient temperature for a period of 1 to 3 days depending on the fermentation temperature (Mutukumira, 1995). A lot of research has been done on Amasi in Zimbabwe; but, amasi is also traditionally and commercially produced in South Africa, Botswana and Namibia. Traditionally, left-over milk is allowed to sour in specialized containers such as clay pots and calabashes depending on the tribe or culture of the processors (Feresu and Muzondo, 1989; Beukes, Bester and Mostert, 2001). Feresu and Muzondo (1989) established from a sensory panel that clay pots were better containers for amasi fermentation compared to glass containers. Due to its popularity, amasi is one of many fermented traditional foods that have been up-scaled to industrial production to meet consumer demands for urban populations (Gadaga, Mutukumira, Narvhus and Feresu, 1999).

2.3.2.2 Lactic acid bacteria used for amasi fermentation

The technology of Amasi production makes use of lactic acid cultures naturally present in raw milk, the fermentation container that may harbour biofilms of lactic acid fermenters, and the surrounding air that may contain other indigenous fermenting bacteria (Gadaga *et al*., 1999). Backslopping with a previous batch is frequently done to speed up the fermentation process. Several lactic acid bacteria have been isolated from traditional Amasi. The predominant ones include *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, *Lactobacillus paracasei* subsp. *paracasei*, *Lb. paracasei* subsp. *pseudoplantarum*, *Lb. helveticus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. plantarum*, *Lb. acidophilus* and *Leuconostoc mesenteriodes* subsp. *mesenteriodes* (Feresu and Muzondo,

1990; Mutukumira, 1996). Other fermenters such as *Enterococcus faecum*, *Enterococcus faecalis* and yeast have been isolated from Amasi (Mutukumira, 1996). Some of these lactic acid bacteria have been developed to be used singly or in combination for industrial Amasi production (Mutukumira, 1996; Gadaga, Mutukumira and Narvhus, 20001). Mutukumira (1996) identified *Lc. lactis* subsp. *lactis* biovar *diacetylactis* of the C1 classification as a potential for Amasi production. In that study, *Lc. lactis* subsp. *lactis* biovar *diacetylactis* was found to coagulate milk within 18 h at 25 °C and it produces sufficient levels of diacetyl, acetaldehyde and lactic acid in modern Amasi. Also Amasi fermented with this strain was judged the most acceptable of all the strains tested by a sensory panel.

2.4 The lactoperoxidase system

The LP system is an antimicrobial system that has been recommended as an alternative to chilling for the preservation of raw milk, especially where lack of capital, unreliable energy supply and high ambient temperatures make chilling of raw milk practically unattainable (IDF, 1988; FAO/WHO, 2006). The antimicrobial action of the LP system involves the catalysis of the peroxidation of thiocyanate (SCN^-) in the presence of hydrogen peroxide (H_2O_2) into antibacterial hypothiocyanite ($OSCN^-$) and unstable thiocyanogen $(SCN)_2$, which hydrolyses quickly into hypothiocyanous acid (HOSCN) and other oxyacids by LP enzyme (Thomas and Aune, 1978; Reiter, 1985; Naidu, 2000). Other halides such as iodide and bromide can also serve as substrates of the LP enzyme.

$$SCN^- + H_2O_2 \xrightarrow{LP} OSCN^- + H_2O$$

$$2SCN^- + H_2O_2 + 2H^+ \xrightarrow{LP} (SCN)_2 + 2H_2O$$

$$(SCN)_2 + H_2O \longrightarrow HOSCN + SCN^- + H^+$$

The LP system has many applications as a natural preservative in the food industry, however, its use has been generally recommended for the dairy industry (Björck, 1987).

This antimicrobial system makes use of the LP enzyme naturally found in milk, saliva and tears of mammals (Wolfson and Sumner, 1993; Kussendrager and van Hooijdonk, 2000). The primary role of the LP enzyme is to protect the mammary gland and the gut of infants against bacterial infections (Naidu, 2000).

2.4.1 Characterization of the LP enzyme

The LP enzyme is a 78 kDa glycoprotein with a heme group at its active site. It also has a covalently bound calcium ion that stabilizes the molecular conformation of the enzyme and thus maintains its structural integrity (Boots and Floris, 2006). LP has high thermal stability in milk whey, permeate and buffer (Kussendrager and van Hooijdonk, 2000). Initial studies reported that LP is relatively heat stable, maintaining its activity at low pasteurization temperatures for extended periods of time (63 °C, 30 min) and at high temperatures for a short time period (72 °C, 15 s) (Barrett, Grandison and Lewis, 1999). However, Marin, Sanchez, Perez, Puyol and Calvo, (2003) showed that LP lost its activity slowly at temperatures below 70 °C with a remarkable decrease in LP activity at 72 °C. According to Korhonen (1980), LP is denatured when heated at 80 °C for 2.5 s. De Wit and van Hooydonk (1996) also reported complete inactivation of LP enzyme at 78 °C for 15 s. For this reason, Barrett *et al.* (1999) suggested that when activated LP milk is pasteurized, the LP system can be reactivated to extend the shelf life of milk. Since LP retains its activity at pasteurization temperatures applied to milk, it can be used in combination with heat treatment for the preservation of milk and milk products. However, at low pH (5.3) the LP enzyme is less heat stable. The loss of the calcium responsible for the structural integrity of LP enzyme has been proposed to be the likely reason for the lower denaturation temperature of LP at low pH (Kussendrager and van Hooijdonk, 2000).

2.4.2 LP activities in milk

Varying LP activities has been reported in bovine, caprine, ewe and buffalo milk. Fonteh,

Grandison and Lewis (2002) reported mean LP activities in cow and caprine milk as 2.3 U/ml and 0.1 U/ml respectively with a large variation in LP activity between individual animals. Higher LP activity has been reported by other authors. Seifu, Buys, Donkin and Petzer (2004) recorded a mean LP activity of 0.79 U/ml in Saanen goat milk while Saad de Schoos, Oliver and Fernandez (1999) reported a mean LP activity of 4.5 U/ml in Creole goat milk. The mean LP activities for buffalo and ewe milk have been reported as 0.9 U/ml (Härnulv and Kandasamy, 1982) and 4.0 U/ml (Chávarri, Santisteban, Virto and de Renobales, 1998) respectively.

2.4.3 Other components of the LP system

Apart from the LP enzyme, exogenous supply of thiocyanate and hydrogen peroxide are needed for a complete and functional LP system. Thiocyanate is naturally found in milk at levels *ca* 24.20 ppm in bovine milk (Fweja, Lewis and Grandison, 2007), *ca* 18.26 ppm in ewe's milk (Chávarri *et al*., 1998) and *ca* 10.29 ppm in goat milk (Saad de Schoos *et al.*, 1999). Although such high concentrations of thiocyanate occur in milk, actual levels of thiocyanate are highly variable. The mean thiocyanate concentrations in common dairying goats have been reported as 4.58 ppm in SAIB goats and 2.78 ppm in Saanen goats (Seifu *et al.,* 2004). Dietary sources of thiocyanate in milk are glucosinolates and cyanogenic glucosides (Reiter, 1985). Fodder and seed meals prepared from the genus *Brassica* are the major source of glucosinolates in ruminant diets (Tripathi and Mishra, 2007). Glucosinolate content and distribution in Brassica originated ruminant feed and fodder however varies with the variety and origin of the pant species, agroeconomic practise and climatic conditions (Tripathi and Mishra, 2007). In some developing countries, goats are fed with leaves and rind of cassava and potatoes which contain cyanogenic glucosides. These components hydrolyse to form thiocyanate in addition to other reaction products (Reiter and Härnuluv, 1984). Kussendrager and van Hooijdonk (2000) also explained that the thiocyanate levels found in milk varies with breed, species, feed and udder health of animal. Chávarri *et al.,* (1998) also reported that thiocyanate concentrations in ewe's milk varied with season of milking. In their study, they recorded

high levels of thiocyanate in ewe's milk in the winter months when the sheep were fed with dry fodder and the lowest levels recorded in summer.

Since the thiocyanate level required for activation of the LP system is 14 ppm (CAC, 1991), exogenous source of thiocyanate has to be added to fully activate the LP system. Unlike thiocyanate, hydrogen peroxide may not be detected in milk under normal conditions (FAO, 1999). However, it can be generated endogenously by polymorpholeucocytes during phagocytosis or by LAB such as lactococci, lactobacilli and streptococci during growth under aerobic conditions (Wolfson and Sumner, 1993; de Wit and van Hooydonk, 1996).

2.4.4 Antimicrobial action of LP system

The LP system has been shown to have a bacteriostatic effect on *E. coli* in goat milk and a bactericidal effect on several other Gram-positive and Gram-negative bacteria (Seifu *et al.,* 2004; Seifu, Buys and Donkin*,* 2005). The antimicrobial effect of the LP system stems from the reaction of unstable hypothiocyanite with sulfhydryl groups in cell membrane proteins and low molecular weight components of cytoplasmic thiols into disulfides, sulfenyl thiocyanates or sulfenic acids (Thomas and Aune, 1978).

$$\text{Protein-SH} + OSCN^- \longrightarrow \text{Protein-S-SCN} + OH^-$$

$$\text{Protein-SH} + (SCN)_2 \longrightarrow \text{Protein-S-SCN} + SCN^- + H^+$$

$$\text{Protein-S-SCN} + H_2O \rightleftharpoons \text{Protein-S-OH} + SCN^- + H^+$$

These reactions inhibit bacterial enzymes responsible for respiration and metabolism (Shin, Hayasawa and Lonnerdal, 2001). These reactions consequently cause alteration of the cell membrane, loss of internal cell components such as leakages of potassium ions, amino acids and polypeptides, and cessation of essential cell functions mainly respiration and metabolism, which result in eventual cell death (Thomas and Aune, 1978; Thomas, 1985; Hirano, Hirano, Oooka, Dosako, Nakajima and Igoshi, 1998). Unlike

pasteurization and fermentation, the LP system does not render raw milk safer for consumption. It however preserves the initial quality of the product (FAO/WHO, 2006).

The LP system has a limited time for preservation of raw milk depending of the temperature of storage. The Codex Alimentarius Commission (CAC) guidelines (1991) indicated the use of LP system at ambient temperatures ranging 15 to 30 °C, however, ambient temperatures may exceed 30 °C during day time in some regions. Several studies have indicated a bactericidal effect of LP system against spoilage and pathogenic bacteria that occur in milk. Seifu *et al.,* (2004) demonstrated a bacteriostatic effect of LP system on *E. coli* and *S. aureus* and a bactericidal effect on *L. monocytogenes* and *Brucella melitensis* in goat milk incubated at 30 °C for 6 h. Both bacteriostatic and bactericidal effects of LP system on *Salmonella* Typhimurium have been demonstrated (Purdy, Tenovuo, Pruitt and White, 1983; Wolfson and Sumner, 1993). The efficacy of LP system against *S.* Typhimurium is dependent on the cell concentration used, the lower the cell concentration, the more lethal the effect of LP, and the permeability of the cell wall, with rough mutants showing higher susceptibility to LP system. Inhibition of other pathogens such as *Campylobacter jejuni* and vegetative cells of *Bacillus cereus* have been reported (Beumer, Noomen, Marijs and Kampelmacher, 1985; Tenovuo, Makinen and Sievers, 1985; Pruitt and Kamau, 1991).

LP system can also be applied in combination with treatments used in preservation of milk. The LP system has been shown to delay proliferation of psychrotrophic spoilage bacteria and thus prolong the keeping quality of raw milk stored under chilling conditions for several days (FAO/WHO, 2006). Zapico, Gaya, Nuñez and Medina (1995) demonstrated a bactericidal effect of LP system on *Pseudomonas fluorescence* for five days at 4 °C and three days at 8 °C in goat milk. This was supported by another study by Lin and Chow (2000) who showed that the activated LP system in combination with chilling at 4 °C extended the keeping quality of raw cow milk for six days. The use of the LP system in combination with heat treatment has also proved effective in eliminating vegetative microorganisms in milk (Marks *et al.,* 2001; Seifu *et al.,* 2004). In another

study, Van Opstal, Bagamboula, Theys, Vanmuysen and Michiels (2006) demonstrated that the LP system in combination with low pH reduced *E. coli* and *Shigella* cell numbers to undetectable levels after 24 h exposure in apple (pH 3.3), orange (pH 3.8) and tomato (pH 4.1) juices as compared to untreated LP samples. Also, Vannini, Lanciotti, Baldi and Guerzoni (2004) investigated the use of high pressure homogenization together with the activated LP system and lysozyme on several bacteria. In their study, *L. monocytogenes*, and *E. coli* showed resistance to high pressure homogenization at 75 MPa, 100 MPa and 130 MPa. However, when these pressures were applied in combination with LP at 37 °C, both *L. monocytogenes* and *E. coli* were reduced to undetectable levels.

An important limitation to the application of the LP system in milk preservation is its effect on the viability and acid production of lactic starter cultures used in dairy fermentations. The biochemical effect that the LP system has on individual lactic cultures varies with inherent resistance of the LAB culture and the type of milk used (Rysstad and Abrahamsen, 1983). In a study by Seifu, Buys and Donkin (2003) to investigate the effect of LP system on several cheese starter cultures in goat milk, they observed different levels of sensitivity to the LP system. While exposure to the LP system caused a general decrease in lactic acid production by a mixed culture composed of *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris, Lc. lactis* subsp. *diacetylactis* and *L. mesenteriodes* subsp. *cremoris,* pure cultures of *Lc. lactis* subsp. *diacetylactis* NCDO 176 and *L. mesenteriodes* subsp. *cremoris* ATCC 33313 were unaffected by the LP. In the same study, a mixed culture LL 50C made up *of Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* showed resistance to the LP system with increased lactic acid production compared to the untreated LP culture. Screening of several strains of LAB for sensitivity to LP system is therefore necessary in order to develop LP resistant lactic starter cultures that can be effectively used in the production of fermented dairy products from activated LP milk.

2.5 *E. coli* general stress response

E. coli strains have developed signal transduction systems that sense physical and chemical challenges that fluctuate in their natural environment. Over the years, these systems have led to the evolution of elaborate adaptive response systems that enable *E. coli* to possess an enhanced survival capacity under several harsh conditions. The general stress response of *E. coli* to unsuitable conditions is characterized by physiological and sometimes, morphological alterations that increase cell resistance (Hengge-Aronis, 2000b). These survival mechanisms are triggered under suboptimal conditions by controlling coordinated expression of cell defence mechanisms (Chung *et al.*, 2006). Hengge-Aronis (2000b) explained that the age old adage 'prevention is better than cure' is a principle that *E. coli* stress response systems very well adhere to. Once *E. coli* senses stresses such as starvation, high osmolarity, high or low temperature, acidic pH and antimicrobial chemicals in the environment, it not only activates but also accumulates stress regulatory systems that induce stress response genes which protect cells under lethal environmental conditions (Chung *et al.,* 2006; Hengge-Aronis, 2000b). The common regulatory systems involve sigma factors. These are small proteins that bind RNA polymerase (RNAP) to direct expression of protective genes in response to external stresses (Chung *et al.,* 2006). In *E. coli*, sigma S, also known as RpoS (σ^s), is the master regulator of stress response genes, and it controls over 60 genes that confer stress tolerance (Hengge-Aronis, 2002). This system is involved in the prevention rather than repair of cellular damage caused by external stresses. RpoS has been extensively studied in enteric bacteria in both exponential and stationary growth phases (for a comprehensive review, see Hengge-Aronis 2002). The current view is that RpoS is elicited by several forms of stress which are often accompanied by reduction or cessation of growth. This allows survival from the apparent stress being experienced in addition to other stresses not yet encountered, a principle known as cross-protection (Hengge-Aronis, 2000b).

2.5.1 Properties and functions of RpoS

The *rpoS* gene encodes the sigma factor known as sigma S or the RpoS protein. The RpoS is sometimes referred to as sigma 38. This is because the molecular mass of RpoS isolated from the common wildtype *E. coli* strains MC4100 and W3110 is 37.8 kDa (Hengge-Aronis, 2000b). Studies have however shown that the *rpoS* locus is highly polymorphic and varying sizes of *rpoS* mutants have been identified in laboratory and natural *E. coli* isolates (King, Ishihama, Kori and Ferenci, 2004; Notley-McRobb, King and Ferenci, 2002; Kolter, 1999).

RpoS is an alternative sigma factor that, like all other sigma factors, binds RNA polymerase holoenzyme ($E\sigma^s$) to increase specificity for promoter regions of stress response genes (Chung *et al.,* 2006). Under normal growth conditions, the housekeeping sigma factor σ^{70}, which is constitutively expressed in enteric bacterial systems, directs transcription of several genes required for optimal growth. However, there are alternative sigma factors that are induced under stress conditions that identify specific promoters for 'stress protection' regulons. These regulons are a group of genes that regulate expression of specific stress response genes (Chung *et al.,* 2006).

RpoS appears to be the most significant stress-induced alternative sigma factor expressed under suboptimal growth conditions. Unlike other alternative sigma factors that are triggered by a specific stress, RpoS is activated under multiple stresses including nutrient limitation, near UV-radiation, high osmolarity, low and high temperature, low pH, ethanol, and antimicrobial compounds (Hengge-Aronis, 2002). In fact, RpoS is sometimes considered a second primary sigma factor. This is because σ^s and σ^{70} are closely related and actually bind similar promoter sites (Becker and Hengge-Aronis, 2001). It has been reported that genes transcribed by $E\sigma^s$ *in vivo* can often be transcribed *in vitro* by $E\sigma^{70}$ (Weber, Polen, Heuveling, Wendisch, Hengge, 2005; Becker and Hengge-Aronis, 2001). Several σ^s–dependent genes are activated when $E\sigma^s$ accumulates in the cell. However, there are other stress response genes that are controlled by RpoS

independent co-regulators or other alternative sigma factors which may be active under a specific stress or environmental condition (Weber *et al.,* 2005). This indicates that RpoS works in conjunction with other regulating factors to modulate stress response in *E. coli* systems. Therefore, the physiological and morphological changes that are elicited under unfavourable conditions may differ depending on the environmental conditions which will in part determine which regulators will modulate the expression of a specific or co-ordinated stress response.

The primary function of the RpoS is to confer a broad range stress response that prevents cellular damage (Hengge-Aronis, 2000b). Other RpoS mediated functions include changes in cell morphology in an unfavorable environment, changes in cell metabolism from optimal growth to maintenance mode metabolism, programmed cell death to increase survival of a fraction of the bacterial population, and activation of virulence genes (Hengge-Aronis, 2002; Hengge-Aronis, 2000a; Bishop, Leskiw, Hodges, Kay and Weiner, 1998).

2.5.2 Regulation of RpoS

Although RpoS plays a vital role during the stationary phase, it is dispensable and in fact present at very low levels during exponential growth (Hengge-Aronis, 2002). In general, activation of alternative sigma factors occurs via a signal transduction system (Hengge-Aronis, 2002). In *E. coli*, the parameter that activates the RpoS-induced stress response is the cellular level of the RpoS protein (Hengge-Aronis, 2002). RpoS regulation is complex involving several global regulatory factors and RNA binding elements that determine RpoS protein concentration at both log and stationary phase growth. RpoS regulation occurs at the transcriptional, translational and post-translational levels (Hengge-Aronis, 2002). A schematic diagram of RpoS regulation is presented in Fig. 2.4.

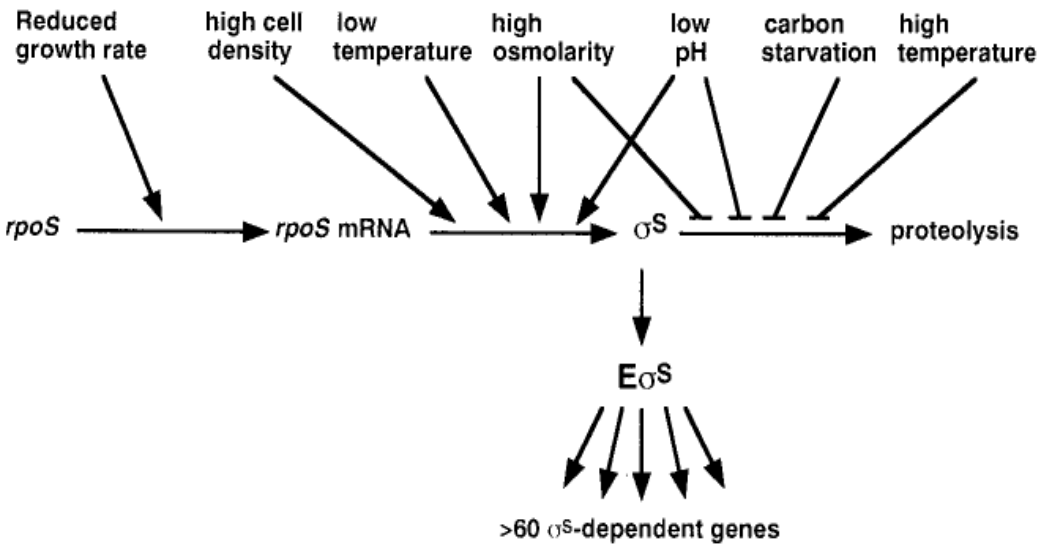

Figure 2.4: RpoS regulation is differentially affected by various stress conditions. An increase in cellular levels of RpoS is modulated by activating *rpoS* synthesis at transcriptional level from the *rpoS* gene or at the translational level from the *rpoS* mRNA. Stabilization of the RpoS protein by inhibition of proteolysis (which occurs rapidly under optimal conditions) is another method used to increase cellular RpoS levels. (Hengge-Aronis, 2002)

Although RpoS occurs at very low levels in log phase growth, studies have shown that the *rpoS* mRNA is present at high levels during the log phase (Hengge-Aronis, 2002). The *rpoS* mRNA levels remain fairly constant during several environmental perturbations. This suggests that *rpoS* mRNAs are routinely synthesized during log phase growth. Nonetheless, transcriptional regulation of *rpoS* appears to be controlled by growth phase and nutrient limitation (Hengge-Aronis, 2002). The major *rpoS* promoter involved in *rpoS* transcription in *E. coli* is reportedly located in the *nlpD* gene. It encodes a monocistronic *rpoS* mRNA (Lange and Hengge-Aronis, 1994). Other trans-acting factors such as the cyclic adenosine monophosphate (cAMP) receptor protein (CRP) and the glucose specific EII component of the phosphotransferase system (EIIA(Glc)) are negative regulators of *rpoS* transcription during the log phase (Hengge-Aronis, 2002). The cAMP-CRP can however act as an activator of *rpoS* transcription during stationary

phase when bound upstream of the *rpoS* promoter site (Hengge-Aronis, 2002). Under conditions of nutrient limitation (starvation for amino acids, carbon, nitrogen and phosphorus) *E. coli* cells accumulate guanosine tetraphosphate (ppGpp) which modulates a change in cellular metabolism culminating in bacterial adaptation (Chatterji and Ojha, 2001). This phenomenon is termed stringent control. Stringent control is known to be one of the factors that regulate RpoS at the transcriptional level, however its mechanism of action has not been fully elucidated. Since ppGpp does not bind to promoter sites, it is suggested that it regulates *rpoS* indirectly by affecting elongation and/or stability of *rpoS* transcripts (Lange, Fischer, and Hengge-Aronis, 1995).

Once transcribed, the *rpoS* mRNA forms a stable secondary structure which is not easily accessible by ribosomes for translation. However, studies have shown that trans-acting factors such as Hfq (host factor 1) and HU proteins alter the *rpoS* mRNA secondary structure to initiate translation, or in the case of Hfq, recruits translational factors that positively regulates *rpoS* translation (Hengge-Aronis, 2002). In contrast, other proteins such as the histone-like proteins (H-NS) and OxyS negatively control *rpoS* translation. Stresses such as temperature and pH downshifts also influence *rpoS* translation (Fig. 2.4).

At optimal growth conditions, RpoS synthesis still occurs but concentrations are maintained at basal level. The low basal levels of RpoS are due to rapid degradation by the ClpXP protease which is recruited by the response regulator RssB to the RpoS protein (Hengge-Aronis, 2002). The RssB directed proteolysis of RpoS depends on phosphorylation of its receiver domain. RssB levels are controlled by feedback regulation based on cellular levels of RpoS (Hengge-Aronis, 2002). Several environmental stresses such as carbon starvation, low pH and high osmolarity also influence RpoS proteolysis by stabilizing the RpoS protein (Lange and Henge-Aronis, 1994; Takayanagi, Tanaka and Takahashi, 1994; Muffler, Traulsen, Lange and Hengge-Aronis, 1996; Bearson, Benjamin, Swords and Foster, 1996).

2.6 *E. coli* response to acid stress

Among the stresses that *E. coli* encounters in its environment, low pH ranks as the most common and most critical barrier, especially for pathogenic *E. coli* that has to breach lethal acidic pH (pH 2) in the stomach (Audia, Webb and Foster, 2001) and volatile fatty acids in the small intestine (Bearson, Bearson and Foster, 1997) to cause disease. Besides being a stress to be endured by pathogenic *E. coli*, acidic pH is also a signal that triggers the induction of several virulence genes because the low pH is an indication that the organism has entered a potential host environment (Audia *et al.,* 2001).

Bearson *et al.* (1997) defined acidic stress as "the combined biological effect of low pH (i.e. H^+ ion) and weak acid concentrations". Though inorganic and organic acids use different mechanisms of microbial inactivation, they both result in acidification of the cytoplasm resulting in inactivation of key proteins and enzymes (Chung *et al.,* 2006). Weak acids are present in the small intestine. They can also be used alone or in combination with low pH in the preservation of foods (Foster, 2000; Beales, 2004). Examples of weak acids include sorbate, butyrate, benzoate, propionate or acetate (Foster, 2000; Beales, 2004). Unlike strong acids, weak acids in their undissociated forms do not reduce the environmental pH, however their protonated forms can diffuse across the cell membrane into the bacterial cell interior where, upon encountering a high internal pH, dissociate to reduce the internal pH (Foster, 2000). This is illustrated in Fig. 2.5. In *E. coli*, the weak acids disrupt proton motive force, which then allows diffusion of H^+ ions into the cell to inhibit metabolic enzymes (Beales, 2004).

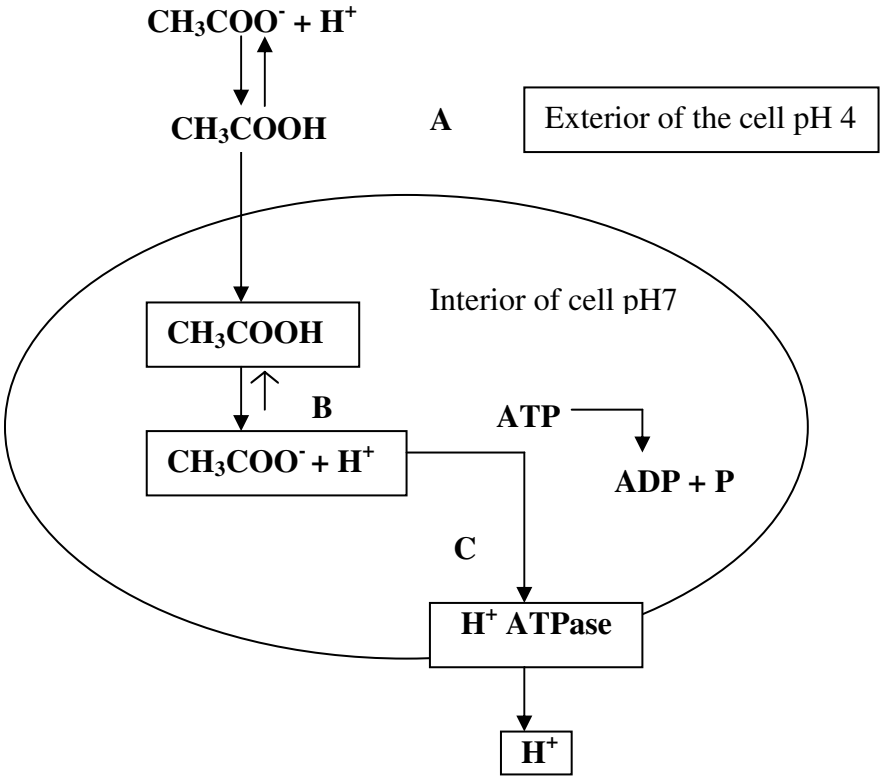

Figure 2.5: Interaction of weak acids in the microbial cell. (A), Exterior of the cell favours undissociated weak acid; (B), On entering the cell, the interior of the cell favours dissociated molecule and acid dissociates; (C), Proton pumps use ATP to remove excess H^+ ions (Beales, 2004).

Under normal growth conditions, the permeability of *E. coli* cell membranes to H^+ ions is low; however, when the external pH is extremely acidic, H^+ ions will be able to leak across the cell membrane to acidify the cytoplasm (Foster, 2000). Nonetheless, *E. coli* has developed a variety of strategies to combat biological effects of acid stress.

E. coli O157:H7 can grow in an environment of pH values ranging 4.5 to 8.0 while maintaining a neutral internal pH (Beales, 2004). Here, *E. coli* uses housekeeping pH homeostatis systems that regulate the internal cell pH at a pH range of 7.6 to 7.8 while the external pH fluctuates between one pH unit above or below optimum growth pH

(Rimon, Gerchman, Olami, Schuldiner and Padan, 1995). Two principal pumps known as the potassium-proton antiporters and the sodium-proton antiporters maintain a stable internal pH. The potassium-proton antiporters regulate internal pH when the external pH shifts to mild acidic environments by pumping out H^+ ions. This process results in alkalinization of the cytoplasm. On the other hand, the sodium-proton antiporter extrudes sodium ions from the cytoplasm in exchange for H^+ ions to acidify the cytoplasm under alkaline conditions (Zilberstein, Agmon, Schuldiner and Padan, 1982; Dibrov, 1991).

Apart from the housekeeping pH homeostatis systems, *E. coli* also possesses an incredible ability to adapt to mild acidic conditions that enable survival in extreme acid conditions (Leyer, Wang and Johnson, 1995). Although *E coli* strains vary in their acid resistance, it has been shown that pathogenic *E. coli* such as *E. coli* O157:H7 are generally more acid tolerant than non-pathogenic strains (Gorden and Small, 1993).

Acid adaptation has been shown to greatly improve survival in several low pH foods. Leyer *et al.,* (1995) demonstrated enhanced survival of acid-adapted *E. coli* O157:H7 in shredded salami at pH 5.0, apple cider at pH 3.4 and during sausage fermentation.

Acid stress response mechanisms have been extensively studied in *Salmonella, Shigella* and *E. coli* systems (Bearson *et al.,* 1997). The acid stress response terminologies that recur in literature include acid habituation (AH), acid tolerance response (ATR) and acid resistance (AR). These terminologies may indicate different acid stress mechanisms that are growth phase and growth medium dependent or may indicate different methods of testing acid stress response in enterobacteriaceae. It is difficult to compare these three acid response systems because some of these terminologies are used interchangeably, especially AH and ATR in log phase acid stress response. Also, factors such as testing methods, media, pH and *E. coli* strain differences contribute to inconsistencies in defining acid stress response in *E. coli*. Nonetheless, AH, ATR and AR systems are interconnected in a complex network of regulatory acid stress response cascade.

2.6.1 Acid tolerance response

Two acid tolerance response systems have been detailed for *Salmonella enterica* serovar Typhimurium. These are the log phase ATR and the stationary phase ATR (Audia *et al.,* 2001). The log phase inducible ATR has been shown to occur in *E. coli* systems as well (Foster, 2000). ATR mainly involves induction of regulatory factors during log phase growth in a mild acid environment, usually in a minimal medium, which activates proteins that protect cells at pH 3.0 for several hours (Foster, 2000). Foster and Hall (1991) illustrated that log phase growth of *Salmonella* in a minimal unsupplemented medium at pH 7.7 and subsequent adaptation at pH 5.8 results in activation of pH homeostasis systems that maintain a near neutral internal pH of cells and are thus protective against low pH macromolecular damage. At a lower adaptation pH of 4.5, cells induce several acid-shock proteins (ASP) that enhance survival not only to acid stress but to unrelated antagonistic environmental conditions as well (Audia *et al.,* 2001). According to Foster (2000), the ASPs are only induced by changes in the internal pH of the cell while other protective systems, for example membrane bound components, respond to changes in external pH. It therefore appears that optimal tolerance to acid treatment in *Salmonella* occurs via a two-step process: the pre-ASPs stimulated at pH 5.5 and the ASPs stimulated at lower pH.

Regulatory elements identified as playing vital roles in log phase ATR in both *Salmonella* and *E. coli* cells include RpoS, PhoP and Fur (Foster, 2000). These three regulatory elements use independent mechanisms to sense acid shock and they each regulate different sets of ASPs. According to Foster (2000), RpoS, PhoP and Fur activate ten, four and five ASPs respectively.

At neutral pH, RpoS is regulated by MviA in *Salmonella* and RssB homolog in *E. coli* which recruits ClpXP to degrade RpoS (see above for regulation of RpoS). The mechanism of sensing low pH is unknown, however, it has been shown that phosphorylation of RssB by acyl phosphate is crucial for the high turnover of RssB

(Bouche, Klauck, Fischer, Lucassen, Jung and Hengge-Aronis, 1998). It therefore appears that at low pH, the enzymes that phosphorylate RssB could be inhibited resulting in accumulation of RpoS in log phase cells (Foster, 2000). RpoS regulates ASPs that confer protection against both inorganic and organic acid stress (Foster, 2000).

PhoP is a response regulator which, together with the membrane bound sensor kinase PhoQ, forms a two-component regulatory system. PhoQ senses Mg^{2+} levels in the periplasm and phosphorylates PhoP (Garcia Vescovi, Soncini and Groisman, 1996; Soncini, Vescovi, Solomon and Groisman, 1996). Phosphorylated PhoP then activates genes that express ASPs. Bearson, Wilson and Foster (1998) suggested that PhoQ has the ability to sense low pH in addition to low Mg^{2+} levels to phosphorylate PhoP. In their study with *rpoS* mutant *Salmonella* cells, *phoP* and *phoQ* mutants showed sensitivity to inorganic acid stress but these genes were not essential for survival of inorganic acid stress in the presence of RpoS.

The third regulatory element, Fur (ferric uptake regulator) represses genes that encode iron transport systems when bound to intracellular Fe^{2+}. It has also been shown to positively regulate a subset of ASPs independent of intracellular concentration of Fe^{2+} (Hall and Foster, 1996; Foster and Hall, 1992; Bagg, and Neilands, 1987). Foster and Hall (1992) found that *Salmonella fur* mutants displayed acid sensitivity due to loss of *fur*-dependent genes that encode a subset of ASPs.

2.6.2 Acid habituation

Acid habituation of *E. coli* cells was first reported by Goodson and Rowbury (1989). They reported an acid-adaptation system very similar to ATR in *Salmonella*. For that reason, most authors refer to log phase acid adaptation as ATR in *Salmonella* systems and AH in *E. coli* systems. Both AH and ATR are induced in minimal medium when cells are exposed to mild acid (pH 5.0) conditions which enhance survival to lethal acid exposure. At a later stage, Raja, Goodson, Chui, Smith and Rowbury (1991) discovered

that when *E. coli* is pretreated at pH levels ranging from pH 4.0 to pH 6.0 in a rich medium, it elicits the same degree of protection as ATR in *Salmonella*. However, Foster (2000) suggested that AH and ATR are mechanistically different since AH occurs in a rich medium (usually Luria Bertani or Nutrient broth) while ATR occurs in minimal medium. He also explained that AH involved short exposures (7 min) of adapted *E. coli* to pH 3.0 unlike in ATR. Further studies by Paul and Hirshfield (2003) indicated that AH in *E. coli* is also a two stage process: the first occurs at milder pH of 5.5 while the second occurs at pH 4.3; both of these AH response showed different profiles on a two dimensional polyacrylamide electrophoresis gel. They also reported that exposure time at mild pH levels had an effect on acid survival at pH 3.0. Olsen (1993) observed that unlike activation of AH at pH 5.5, AH at pH 4.3 induces heat shock proteins such as GroEL, DnaK, HtpG and HtpM (Heyde and Portalier, 1990) in addition to ASPs. These studies indicate that AH at pH 5.5 may induce protective mechanisms to protect cells at lethal pH levels only, while AH at 4.3 may induce cross-protection to lethal pH as well as other stress that *E. coli* may encounter in its environment.

Regulation of AH has not been fully unraveled however, compounds such as glucose, glutamate, aspartate, iron chloride, potassium chloride and L-proline can induce AH in log phase cells at neutral external pH (Foster, 2000). Also, studies have shown that CysB, phosphate and cAMP influence AH regulation (Rowbury and Goodson, 1998; Rowbury and Goodson, 1997; Rowbury and Goodson, 1993).

2.6.3 Acid resistance of *E. coli*

According to Foster (2000), the most dramatic acid stress response occurs at the stationary phase. Stationary phase acid stress response is also termed acid resistance (AR). Once *E. coli* is exposed to mild acidic conditions (pH 4.5 to 5.5) in a complex medium, it induces an acid stress response that confers AR at subsequent lethal pH levels ($4.5 > pH \leq 2.5$) for extended periods of time (Cheng, Yu and Chou, 2003; Foster, 2000). Both log phase and stationary phase *E. coli* can induce AR under mild acidic conditions,

though stationary phase AR varies greatly from strain to strain (Buchanan and Edelson, 1996). Three complex cellular AR systems have been extensively studied in stationary phase *E. coli* (Lin, Lee, Frey, Slonczewski, and Foster, 1995). These are the oxidative AR, glutamate-dependent AR and arginine-dependent AR systems. The lysine and ornithine decarboxylases have also been identified to contribute, though poorly, to acid resistance in *E. coli* (Foster, 2000; Diez-Gonzalez and Karaibrahimoglu, 2004).

2.6.3.1 The oxidative acid resistance system

The oxidative AR system, also known as the glucose repressed system, is induced at late exponential phase and at stationary phase (Dodd and Aldsworth, 2002). It occurs in oxidative metabolizing cells in the absence of glucose (i.e. during starvation). Induction of the oxidative AR system is highly dependent on the expression of the alternative sigma factor RpoS however the mechanism of reaction is unknown (Lange and Hengge-Aronis, 1991). RpoS is maximally activated upon entry into stationary phase (Lange and Hengge-Aronis, 1991; Dodd and Aldsworth, 2002). It controls several *E. coli* stress response genes including acid stress response genes (Dodd and Aldsworth, 2002) by binding to RNAP which results in increased specificity for promoter regions for stress response genes (Chung *et al.,* 2006). In addition to inducing AR at moderate acidic conditions (pH > 3) at stationary phase (Chung *et al.,* 2006), RpoS also induces stress response to heat and osmotic stress (Hengge-Aronis, Klein, Lange, Rimmele and Boss, 1991), oxidative stress (Loewen and Hengge-Aronis, 1994) as well as other genes that participate in starvation resistance, cell wall synthesis, cell division and protection of DNA (Hengge-Aronis, 1996).

2.6.3.2 The pH homeostasis systems

The glutamate-dependent AR and arginine-dependent AR are pH homeostasis systems that protect *E. coli* cells at low pH levels (pH ≥ 2.5) (Lin *et al.,* 1996). It has been

reported that the glutamate dependent AR system is more effective in protecting *E. coli* cells at low pH levels compared to arginine dependent AR system. However, both AR systems require the extracellular glutamate and arginine for complete activation (Lin *et al*., 1995; Chung *et al*., 2006).

2.6.3.2.1 Glutamate decarboxylase acid resistance system

The glutamate dependent AR system is composed of three genes: *gadA, gadB* and *gadC* (Smith, Kassam, Singh and Elliot, 1992). The *gadA* and *gadB* genes encode isoforms of glutamate decarboxylase that catalyze the conversion of glutamate to γ-aminobutyric acid (Smith *et al.,* 1992). In their study, Castanie-Cornet, Penfound, Smith, Elliot and Foster (1999) discovered that while a single GAD isoform will protect *E. coli* cells at pH 2.5, both GadA and GadB are required for acid resistance at pH 2.0. The third gene, *gadC*, encodes a transmembrane glutamate: γ-aminobutyric acid antiporter. The glutamate dependent AR system maintains a near neutral internal pH by consuming a proton during glutamate decarboxylation and exchanges the end product (γ-aminobutyric acid) with glutamate via the glutamate: γ-aminobutyric acid antiporter (Chung *et al.,* 2006). This process increases the internal pH of the cell. The *gadB* and *gadC* occur as a linked operon on the *E. coli* chromosome while the *gadA* gene is transcribed from a separate promoter (Audia *et al*., 2001).

Regulation of the glutamate-dependent AR is complex. While some authors report that the glutamate-dependent AR system is induced in log phase under acid conditions, others suggest that the *gad* genes are highly expressed in log phase but remain inactive (Audia *et al.*, 2001; Castanie-Cornet *et al.,* 1999). The *gad* genes are regulated by the catabolyte repressor protein (CRP, encoded by *gadY*), H-NS and cAMP (Castanie-Cornet and Foster, 2001; Castanie-Cornet *et al.,* 1999, De Baise, Tramonti, Bossa and Visca, 1999). Audia *et al.,* (2001) proposed a model that attempts to elucidate regulation of the glutamate-dependent AR system. The authors explained that in a complex medium, when

CRP-cAMP levels are high, RpoS is required to induce the expression of *gad* genes. This occurs at stationary phase where RpoS is maximally expressed. Here, glutamate-dependent AR is induced irrespective of the media pH. Alternatively, in a minimal glucose media when CRP is absent or cAMP levels are low, the *gad* genes are induced by sigma 70 (housekeeping sigma factor). This induction occurs in log phase cells and requires low pH to negate GadY repression which occurs at pH 8.0.

2.6.3.2.2 Arginine decarboxylase acid resistance system

The arginine dependent AR system uses a mechanism similar to the glutamate dependent AR system. The arginine decarboxylase is encoded by *adiA* and it also elevates internal pH by consuming protons during arginine decarboxylation. It then exchanges the end product (agmatine) with new substrate (extracellular arginine) via the arginine: agmatine antiporter encoded by *adiC* (Gong, Richard and Foster, 2003; Chung *et al.,* 2006). The arginine-dependent AR is thought to be less protective at pH 2.5 compared to the glutamate-dependent AR system (Audia *et al.,* 2001; Foster, 2000). Regulation of *adi* genes seems elusive. Nonetheless, studies have indicated that the *adi* genes are activated by CysB regulatory protein (Castanie-Cornet *et al.,* 1999; Shi and Bennett, 1994)

Lin *et al.* (1996) illustrated in their study that the glutamate-dependent and arginine-dependent AR systems function independent of RpoS although their activities were reduced by 20 to 100 fold in *rpoS* mutant *E. coli* challenged at pH 2.5. They explained that the amino acid decarboxylase systems may not be regulated by RpoS, however, RpoS may be required to activate other proteins needed to prevent macromolecular damage to *E. coli* in extreme acidic conditions. On the other hand, Castanie-Cornet *et al.,* (1999) reported that acid induced amino acid decarboxylase systems (in particular the *gad* genes) are highly expressed in log phase though they remain inactive. Their report suggests that the *gad* genes are constitutively expressed in log phase growth and are driven by $E\sigma^{70}$. Since RpoS recognizes similar promoter sites as sigma 70, it is possible

that $E\sigma^s$ recognizes the same transcription start site as $E\sigma^{70}$ during stationary-phase GAD induction. It is also likely that regulation of GAD occurs at the translational or post-translational level.

2.6.4 Contribution of other macromolecular components to acid resistance

Gram-negative bacteria are characterized by their double cell membranes: the outer membrane and the inner membrane separated by the periplasmic compartment. These membranes are composed of a phospholipid bilayer that serves as an important barrier to the external environment. To a large extent, the lipid bilayers restrict transport of hydrophilic solutes, including some nutrients, into the cell (Nikaido, 2003). For that reason, there are outer membrane proteins that form channels in the lipid bilayer to allow influx of nutrients and transport of toxic waste out of the cell (Nikaido, 2003). These outer membrane proteins were originally identified as non-specific diffusion channels that span the outer membrane and were thus termed porins (Nikaido, 2003). However later studies broadened the scope of the term porins to include channels with selected specificity (Nikaido, 2003). Recent studies have indicated that in *E. coli*, the outer membrane proteins and the fatty acids in the outer phospholipids bilayer contribute to cell survival under stressful conditions (Brown, Ross, McMeekin and Nichols, 1997; Chang and Cronan, 1999; Sainz, Perez, Villaseca, Hernandez, Eslava, Mendoza and Wacher, 2005). Therefore, the ability of *E. coli* cells to modulate changes in the cell membrane in order to restrict solute transport and increase selectivity for preferred compounds during disturbances in the external environment is critical to survival.

2.6.4.1 Outer membrane fatty acids

At optimum growth conditions, bacteria maintain a fluid cell membrane with balanced levels of monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs). This is to enable easy exchange of molecules into and out of

the cell respectively. Under unfavourable environmental conditions, bacteria increase the levels of SFAs which results in increased rigidity and plasticity of membranes due to compact packing of the unbranched fatty acid chains. This change is essential to reduce passive transport of unwanted molecules into the cell (Beales, 2004). Several investigations of stressed bacterial membranes indicate that changes in bacterial membrane lipids occur under various stress exposures including antimicrobial compounds, thermal treatments, cold shock and low pH (Beales, 2004; Yuk and Marshall, 2004; Ingram, 1977).

During acid stress response, *E. coli* converts a proportion of the MUFA to cyclopropane fatty acids (CFAs) or replaces them with SFAs (Brown *et al.,* 1997). Of all the SFAs, palmitic acid typically increases in Gram-negative cells at sub-optimal conditions (DiRusso and Nyström, 1998). Studies by Brown *et al.,* (1997) indicate that changes in the membrane lipids occur during acid habituation. This feature was further confirmed by Yuk and Marshall (2004) who observed increased levels of CFAs and SFAs with a corresponding decrease in MUFAs after acid-adaptation. Recent studies have shown that the formation of CFAs is critical to acid resistance in *E. coli* (Chang and Cronan, 1999).

2.6.4.2 Cyclopropane fatty acid

Cyclopropane fatty acid (CFA) was first identified in *Lactobacillus arabinosus* in 1950 when Hofmann and Lucas identified a novel fatty acid in the cell membrane of *L. arabinosus* (Hofmann and Lucas, 1950). This fatty acid was subsequently identified as the 19-carbon cyclopropane analog of *cis*-vaccenic acid, which is the dominant unsaturated fatty acid (UFA) in the phospholipids of *L. arabinosus.* Since then, there have been several studies on CFAs in the cell membranes of bacteria. CFAs have been identified as components of phospholipids in the cell membranes of major Gram-positive and Gram-negative bacterial lineages, including eukaryotes (Grogan and Cronan, 1997). The predominant natural CFAs in bacterial membrane lipids include *cis*-9,10-methylene

hexadecanoic acid, *cis*-11,12-methylene octadecanoic acid and *cis*-9,10-methylene octadecanoic acid (Grogan and Cronan, 1997).

2.6.4.2.1 Biosynthesis of CFAs

CFAs are post-synthetic modifications of UFAs that occur as components of mature phospholipids present in cell membranes (Chang and Cronan, 1999). According to Cronan, Nunn and Batchelor (1974), the natural CFAs have a *cis* conformation and are only present in bacteria that have the homologous UFAs with *cis* double bonds. These are palmitoleic (*cis*-9-hexadecanoic acid), *cis*-vaccenic (*cis*-11-octadecenoic) and oleic (*cis*-9-octadecenoic) acids. In their study, Cronan *et al.* (1974) observed a decrease in UFA concentration as CFA concentration increased *in vivo*, showing evidence that CFAs are directly synthesized from UFAs.

In their study to determine the potential role of lipid modification of acid habituated *E. coli*, Brown *et al.* (1997) determined the fatty acid profile of several acid habituated, non-acid habituated and dehabituated *E. coli* strains. They observed a marked increase in C-17 and C-19 CFAs in acid habituated cells with a corresponding decrease in homologous MUFA. They also showed that strains with high intrinsic tolerance to environmental stresses such as *E. coli* O157:H- and M23 had higher concentrations of CFAs compared to commensal strains.

The mechanism of CFA biosynthesis has not been fully established. Grogan and Cronan (1997) explained that CFAs are synthesized via addition of a methyl group, supplied by *S*-adenosyl-$_L$-methionine (AdoMet), across the carbon-carbon double bond of UFAs to form the cyclopropane ring (Fig. 2.6 & 2.7). When *E. coli* was infected with wild type phage T3 that encodes the enzyme AdoMet hydrolase, which destroys AdoMet, CFA synthesis was blocked while CFA synthesis was unaffected in *E. coli* strains infected with phase T3 that had a mutation in the AdoMet hydrolase gene (Cronan, Reed, Taylor and

Jackson, 1979). This suggests that AdoMet is essential for CFA synthesis. In another study, *Enterobacter aerogenes* was fed with exogenously supplied radiolabelled [*methyl*-^{14}C]AdoMet. This resulted in the formation of radiolabelled CFA indicating that AdoMet is the C_1 donor for the formation of the cyclopropane ring in CFAs (O'Leary, 1962).

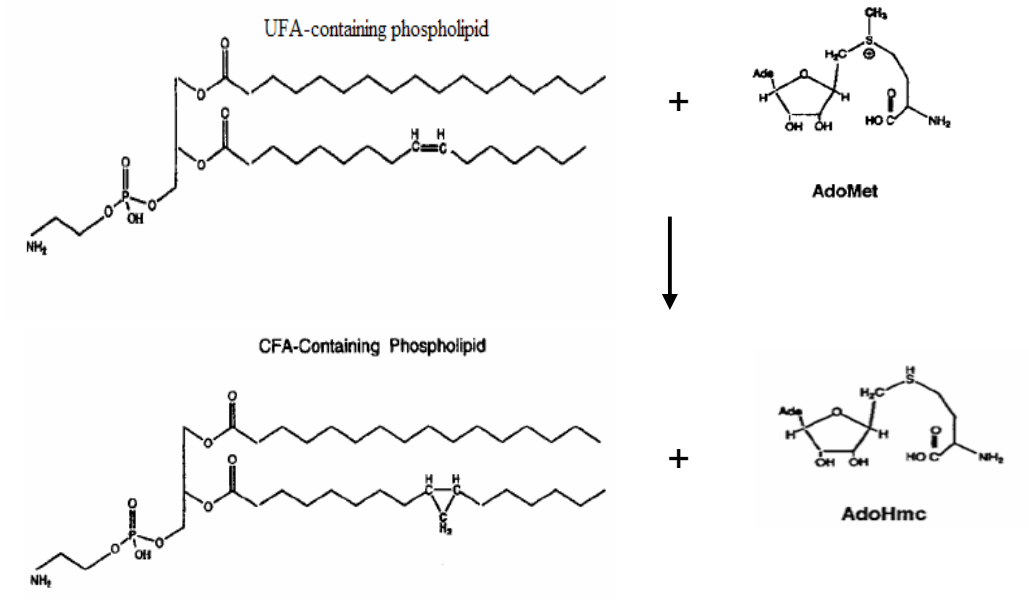

Figure 2.6: Structures of CFA synthase substrates and products. The phospholipids shown (phosphatidylethanolamines) are typical components of membrane lipids of Gram-negative bacteria. AdoMet, S-adenosyl-L-methionine; AdoHme, S-adenosyl-L-homocysteine (Grogan and Cronan, 1997)

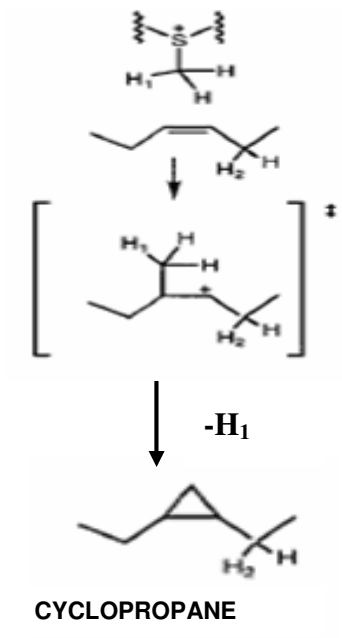

Figure 2.7: Probable mechanism for C1 addition to double bonds for sterol methyltransferases (Grogan and Cronan, 1997)

Currently, the accepted mechanism for cyclopropane ring formation is shown in Fig. 2.7. Once the AdoMet sulfonium group is separated from its counter ion, the sulfonium cation becomes susceptible to attack by the π electrons of the double bond which results in carbonation of the fatty acid. Removal of the methyl proton (H_1) leads to the formation of the cyclopropane ring (Grogan and Cronan, 1997)

2.6.4.2.2 Mechanism of action of CFAs

The physiological role of CFAs has not been fully elucidated, but, considering that the synthesis of CFAs in cell membranes is energetically expensive, it is believed to have an indispensable role in cell adaptation to stresses normally encountered in stationary phase (Grogan and Cronan, 1997). In *E. coli*, conversion of each molecule of UFA to CFA via AdoMet requires three molecules of ATP (Knivett and Cullen, 1965) and almost all UFAs are converted to CFAs during the transition from late exponential phase to

stationary phase (Chang and Cronan, 1999). Chang and Cronan (1999) demonstrated that CFAs protect *E. coli* against acid shock. In their study, *E. coli* exogeneously supplemented with C-17 CFA were better protected against acid shock at pH 3.0 compared to those supplemented with C-19 homologue. Brown *et al*. (1997), also observed that *E. coli* strains having high levels of UFAs had a corresponding high level of CFA in the membrane phospholipid. Also, the *E. coli* strains that had high levels of CFAs showed better resistance to acid shock compared to strains with lower levels of CFA.

According to Grogan and Cronan (1997) the combination of energetic expense of CFA synthesis, its timing and sensitivity of culture conditions has been used as evidence to suggest that CFAs adapts cells for adverse conditions in stationary phase. It has been hypothesized that since the CFAs are much less reactive compared to their UFA counterparts, their primary functions could potentially be to modulate changes in chemical properties of bacterial membranes without changing its physical properties (Grogan and Cronan, 1997). Other suggestions for the physiological role of CFAs in bacterial membranes include protection from chemical destruction at the site of unsaturation (Law, 1971), stabilization of structural properties of biological membranes (Dufourc, Smith and Jarell, 1984) and increase in membrane rigidity which leads to decrease in membrane permeability to protons (Dunkley, Guffanti, Clejan and Krulwich, 1991). Finally, Chang and Cronan (1999) suggested that CFAs contribute indirectly to *E. coli* AR by binding to membrane proteins to increase proton efflux (Chang and Cronan, 1999).

2.6.4.2.3 Regulation of CFA synthesis

CFA synthesis is catalyzed by CFA synthase during *E. coli* transition from late exponential phase to stationary phase (Wang and Cronan, 1994). Wang and Cronan (1994) explained that expression of CFA synthase is regulated by RpoS at its proximal

promoter, P2 during late exponential to stationary phase, while its distal promoter, P1 is regulated by standard σ^{70} throughout *E. coli* growth. During exponential phase, σ^{70} dependent CFA synthesis occurs at low levels (Wang and Cronan, 1994) while maximal induction of CFA synthase induced by RpoS occurs at the stationary phase (Grogan and Cronan, 1997).

There are three mechanistic approaches to CFA synthase regulation in *E. coli*. These include stringent control, RpoS control and proteolysis of CFA synthase. The stringent response and RpoS regulation are interrelated in that they both regulate CFA synthase indirectly and they both affect RpoS concentrations in the cell (Grogan and Cronan, 1997; Eichel, Chang, Reisenberg and Cronan, 1999). Studies by Grogan and Cronan (1997) suggest that CFA synthase is unstable and has a half-life of less than 5 minutes *in vivo*. It is proposed that the sharp decline in CFA synthase activity and high turnover is due to proteolysis of CFA synthase (Grogan and Cronan, 1997). However, the mechanism of proteolysis of CFA synthase needs further investigation.

2.6.4.3 Outer membrane proteins

Transport of protons and other polar solutes into the cell can also be controlled by regulating outer membrane porins (Omps) in *E. coli* systems (Sainz *et al.,* 2005). The major outer membrane porins present in *E. coli* cells are the larger OmpF, the smaller OmpC, and the glycoprotein LamB channels (Liu and Ferenci, 1998). These porins are hydrophilic channels with a homo-trimeric structure. Each of the three monomeric transmembrane channels is composed of 16 β sheets making up the β-barrel. The interaction between the β-barrels is stabilized by hydrophobic and polar interactions (Nikaido, 2003). These β-barrels are connected to neighbouring monomers by the extracellular loop indicated as loop 2 in Fig. 2.8 below. The third loop, called the periplasmic turn, folds within the β-barrels to narrow the channel (Lui and Delcour, 1998).

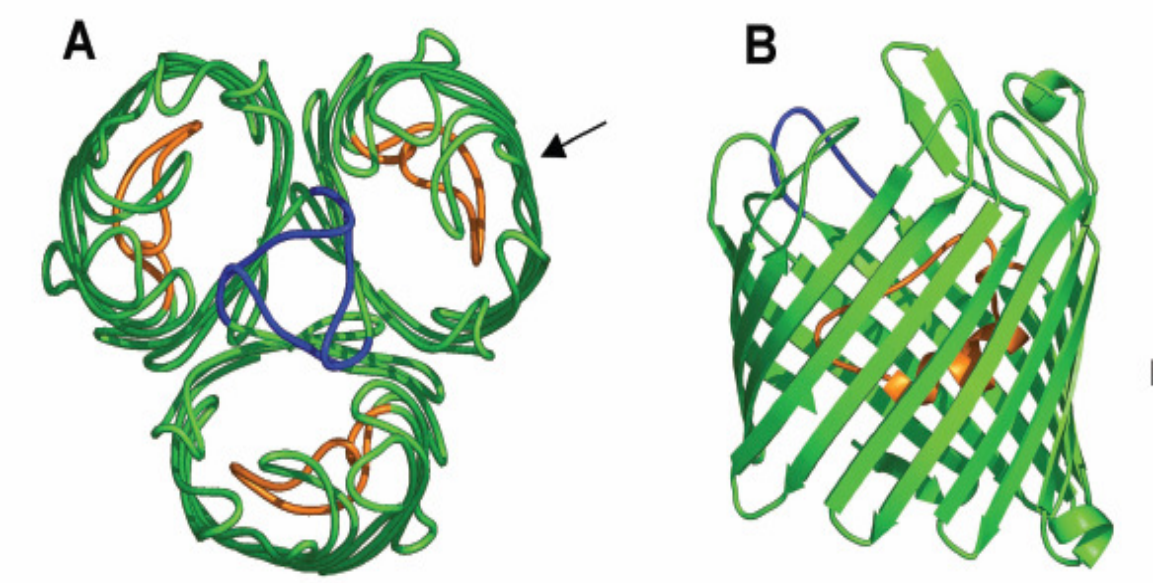

Figure 2.8: The OmpF porin of *Escherichia coli.* (A) View of the trimer from the top, i.e., in a direction perpendicular to the plane of the membrane. Loop 2, coloured blue, plays a role in interaction of the monomer with its neighbouring unit. Loop 3, coloured orange, narrows the channel. (B) View of the monomeric unit from the side, roughly in the direction of the arrow in panel A. Loops 2 and 3 are coloured as in panel A (Nikaido, 2003).

According to Baslè, Rummel, Storici, Rosenbusch and Schirmer (2006), OmpC is not structurally distinguishable from OmpF and that the pore size and the constriction region of OmpC and OmpF are similar. Data generated from rates of diffusion of organic molecules through OmpF and OmpC indicates that OmpC is a smaller porin (Nikaido, 2003). Schultz (2002) suggested that the greater number of charged residues in the lumen of the OmpC β-barrel could be responsible for reducing the size of the OmpC. However, studies by Baslè *et al*., (2006) indicated that differences in ompF and OmpC could be attributed to the electrostatic potential of the OmpC pore and not the size of the pore.

These classical porins allow non-specific diffusion of small molecules across the outer membrane (Molloy, Herbert, Slade, Rabilloud, Nouwens, Williams, and Gooley, 2000).

The proportion of OmpF and OmpC in the cell membrane depends on external factors such as the growth phase of the cell, osmolarity, temperature, nutrient limitation and antibacterial molecules in the growth medium (Liu and Ferenci, 1998).

At high temperatures, osmolarity and high concentration of antimicrobials, *E. coli* regulates the levels of OmpC and OmpF in the outer membrane. Liu and Ferenci (1998) explained that under medium glucose limitation, LamB is optimally expressed followed by OmpF induction to scavenge the remaining glucose in the medium. Upon depletion of glucose, *E. coli* switches off LamB and OmpF and in turn induces OmpC expression when in protective mode (stationary phase). Studies have shown that at low pH, *E. coli* represses OmpF and increases expression of OmpC (Heyde and Portalier, 1987). Although this regulation of OmpF and OmpC at low pH is undeniable, Nikaido (2003) suggested that increased expression of OmpC has little relevance in terms of influx of protons into the cell since both OmpF and OmpC transport protons with similar efficiencies. Notwithstanding, she suggested that the increased expression of OmpC at the expense of OmpF may enhance pH homeostasis by preventing efflux of homeostatic molecules such as glutamate and arginine out of the cell. She also proposed that the closure of porins by constriction of the β-barrel to prevent influx of protons into the cell could contribute to survival at low pH.

Regulation of Omps is complex involving several global regulators including RpoS, cyclic adenosine monophosphate (cAMP)/Crp (Catabolite repressive protein), other protein complexes such as OmpR/EnvZ, MarA, Histone-like DNA binding proteins (HNS), integration factors and Lrp (Scott and Harwood, 1980; Tsui, Helu and Fruendlich, 1988; Ferrario, Ernsting, Borst, Wiese, Blumenthal and Mathews, 1995; Pratt and Silhavy, 1996; Suzuki, Ueguchi and Mizuno, 1996; Sainz *et al.,* 2005).

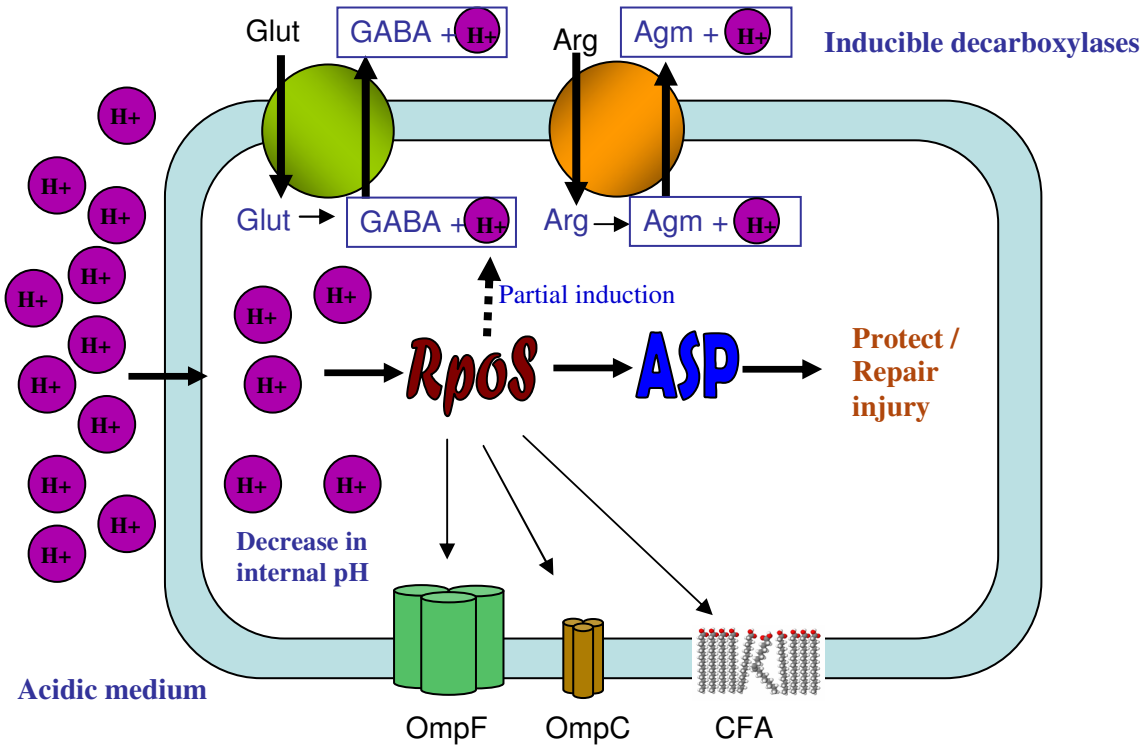

Figure 2.9: Proposed model for mechanisms of *Escherichia coli* survival under low pH stress. Glut-glutamine, GABA–γ-amino butyric acid; Arg-arginine; AGM-agmatine; green circle is the transmembrane glutamate: γ-aminobutyric acid antiporter; orange circle is the arginine: agmatine antiporter; RpoS-alternative sigma factor s; ASPs-acid shock proteins; CFA-cyclopropane fatty acids; OmpC-outer membrane porin C; OmpF-outer membrane porin F (Adapted from Lin *et al*., 1995; Grogan and Cronan, 1997; Chung *et al*., 2006)

2.7 *E. coli* tolerance to lactoperoxidase system

In addition to Omps, *E. coli* possesses integral membrane protein CorA that functions as a Mg^{2+} transporter (Kehres and Maguire, 2002). Sermon, Vanoirbeek, De Spiegeleer, Van Houdt, Aertsen, and Michiels (2005) showed that *corA* knockout *E. coli* mutants were hypersensitive to LP system; however this mutant did not show sensitivity to

hydrogen peroxide nor superoxide generator plumbagin. CorA also mediates influx of bivalent cations including transition metal ions that have been linked to safeguard cells against oxidative stress (Sermon *et al*., 2005). The mechanism of CorA protection against LP stress is not understood, however, it is suggested that CorA influences LP resistance by mediating cytoplsmic concentrations of metals ions that affect toxicity of the LP system (Sermon *et al.,* 2005).

2.8 Cross-protection of acid adapted *E. coli*

Once *E. coli* becomes adapted to one stress, it develops enhanced resistance against other stresses, a phenomenon called cross-protection (Chung *et al.,* 2006). Cross-protection of acid-adapted *E. coli* and *Salmonella* to heat, salt, H_2O_2, crystal violet and polymixin B has been reported (Leyer and Johnson, 1993; Lin *et al.,* 1995; Rowe and Kirk, 1999). In a study by Rowe and Kirk (1999), pre-stressed *E. coli* showed increased resistance to 20 % (w/v) NaCl. The highest resistance was observed in *E. coli* cells pre-stressed at pH 5.0 for 1 h compared to those pre-stressed at pH 4.0 and 3.5. In fact, after 15 min exposure to moderately acidified tryptone soy broth (TSB), cross-protection against NaCl had been fully induced. Also, *E. coli* cells adapted at pH 4.0 demonstrated an increased resistance against heat (56 °C) compared to the non-adapted cells. Cross-protection against heat was also demonstrated for stationary phase acid-adapted cells while those adapted in log phase showed significant reduction in cross-protection against heat (Rowe and Kirk, 1999). Cross-protection was attributed to RpoS which regulates several stress response genes. However, studies with *rpoS* mutants indicated that other RpoS-independent mechanisms are involved in cross-protection of acid-adapted *E. coli* against salt stress (Rowe and Kirk, 1999). Cheville, Arnold, Buchrieser, Cheng and Kaspar (1996) demonstrated that stationary-phase *rpoS* mutant *E. coli* cells were sensitive to heat (55 °C), 2.5 M salt and dry fermented sausage (pH 4.6 to 4.8, 1.2 % salt and 1.9 % moisture) compared to the wild-type cells. Also, stationary phase cells were more resistant to simulated gastric fluid (pH 1.5), acidified TSB (pH 2.0) and 2.5 M salt compared to log

phase cells. Starvation induced ATR was also absent in *rpoS* mutant cells. This suggests that cross-protection is mediated at least in part by RpoS.

Cross-protection occurs because some stress response systems share the same/overlapping regulatory pathways (Rowe and Kirk, 1999). Stationary phase RpoS, for example, regulates about 60 stress response genes (Hengge-Aronis, 2002) that provide protection against chemical and physical challenges. Other regulatory systems that are coordinated with specific stresses include, but are not limited to Lrp, cAMP and HNS at stationary phase (Hengge-Aronis, 2002). Most of these stress response genes encode chaperones that prevent denaturation or loss of enzyme activity, DNA binding proteins that stabilize nucleic material under physical and chemical challenges, and DNA repair factors that repair damages to DNA materials by external stresses as they occur (Slonczewski and Foster, 1996).

In another study, Wang and Doyle (1998) demonstrated cross-protection of heat shocked *E. coli* cells against low pH (pH 2.5). Survival of heat shocked *E. coli* cells were actually similar to acid-adapted (at pH 5.0) *E. coli* cells to lethal pH. Although cross-protection of heat shocked cells against low pH has been shown to occur, there is no evidence that heat shock confers broad range cross-protection against other unrelated environmental stresses as with acid-adaptation and entry into stationary phase. Relevant to heat shock is the activation of heat shock regulons sigma 32, sigma E, and sigma N (Chung *et al*., 2006). These sigma factors induce several heat shock proteins (HSPs), mainly chaperones that bind and stabilize several proteins essential for survival, as well as proteases that digest denatured proteins in order to supply amino acids for protein synthesis. These chaperones and proteases include DnaK, GroE, GroEL and GroES (Arsène, Tomoyasu and Bukau, 2000). Other heat shock proteins are involved in cell wall synthesis, proteolysis and DNA replication (Chung *et al.,* 2006). Some of the HSPs, may be required for stabilization of nucleic material and proteins during cell exposure to other stresses. For example, DnaK is also influenced by RpoS during *E. coli* acid-adaptation (Hengge-Aronis, 1996; Slonczewski and Foster, 1996).

Nonetheless, one stress does not always provide cross-protection against subsequent stress (Chung *et al.,* 2006). This is the case with stresses that have distinct response pathways. In a study by Riordan, Duffy, Sheridan, Whiting, Blair and McDowell (2000) that compared survival of acid-adapted and non-adapted *E. coli* O157:H7 in pepperoni fermentation, the authors observed that the acid-adapted cells were significantly more sensitive to heating compared to the non-adapted cells. In another study by Hsin-Yi and Chou (2001), non-adapted *E. coli* O157:H7 displayed significantly better survival in yakult (pH 3.6) and low fat yoghurt (pH 3.9) for extended periods of time at 7 °C. This lack of cross-protection to heat and low pH of acid-adapted *E. coli* cells has not been reported in broth. It therefore appears that the complexity of food matrix influences cross-protection of acid-adapted *E. coli* O157:H7 against subsequent stresses (that have already been reported in broth studies). The lack of cross-protection in some food systems present a potential for application of combined treatments to sufficiently reduce *E. coli* O157:H7 numbers in foods.

E. coli adaptation to other stresses, for example thermal, osmotic or oxidative stresses may not induce significant acid-resistance or even cross-protection possibly because, acid-stress may be perceived as a general stress indicator whereas heat, salt and H_2O_2 may be more specific stress signals (Bearson *et al.,* 1997). Moreover induction of cross-protection during acid-adaptation prepares cells undergoing acid-shock in the stomach for subsequent environmental stresses yet to be encountered in the intestine (Bearson *et al.,* 1997).

2.9 Hypotheses

- Acid-adapted *E. coli* will exhibit cross-protection against activated LP. When stationary phase *E. coli* are exposed to mild acidic environments, it activates RpoS which protects *E. coli* by inducing decarboxylase systems that consume

protons as they enter the cell, and by reducing influx of protons into the cell via regulation of outer membrane protein channels and modification of fatty acids in the cell membrane lipid bilayer (Beales, 2004). Changes in cell membrane proteins and lipids protect the cell by reducing entry of antimicrobial agents into the cell.

- Acid-adapted *E. coli* subjected to activated LP will have increased levels of expressed acid resistance genes (*rpoS, gadA* and *adiA*) and LP inducible gene (*corA*). When stationary phase *E. coli* encounters acidic conditions in its environment, it activates expression of acid resistance genes at different levels depending on the severity of the stress to maintain internal pH homeostasis. It also regulates concentration of outer membrane proteins to control influx of protons into the cell. In an activated LP system, *E. coli* synthesizes CorA that mediates cytoplasmic concentrations of metal ions that enhance LP toxicity in *E. coli.*

- Activated LP in combination with heat, and low pH, will be less effective in eliminating acid-adapted *E. coli* O157:H7 compared to non-adapted *E. coli* O157:H7 because the expression of several acid shock proteins and changes in cell membrane of acid-adapted *E. coli* increases tolerance to heat treatment and LP mediated cell death respectively (Leyer and Johnson, 1993).

- Activated LP will reduce acid production of single strains of commercial lactic starter cultures in goat milk because activated LP has been shown to inhibit starter cultures and subsequently, acid production in mixed starter cultures in goat milk (Seifu *et al.,* 2003). Reduction of acid production during fermentation of activated LP goat milk may provide a suitable environment for *E. coli* O157:H7 to thrive.

2.10 Objectives

- To determine the survival of acid-adapted *E. coli* O157:H7 in activated LP goat milk.

- To determine whether acid-adapted *E. coli* O157:H7 exhibits cross-protection against activated LP in combination with low pH in Tryptone Soy Broth.

- To determine the levels of gene expression of acid resistance genes (*rpoS, gadA, adiA*), LP system inducible gene (*corA*) and genes for outer membrane proteins (*ompC* and *ompF*) in acid-adapted *E. coli* O157:H7 subjected to activated LP.

- To determine whether acid-adapted *E. coli* O157:H7 exhibits cross-protection against activated LP in combination with heat and lactic acid treatments in raw goat milk.

- To determine whether the activated LP system affects acid production of single strain starter cultures and how that impacts survival of *E. coli* O157:H7 during processing of commercial and traditional fermented goat milk.