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LIST OF ABBREVIATIONS

AA	Acid-adapted
ABTS	2,2'-Azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid
AdiA	Arginine decarboxylase
AdiC	Arginine: agmatine antiporter
AdoHme	S-adenosyl-L-homocysteine
AdoMet	S-adenosyl-L-methionine
AGM	Agmatine
AH	Acid habituation
ANOVA	Analysis of variance
AR	Acid resistance
ASP	Acid shock proteins
ATA	Arginine tetrazolium agar
ATP	Adenosine triphosphosphate
ATR	Acid tolerance response
BHI	Brain Heart Infusion
CAC	Codex Alimentarius Commission
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CDSC	Communicable Disease Surveillance Centre
CFA	Cyclopropane Fatty Acid
cfa	Cyclopropane fatty acid synthase gene
ClpXP	Serine protease complex responsible for ATP-dependent degradation of proteins
CorA	Magnesium transporter
CRP	Cyclic adenosine monophosphate receptor protein
DNA	Deoxyribonucleic acid
DnaK	*Escherichia coli* chaperone protein
$E\sigma^s$	RNA polymerase holoenzyme

EHEC	Enteroheamorrhagic *Escherichia coli*
EIIA(Glc)	Glucose specific EII component of the phosphotransferase system
FAO	Food and Agriculture Organization
Fur	Transcriptional repressor of iron-regulated promoters
GAD	Glutamate decarboxylase
GABA	Gamma aminobutyric acid antiporter
GroE, GroEL, GroES	A group of chaperone proteins required for high temperature growth/ viability
HC	Haemorrhagic colitis
Hfq	host factor 1
HNS	Histone-like DNA binding proteins
HPLC	High performance liquid chromatography
HST	Heat shock proteins
HTST	High temperature short time
HU	Major DNA binding protein of *Escherichia coli*
HUS	Haemolytic uremic syndrome
IDF	International Dairy Federation
LA	Lactic acid
LAB	Lactic acid bacteria
LamB	Maltoporin of *Escherichia coli*
LEE	Pathogenicity island in *Escherichia coli* O157:H7 genome
LP	Lactoperoxidase
LTLT	Low temperature long time
MarA	Transcriptional activator in *Escherichia coli*
MOPS	Morpholinemethanesulfonic acid
MRS	de Mann Rogosa Sharpe
mRNA	Messenger ribonucleic acid
MTC	Medium chain triglycerides
MviA	Mouse virulence gene in *Salmonella*; plays a central role in facilitating sigma S degradation by ClpXP

MUFA	Monounsaturated fatty acid
NA	Non-adapted
Omp	Outer membrane porins
OmpR/EnvZ	Regulator proteins for the *Escherichia coli* outer membrane
PCR	Polymerase chain reaction
PhoP	Protein that phosphorelates and regulates the expression of a large collection of genes in enteric bacteria
ppGpp	Guanosine tetraphosphate
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
RpoS	Alternative sigma factor S
RSA	Republic of South Africa
RssB	*Escherichia coli* response regulator
qRT-PCR	quantitative real time polymerase chain reaction
SFA	Saturated fatty acid
SH	Sulfhydryl group
SMAC	Sorbitol MacConkey agar
STEC	Shiga toxin producing *Escherichia coli*
stx	Shiga toxin gene
TSA	Tryptone soy agar
TSB	Tryptone soy both
TSBG	Tryptone soy broth supplemented with 1 % glucose
WHO	World Health Organization

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).

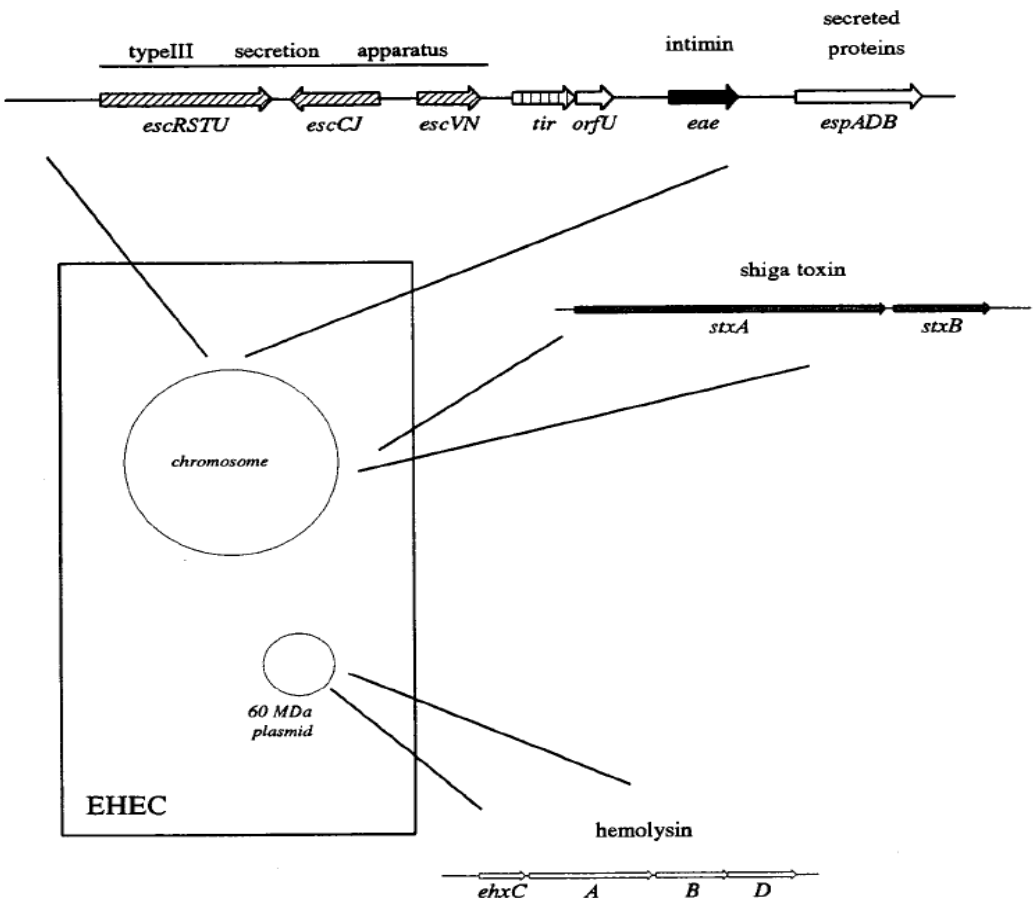


Figure 2.1: Genes involved in pathogenicity of Enterohaemorrhagic *Escherichia coli* (Nataro and Kaper, 1998)

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.

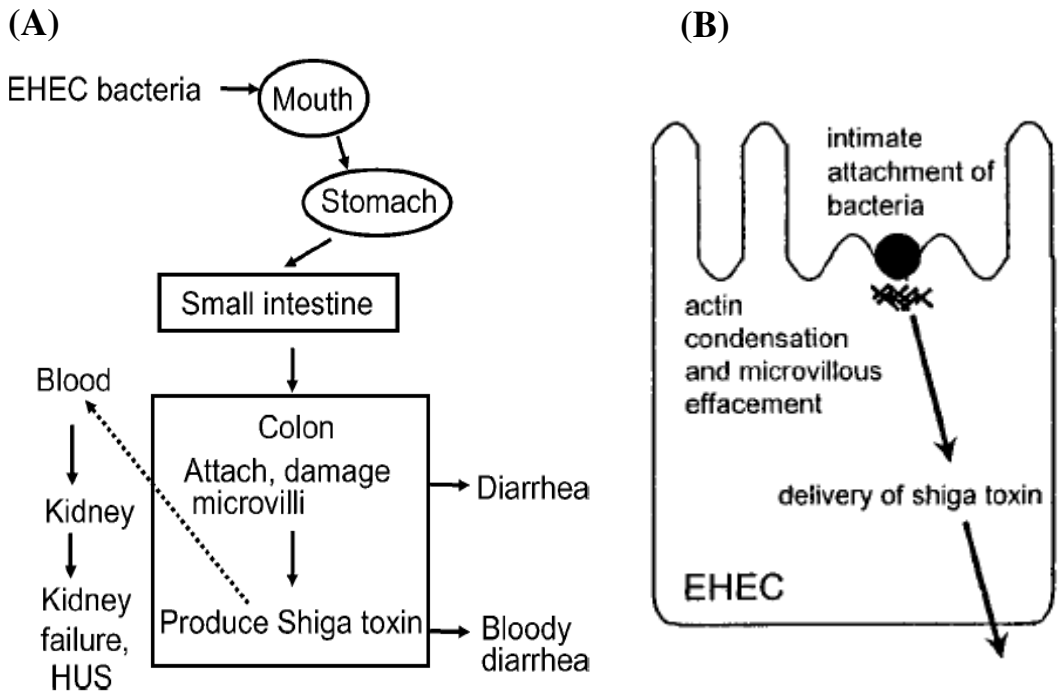


Figure 2.2: (A) The invasion pathway and diseases caused by Enterohaemorrhagic *Escherichia coli* (EHEC) (Gyles, 2007);
(B) Interaction of EHEC with the mucosal cells in the large intestine (Nataro and Kaper, 1998)

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).

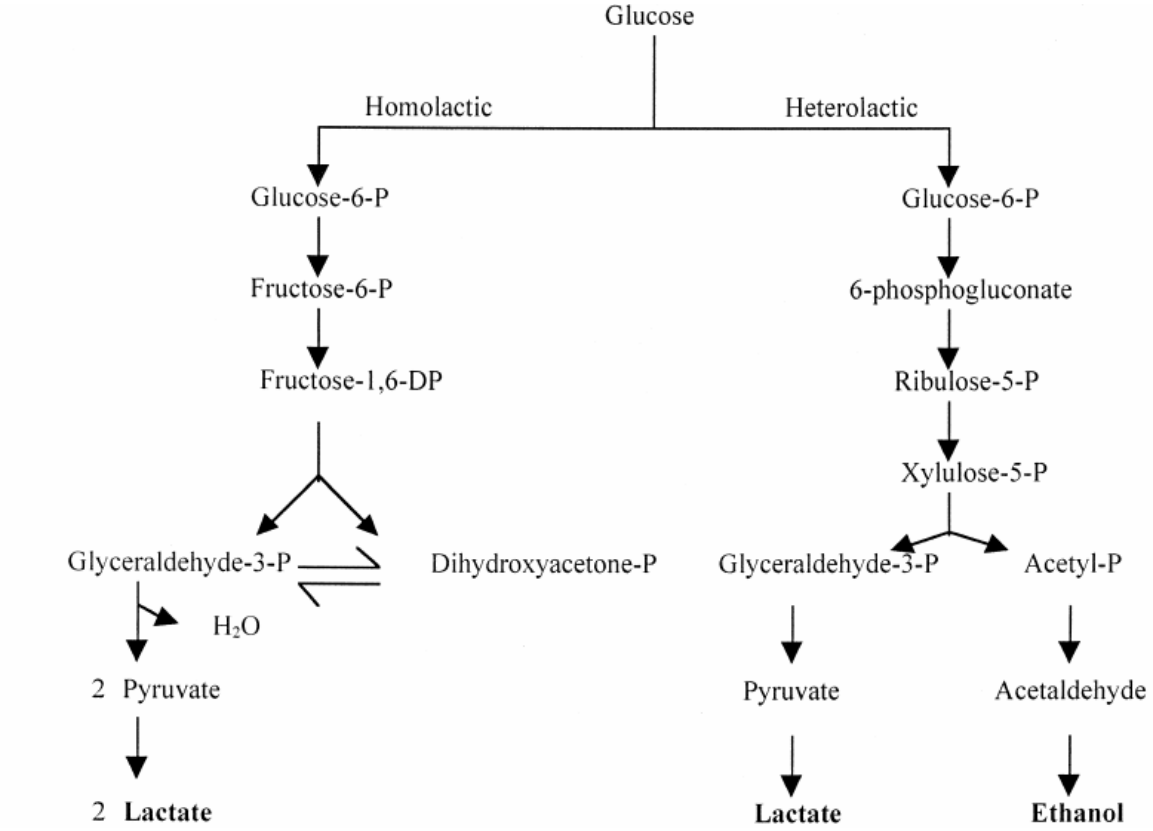


Figure 2.3: Glucose fermentation in homofermentative and heterfermentative lactic acid bacteria (Caplice and Fitzgerald, 1999)

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.

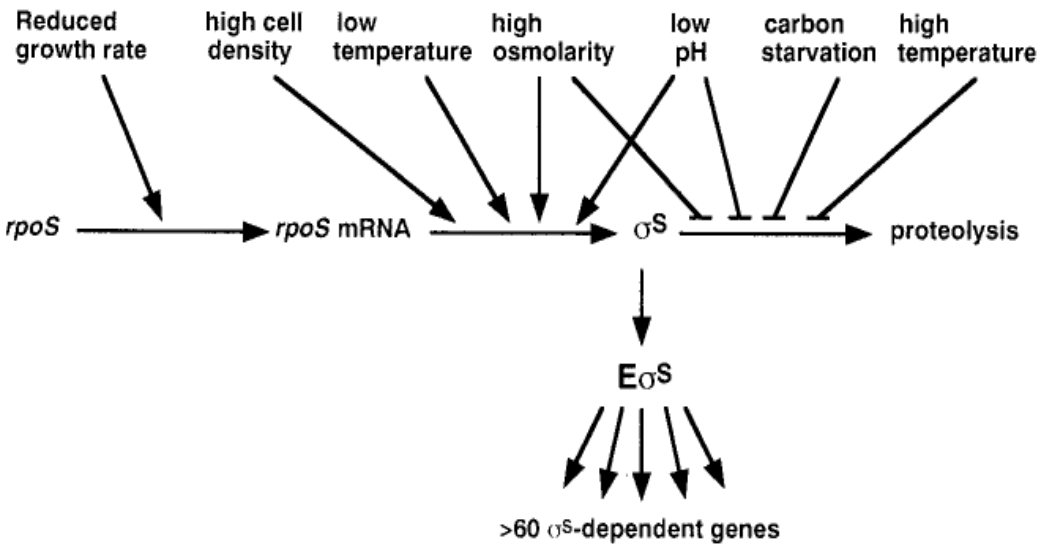


Figure 2.4: RpoS regulation is differentially affected by various stress conditions. An increase in cellular levels of RpoS is modulated by activating *rpoS* synthesis at transcriptional level from the *rpoS* gene or at the translational level from the *rpoS* mRNA. Stabilization of the RpoS protein by 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).

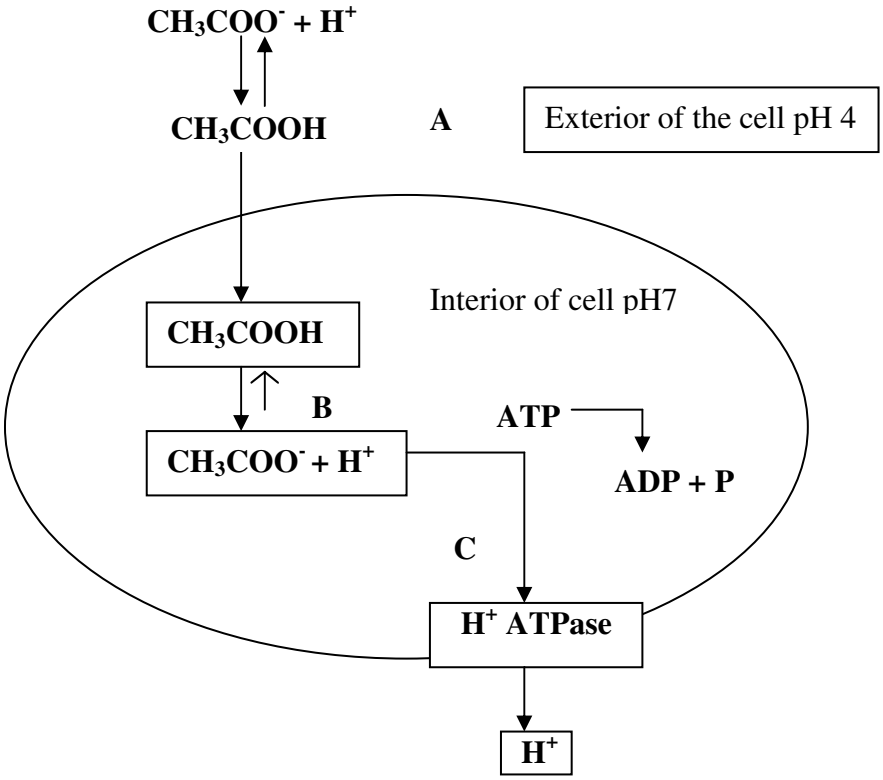


Figure 2.5: Interaction of weak acids in the microbial cell. (A), Exterior of the cell favours undissociated weak acid; (B), On entering the cell, the interior of the cell favours dissociated molecule and acid dissociates; (C), Proton pumps use ATP to remove excess H^+ ions (Beales, 2004).

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).

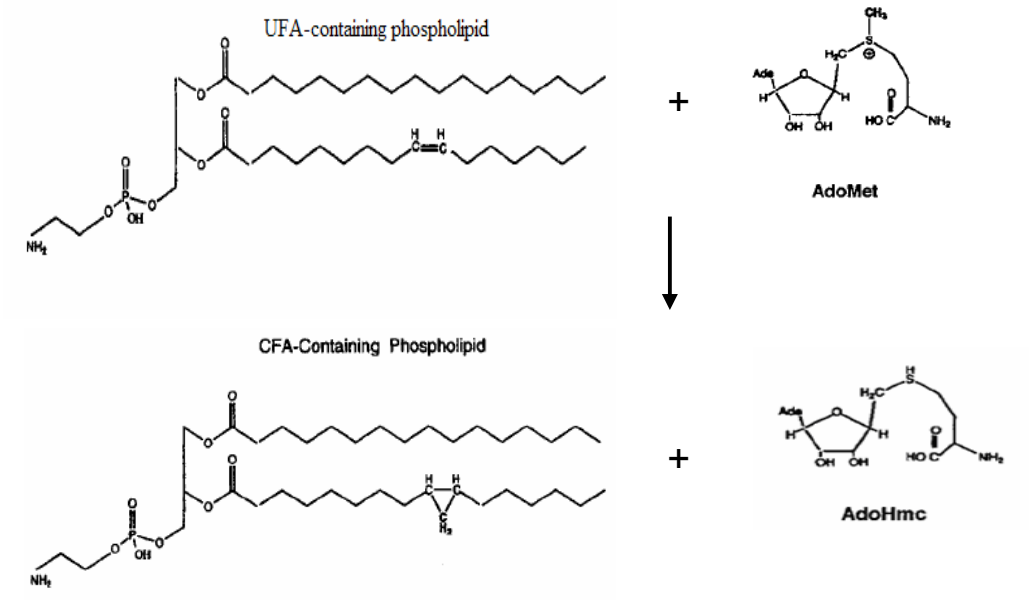


Figure 2.6: Structures of CFA synthase substrates and products. The phospholipids shown (phosphatidylethanolamines) are typical components of membrane lipids of Gram-negative bacteria. AdoMet, S-adenosyl-L-methionine; AdoHme, S-adenosyl-L-homocysteine (Grogan and Cronan, 1997)

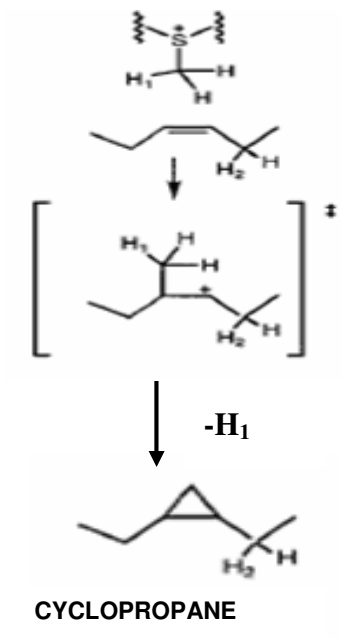


Figure 2.7: Probable mechanism for C1 addition to double bonds for sterol methyltransferases (Grogan and Cronan, 1997)

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).

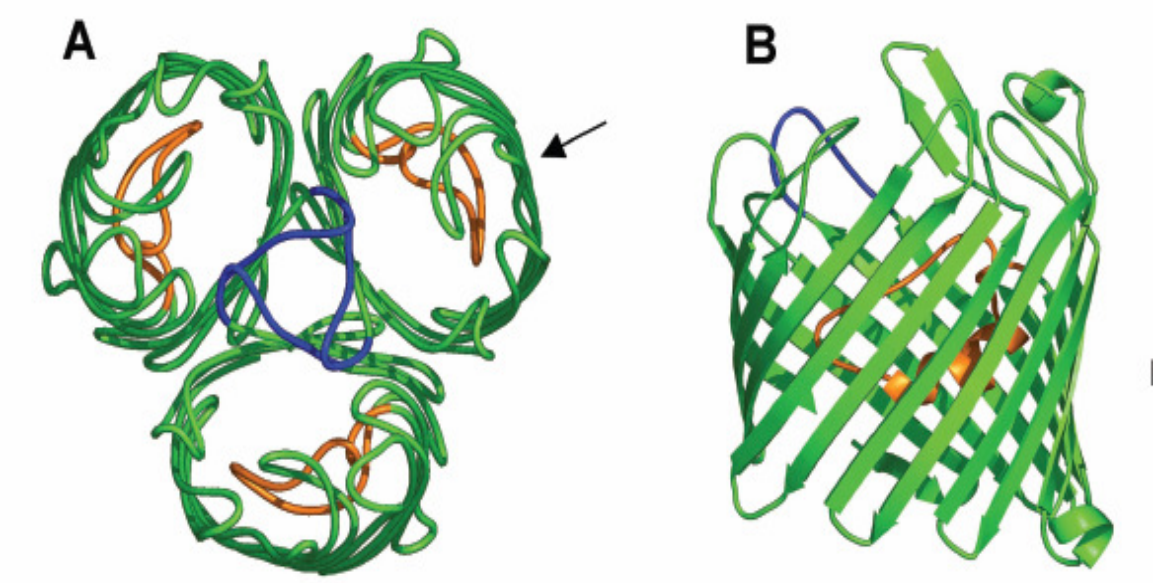


Figure 2.8: The OmpF porin of *Escherichia coli.* (A) View of the trimer from the top, i.e., in a direction perpendicular to the plane of the membrane. Loop 2, coloured blue, plays a role in interaction of the monomer with its neighbouring unit. Loop 3, coloured orange, narrows the channel. (B) View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

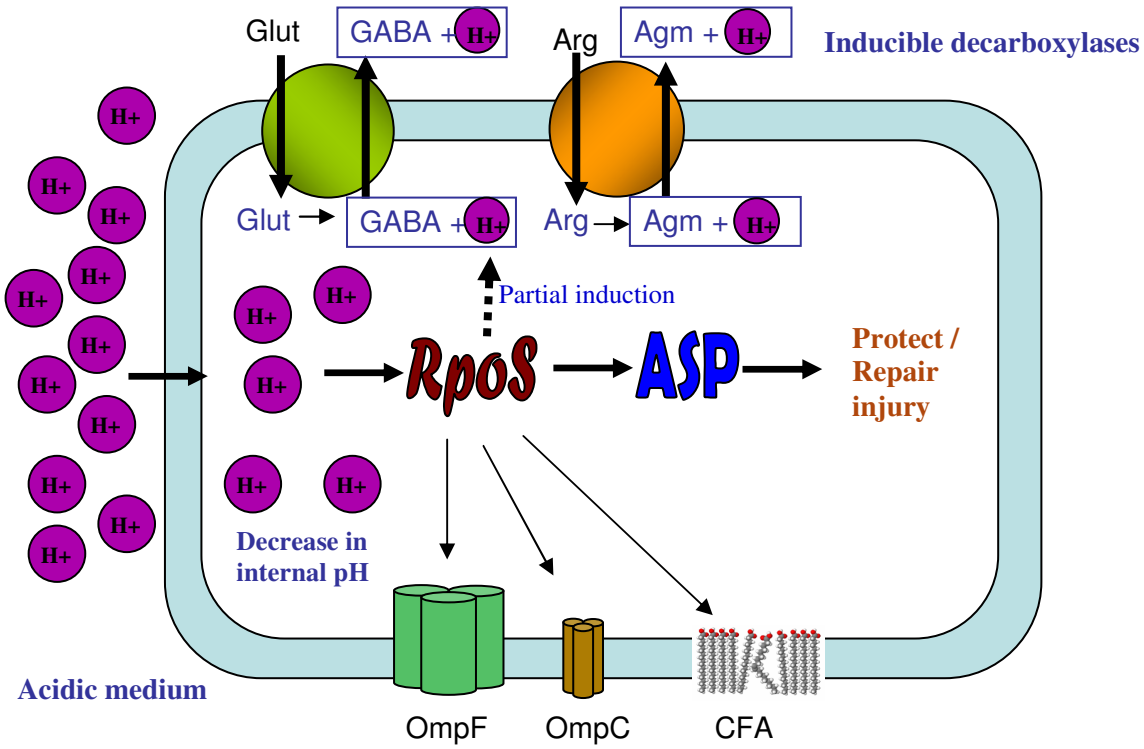


Figure 2.9: Proposed model for mechanisms of *Escherichia coli* survival under low pH stress. Glut-glutamine, GABA–γ-amino butyric acid; Arg-arginine; AGM-agmatine; green circle is the transmembrane glutamate: γ-aminobutyric acid antiporter; orange circle is the arginine: agmatine antiporter; RpoS-alternative 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.

Chapter 3:

RELATIVE GENE EXPRESSION IN ACID-ADAPTED ESCHERICHIA COLI *O157:H7 DURING LACTOPEROXIDASE AND LACTIC ACID CHALLENGE IN TRYPTONE SOY BROTH*

Submitted to Microbiological Research

Abstract

Cross-protection of acid-adapted *Escherichia coli* O157:H7 against inimical stresses is mediated by the glucose-repressed sigma factor RpoS. However, many food systems in which *E. coli* O157:H7 occurs are complex and contain glucose. This study was aimed at investigating the contribution of acid and lactoperoxidase (LP) inducible genes to cross-protection of *Escherichia coli* O157:H7 against the LP system and lactic acid (LA) in Tryptone Soy Broth (TSB). Acid-adapted and non-adapted *E. coli* O157:H7 were challenged against activated LP in TSB at pH 7.4 and 4.0 and against LA in TSB at pH 4.0 and 5.0 for 6 h at 25°C followed by extraction of expressed acid and LP inducible genes. Acid-adapted *E. coli* showed cross-protection against activated LP and LA. All the acid inducible genes tested were repressed at pH 4.0 with or without the activated LP system. At pH 7.4, *gadA, ompC* and *ompF* were induced in acid-adapted cells. Induction of *corA* occurred in non-adapted cells but was repressed in acid-adapted cells. Although acid-inducible genes were repressed at pH 4.0, high resistance of acid-adapted cells indicates that expression of acid inducible genes occurred during acid-adaptation and not during the actual challenge. Repression of *rpoS* indicates that RpoS-independent systems contribute to cross-protection in acid-adapted *E. coli* O157:H7.

Keywords: Cross-protection, *Escherichia coli* O157:H7, lactoperoxidase, acid-adaptation and real-time PCR

3.1. Introduction

Escherichia coli O157:H7 is ubiquitous in nature, has a low infectious dose and causes acute illness with long term sequelae (Paton and Paton, 1998; Bell, 2002). The control of *E. coli* O157:H7 survival in acidic foods, especially fermented dairy products made from unpasteurized milk, is one of the greatest microbiological challenges facing the food industry. The lactoperoxidase (LP) system is a natural antimicrobial system that has been found effective in controlling growth of *E. coli* O157:H7 in raw milk (Seifu *et al.,* 2004) and has been recommended in raw milk preservation particularly in rural areas where ambient temperatures are high and where facilities for refrigeration of milk are not available (FAO/WHO, 2006). LP system in combination with low pH can be potentially applied in dairy technology to minimize *E. coli* O157:H7 survival in dairy foods.

Notwithstanding, *E. coli* O157:H7 can adapt to mild acid conditions which confer acid resistance under subsequent lethal acidic conditions (< pH 4.5) (Seputiene *et al.,* 2005). There is also evidence that once *E. coli* becomes acid-adapted, it exhibits cross-protection against inimical food processes including high salt concentration (Rowe and Kirk, 1999) and heat (Ryu and Beuchat, 1998). Leyer and Johnson (1993) also demonstrated that acid-adaptation of *Salmonella* Typhimurium confers cross-protection against heat, salt, crystal violet, polymixin B and LP stresses in Brain Heart Infusion broth.

The mechanisms of acid-adaptation and acid resistance have been extensively studied in *E. coli* systems. However, there is insufficient understanding of cross-protection of acid-adapted *E. coli* O157:H7 using molecular technology. Cross-protection of acid-adapted *E. coli* against environmental stresses is mediated by the alternate sigma factor, RpoS (Hengge-Aronis, 2002). Studies have however indicated that *rpoS* is repressed in the presence of glucose. For that reason, most molecular studies on acid resistance mechanisms in *E. coli* have been conducted in minimal media. However, food systems themselves in which *E. coli* occurs are complex and in many cases, have glucose present. To effectively control stress adapted *E. coli* O157:H7 in food, it is necessary to determine

their mechanism of resistance using molecular and physiological studies in stressful environments (Chung *et al.,* 2006). Their changes in cell membrane profiles in stressful environments are also important in designing methods to effectively control their presence in food systems. Thus the objective of this study was to investigate whether acid-adapted *E. coli* O157:H7 elicits cross-protection against activated LP and lactic acid (LA) in Tryptone Soy Broth (TSB), and to determine the relative gene expression of acid and LP inducible genes during LA and LP challenge via quantitative real time PCR (qRT-PCR).

3.2 Materials and Methods

3.2.1 Bacterial cells and culture conditions

E. coli O157:H7 strain UP10 obtained from Onderstepoort Agricultural Research Institute, Republic of South Africa (RSA) was used in this study. *E. coli* O157:H7 cultures were grown aerobically in TSB (Biolab, Wadeville, RSA) at 37 °C for 24 h. Two successive transfers of 1 ml *E. coli* O157:H7 culture were made into 100 ml sterile TSB to generate an active culture, which was used as inoculum for acid-adaptation.

3.2.2 Acid-resistance assay and viability of *E. coli* O157:H7

Acid-adapted and non-adapted *E. coli* O157:H7 cultures were prepared according to procedure described by Buchanan and Edelson (1996). This entailed transferring 1 ml of an active culture of *E. coli* O157:H7 into 100 ml of sterile TSB supplemented with 1 % glucose (TSBG, Associated Chemical Enterprises, Glenvista, RSA). The culture was incubated at 37 °C for 18 h until the stationary phase was reached. The growth phase was monitored by measuring the optical density in a spectrophotometer (Milton Roy Spectronic 20D) at a wavelength of 600 nm. For non-adapted controls, 1 ml of *E. coli* O157:H7 cells was transferred to 100 ml of TSB and buffered with 100 mM

morpholinemethanesulfonic acid (MOPS, Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 37 °C for 18 h.

Acid resistance was tested by inoculating 1 % of acid-adapted and non-adapted cultures into TSB acidified with 6 mol/l LA (Saarchem, Wadeville, RSA) to pH 4.0 and 5.0 and incubating at 37 °C for 6 h.

To test cross-protection of acid-adapted *E. coli* to combined LP system and acidification, the LP system was activated in TSB (pH 7.4) and in acidified TSB (pH 4.0) by adding 10 µg ml^{-1} LP enzyme (39 U/ml, Sigma-Aldrich, Steinheim, Germany) and 0.25 mmol/l sodium thiocyanate (Saarchem, Krugersdorp, South Africa). As a source of hydrogen peroxide 0.25 mmol/l sodium percarbonate (Aldrich Chemical Company Inc., Milwaukee, USA) was added. Acid-adapted and non-adapted cultures were challenged against the LP system in TSB (pH 7.4) and in acidified TSB (pH 4.0) for 6 h at 25 °C.

The viability of acid-adapted and non-adapted *E. coli* O157:H7 cells were quantified on Tryptone Soy Agar (TSA, Biolab, Wadeville, RSA) after being subjected to combined LP system and LA at pH levels of 4.0 and 5.0. Inoculated plates were incubated at 37 °C for 24 h before enumeration.

3.2.3 Fatty acid analysis

The method of Brown *et al.* (1997) was used to determine the fatty acid profile of *E. coli* O157:H7. The 18 h acid-adapted and non-adapted *E. coli* O157:H7 cultures were harvested by centrifugation at 9,000 x g for 10 min (Hettich Zentrifugen Rotanta 460R, Tultligen, Germany). The supernatant was discarded and the pellet washed twice with sterile saline water (0.85 % [w/v]). The concentrated cells were then stressed for 6 h by inoculating them into acidified TSB at pH levels of 4.0 and 5.0. and 7.4, and LP system only at pH 7.4. The cells were centrifuged once again at 9,000 x g for 10 min and washed twice in sterile saline water. The modified one-phase $CHCl_3$-MeOH-H_2O Bligh and Dyer

method (Bligh and Dyer, 1959) was used for lipid extraction and purification. The fatty acid methyl esters (FAME) were analyzed in a Varian 3300 gas chromatograph (Varian Associates Inc., 1985, USA). The peak areas were quantified with Empower build 1154, and identified by comparing them with the retention times of standard FAME mixture.

3.2.4 RNA extraction and cDNA synthesis

Acid-adapted and non-adapted *E. coli* O157:H7 challenged with LA at pH 4.0 only, LP system only and the combination of LA and the LP system at pH 4.0 for 6 h at 25 °C was harvested by centrifugation (5,000 x g for 10 min). Total RNA was extracted and purified using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The RNA concentrations and purity were determined by spectrophotometry at 260 nm. The total RNA for all samples were reverse transcribed into cDNA after further elimination of genomic DNA from isolated RNA. Reverse transcription was conducted with the Qiagen QuantiTect Reverse Transcription Kit using random hexamer primers (Qiagen) in accordance with the manufacturer's protocol.

3.2.5 Quantitative real-time PCR

Target specific primers used for qRT-PCR are shown in Table 4. The 16SrRNA primers were used for amplification of the reference gene. Real time PCR conditions were optimized in a gradient cycler (Chromo4 light cycler, BioRad).

The light cycler mastermixes were prepared to a final volume of 25 µl with the following components: 12.5 µl of iQ SYBR Green supermix (BioRad), 5 µmol/l primers, and 5.5 µl nuclease free water (Fermentas). PCR strips were filled with RT-PCR mastermix (23.0 µl) and 2.0 µl of cDNA (10 ng). The following thermal cycling parameters were used for real-time PCR: denaturation at 95 °C for 3 min; and 30 cycles of amplification and quantitation at 95 °C for 15 s, 58–64 °C for 15 s and 72 °C for 60 s followed by fluorescence reading. The melting curve of the amplified products was generated at the

end of each amplification assay at 60–95 °C at a heating rate of 0.1 °C/s. All experiments were conducted in duplicate and normalized with the reference gene. The Relative Expression Software Tool (REST, version 1.9.12, 2005) was used to determine whether there were significant differences in the expression of target genes between challenged cells and the control (untreated *E. coli* O157:H7 cells).

Table 4: Oligonucleotide primers used for quantitative real time-PCR

Gene	Gene product	Forward primer (5'-3')	Reverse primer(5'-3')
16SrRNA	House keeping gene (reference gene)	GAATGCCACGGTGAA TACGTT	ACCCACTCCCAT GGTGTGA
rpoS	Alternate sigma factor S	GAATAGTACGGGTTT GGTTCATAAT	GCGTTGCTGGACC TTATC
gadA	Glutamate decarboxylase isozyme	CTTTCGCCATCAACT TCT CC	AGGGTGTATCCCG GATCTTC
ompC	Outer membrane porin	GAAACTGCAGCACCG AT	CTTTGCTGTTCAGT ACCAGG
ompF	Outer membrane porin	TTAGAGCGGCGTGCA GTGTC	CGCTGACGTTGGT TCTTTCG
cfa	Cyclopropane fatty acyl phospholipids synthase	TTGATGGCGTGGTAT GAACG	AGAACACCACCTG CCAGAGC
corA	Magnesium transporter	GATGACGGCCTGCAT ATTC	GGGCACGCATACG ATACA

3.2.6 Statistical analysis

Analysis of variance (ANOVA) was used to determine whether the activated-LP system and low pH had a statistically significant effect on the survival of acid-adapted and non-adapted *E. coli* O157:H7 in TSB. The significant level of ANOVA was set at $P \leq 0.05$. Duplicate samples were evaluated in each analysis and the experiment was repeated three times. ANOVA was performed using Statistica (Tulsa, Oklohama, USA, 2008). Two

experimental determinations in duplicate were conducted for the relative gene expression using Rt-PCR.

3.3 Results

3.3.1 Resistance of *E. coli* O157:H7 to lactoperoxidase in combination with lactic acid

The acid-adapted *E. coli* O157:H7 cells survived significantly ($P \leq 0.05$) better when challenged against the LP system in combination with LA stress over 24 h (Fig. 3.1.1A&B). At pH 5.0, counts of acid-adapted cells subjected to LP treatment, and control cells (no LP stress) remained unchanged during the first 6 h. Subsequently, growth of acid-adapted control cells occurred with cell concentrations increasing from 6.3 $\log_{10}$ cfu/ml to 7.6 $\log_{10}$ cfu/ml between 6 to 24 h (Fig. 3.1.1A). Similarly, growth of LP treated acid-adapted cells was observed although cell concentrations increased by 0.5 $\log_{10}$ cfu/ml between 6 and 24 h (Fig. 3.1B). Acid-adapted cells challenged at pH 4.0 with and without LP followed the same trend as acid-adapted cells challenged at pH 5.0 during the first 6 h. However, when acid-adapted cells were challenged with LP at pH 4.0, their cell numbers declined by 0.5 $\log_{10}$ cfu/ml while the control cell concentration at pH 4.0 increased by 0.5 $\log_{10}$ cfu/ml between 6 and 24 h (Fig. 3.1.1C&D).

Unlike the acid-adapted cells, LP in combination with LA had a synergistic effect on the survival of non-adapted *E. coli* O157:H7 cells over time at pH 5.0 ($P \leq 0.05$), the combined effect being bacteriostatic. This was evident between 6 and 24 h of incubation where growth of non-adapted control cells occurred after 6 h while the cell concentrations of LP treated non-adapted cells remained unchanged throughout the 24 h of incubation at 25 °C (Fig. 3.1.1C&D). At pH 4.0, non-adapted cell numbers from both LP-treated and control samples declined to undetectable levels after 24 h. The detection limit was 10 cfu/ml. However, the rate of decline of cell numbers was slower in control samples compared to LP-treated samples (Fig. 3.1.1C&D). After 6 h of challenge at pH

4.0, the non-adapted control cell concentration reduced by 2.4 $\log_{10}$ cfu/ml while LP-treated cells had a 3.9 $\log_{10}$ cfu/ml reduction in cell concentration.

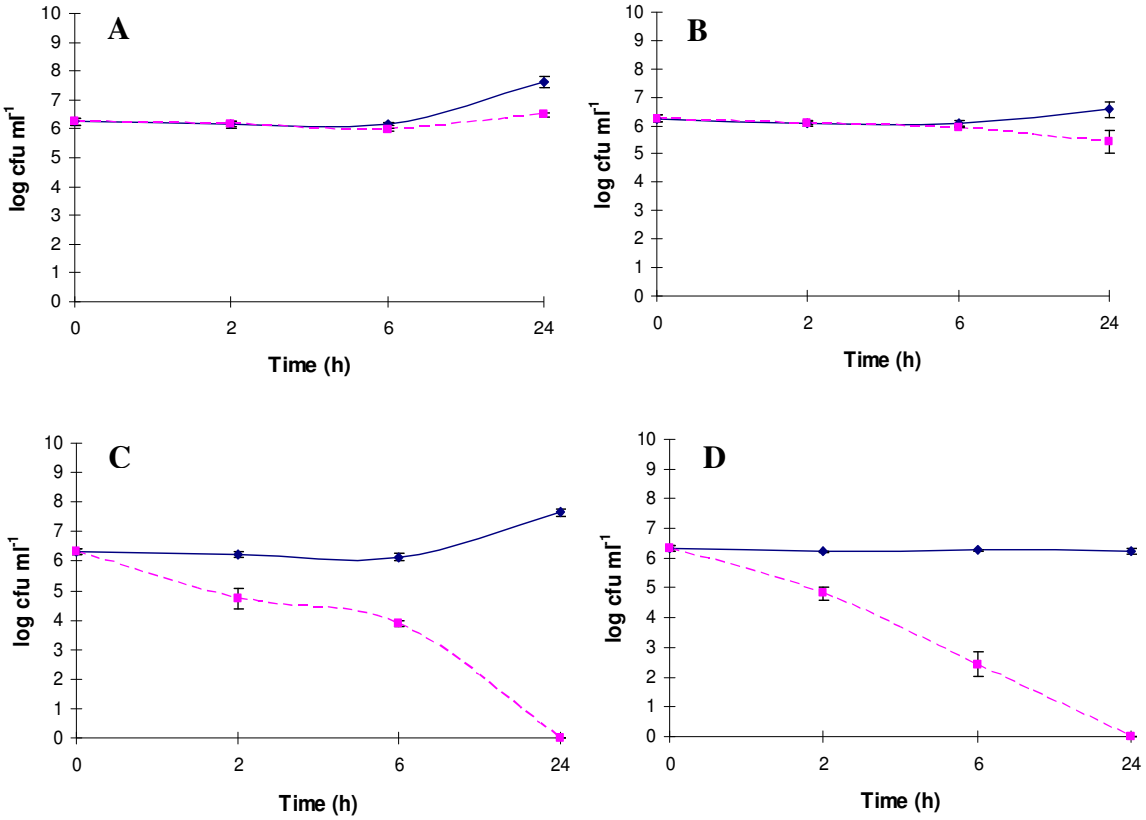


Figure 3.1: The effect of lactic acid and activated lactoperoxidase (LP) system on survival of acid-adapted (A, B) and non-adapted (C, D) *Escherichia coli* O157:H7 inocula in Tryptone Soy Broth. (A, C) Lactic acid challenge only, (B, D) Activated LP in combination with lactic acid challenge. ♦, pH 5.0; □, pH 4.0. The error bars represent standard errors of the mean from three experimental determinations

3.3.2 Effect of acid- adaptation on the fatty acid profile of *E. coli* O157:H7

The fatty acid (FA) profile of acid-adapted and non-adapted *E. coli* cells challenged against LP and LA treatments at pH levels 4.0, 5.0 and 7.4 is shown in Table 5. The FAs that were most influenced by acid-adaptation were palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1n9c) and linoleic acid (C18:2n6c).

Table 5: Outer membrane fatty acid profile of acid-adapted and non-adapted *Escherichia coli* O157:H7 challenged to lactic acid pH levels 4.0, 5.0 or 7.4 or activated lactoperoxidase at pH 7.4

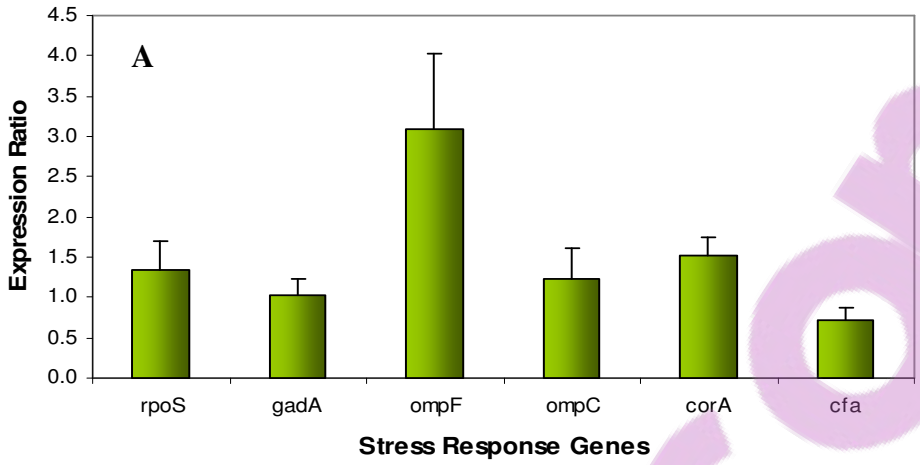
Fatty acid (%)	**A + pH7**	**N + pH7**	**A + pH5**	**N + pH5**	**A + pH4**	**N + pH4**	**A + LP**	**N + LP**
Lauric acid (C12:0)	1.98 (±1.57)	1.9 (±0.25)	ND	1.64 (±1.12)	1.11 (±0.05)	ND	1.37 (±0.43)	4.61 (±3.86)
Myristic acid (C14:0)	15.54 (±1.68)	11.74 (±2.38)	14.51 (±1.08)	9.75 (±2.11)	16.30 (±0.09)	10.42 (±0.13)	13.83 (±1.00)	12.57 (±4.11)
Palmitic acid (C16:0)	61.87 (±6.20)	53.37 (±0.21)	66.30 (±1.98)	50.03 (±0.3)	64.01 (±3.50)	51.04 (±1.40)	62.81 (±1.81)	47.00 (±4.99)
Stearic acid (C18:0)	2.63 (±1.49)	2.23 (±1.32)	2.33 (±1.32)	2.73 (±1.71)	1.04 (±0.41)	1.58 (±1.04)	2.08 (±0.72)	1.35 (±0.30)
Palmitoleic acid (C16:1)	2.28 (±0.22)	22.68 (±1.06)	2.85 (±0.09)	20.09 (±1.81)	2.71 (±0.13)	23.10 (±2.51)	3.60 (±0.19)	21.01 (±1.76)
Oleic acid (C18:1n9c)	2.94 (±0.65)	4.47 (±4.47)	4.42 (±0.09)	11.15 (±1.70)	3.95 (±0.79)	10.63 (±0.84)	6.50 (±2.04)	10.58 (±1.11)
Linoleic acid (C18:2n6c)	10.35 (±0.11)	1.13 (±0.50)	9.61 (±0.42)	3.56 (±1.11)	10.38 (±1.95)	2.00 (±1.25)	9.43 (±1.86)	2.00 (±0.15)

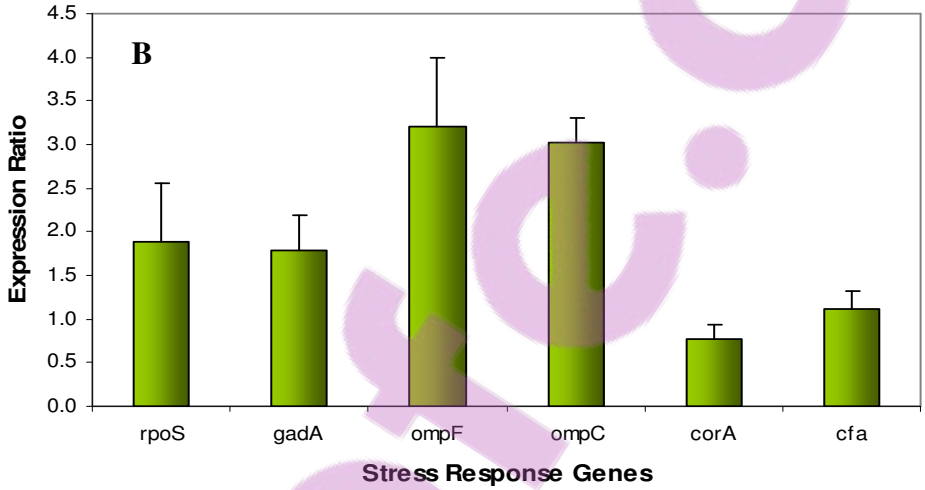
Each value presented as the mean of replicate determinations ($n = 2$) of outer membrane fatty acids ± standard deviation of replicate values; A: acid-adapted *E. coli* O157:H7; N: non-adapted *E. coli* O157:H7; LP: activated lactoperoxidase system; ND: not detected

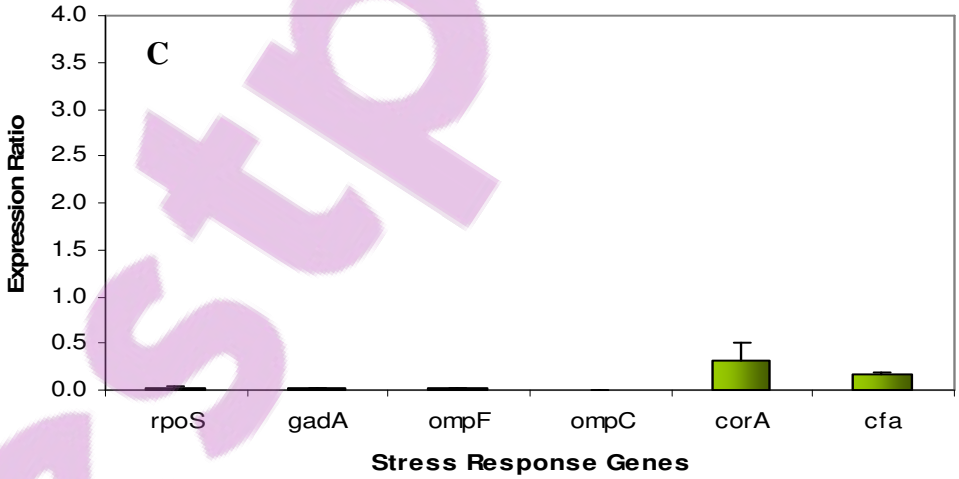
An increase in palmitic acid with a corresponding decrease in palmitoleic FAME was evident with all acid-adapted cells at pH 4.0 and pH 5.0. The acid-adapted cells had significantly higher levels of palmitic acid ($P \leq 0.05$) and significantly lower levels of palmitoleic acid ($P \leq 0.05$) compared to the non-adapted cells. It was however interesting to note selective synthesis of polyunsaturated fatty acids (PUFAs) in both acid-adapted and non-adapted *E. coli* O157:H7 cells. In acid-adapted cells, the levels of oleic acid remained unchanged. Nevertheless, significantly higher levels ($P \leq 0.05$) of linoleic acid were observed for all acid-adapted cells following LA and LP treatments.

3.3.3 Relative expression levels of lactoperoxidase and acid-inducible genes in *E. coli* O157:H7

To further understand cross-protection of acid-adapted *E. coli* O157:H7 against LP and LA challenged at pH 4.0 in a rich medium, the relative expression of acid inducible genes were assessed. Acid-adapted *E. coli* O157:H7 cells challenged against activated LP only at pH 7.4 revealed a significant ($P \leq 0.05$) increase in the expression of *gadA*, *ompC* and *ompF* compared to the control cells (Fig 3.1.2B). Although *rpoS* was expressed 1.8 times more than in the untreated control, this increase was not statistically significant ($P > 0.05$). The *corA* and *cfa* genes were expressed at basal level. The LP-treated non-adapted cells showed a significant ($P \leq 0.05$) increase in the expressions of *ompF* and *corA* (Fig. 3.1.2A). When acid-adapted cells were challenged in acidified TSB at pH 4.0 with or without LP treatment, the results revealed a significant ($P \leq 0.05$) decrease in the expressions of *rpoS*, *gadA*, *ompF*, *ompC* and *cfa* genes (Fig. 3.1.2C&D). The expression of *corA* was not significantly affected by LA and LP treatments at pH 4.0.


A
Expression Ratio
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
rpoS
gadA
ompF
ompC
corA
cfa
Stress Response Genes


B
Expression Ratio
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
rpoS
gadA
ompF
ompC
corA
cfa
Stress Response Genes


C
Expression Ratio
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
rpoS
gadA
ompF
ompC
corA
cfa
Stress Response Genes

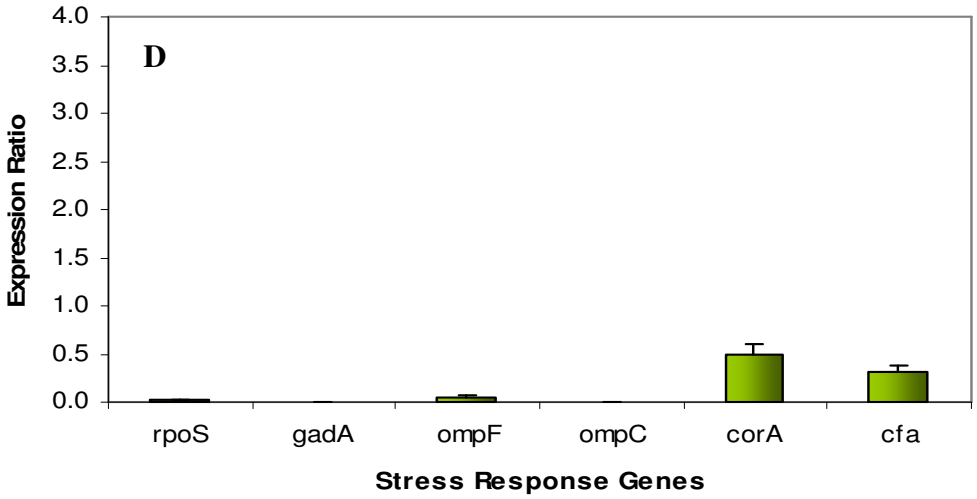


Figure 3.2: Expression of lactoperoxidase (LP) and acid inducible genes in *Escherichia coli* O157:H7 challenged against LP system and lactic acid for 6 h in Tryptone Soy Broth for 6 h at 25 °C. The bars represent the expression ratio of genes compared to untreated non-adapted *E. coli* O157:H7 cells. Error bars represent standard error of the mean from two experimental determinations.

(A) Non-adapted *E. coli* O157:H7 challenged to LP system (pH 7.4) only;

(B) Acid-adapted *E. coli* O157:H7 challenged to LP system (pH 7.4) only;

(C) Acid-adapted *E. coli* O157:H7 challenged to lactic acid at pH 4.0 only;

(D) Acid-adapted *E. coli* O157:H7 challenged to LP system in combination with lactic acid at pH 4.0.

3.4 Discussion

It has been established that *E. coli* possesses three stationary phase acid resistance systems. These include the oxidative, glutamine and arginine acid resistance systems (Lin *et al.,* 1995). In this study, expression analysis of acid-inducible genes in LP treated acid-adapted *E. coli* O157:H7 cells in TSB at pH 7.4 indicated the induction of *gadA*, *ompC,* and *ompF* genes. Increased expression of *gadA* suggests that the glutamate decarboxylase (GAD) system was induced in acid-adapted cells. The GAD system is partially regulated by RpoS (Audia *et al*., 2001). However in the absence of RpoS, GAD can be induced at

low pH and requires only 0.9 mM of glutamate for activation (Hersh *et al*., 1996). In this instance, induction of GAD expression was most likely triggered by the decrease in external pH. Since *rpoS* was not induced, it is quite clear that GAD system was induced by the house keeping sigma factor (sigma 70). This occurs at low pH in minimal glucose media (Audia *et al.,* 2001) and requires 0.9 mM of glutamate for activation (Hersh *et al.,* 1996). TSB contains 22.2 mM glutamate, which is more than enough for activation of GAD in acid-adapted *E. coli* O157:H7. The lack of induction of *rpoS* and increased expression of *gadA* in acid adapted *E. coli* O157:H7 suggest that the GAD system was at least, in part responsible for acid resistance and may have contributed to cross-protection against LP and LA stresses.

It is not clear what regulator was responsible for cross-protection against activated LP and hence increased *ompC* and *ompF* expression. The *ompC* and *ompF* genes encode outer membrane porin channels with the *ompF* being the larger of the two porins. During medium glucose limitation, the *ompF* is induced to scavenge the remaining glucose in the medium (Liu and Ferenci, 1998). However it has been reported that in a low pH medium, *ompC* is expressed at the expense of *ompF* (Heyde and Portalier, 1987). In this study, there was increased expression of both *ompC,* to limit influx of protons and *ompF,* to scavenge for glucose in the medium. In non-adapted *E. coli* O157:H7, *corA* was induced after activated LP challenge at pH 7.4. The *corA* gene encodes a magnesium transporter that has been suggested to contribute to LP resistance in *E. coli* O157:H7. Sermon *et al.* (2005) reported that a knockout mutation of *corA* gene in *E. coli* caused hypersensitivity to the LP system. In this study, the *corA* gene was however not induced in LP-treated acid-adapted cells at pH 7.4 indicating that it may be repressed during acid-adaptation.

Resistance of acid-adapted cells at pH 4.0 was due to acid-adaptation resulting from pre-exposure to mild acid stress from natural fermentation of glucose in TSBG (Buchanan and Edelson, 1996). Leyer *et al.* (1995) observed acid resistance in acid-adapted *E. coli* O157:H7 strains in broth acidified with LA. Conner and Kotrola (1995) also established that pH 4.0 prevents *E. coli* growth at 25 °C. In this study, TSB acidified to pH 4.0 with

LA inactivated non-adapted *E. coli* O157:H7. Acid-adapted cells also showed remarkable resistance to combined LP and LA treatments at pH 4.0. This resistance was due to adaptation which may have contributed to cross-protection against LP system. Cross-protection of acid-adapted *Salmonella* Typhimurium against activated LP has been reported (Leyer and Johnson, 1993). However, to the knowledge of the authors, cross-protection of acid-adapted *E. coli* O157:H7 against LA and the activated LP system has not been reported.

Expression analysis of LP and LA (pH 4.0) challenged *E. coli* O157:H7 cells revealed that the *rpoS*, *gadA*, *ompC*, *ompF* and *cfa* genes were down-regulated. Earlier studies have indicated that low pH triggers the induction of the amino acid decarboxylase systems and the sigma factor *rpoS* during log phase (Audia *et al.,* 2001; Hengge-Aronis, 2002). Regardless of down-regulation of acid resistance genes, the acid-adapted cells survived LP and LA treatments for 6 h. It is possible that during the acid challenge, the acid-inducible genes had already been translated into proteins which protected the acid-adapted *E. coli* O157:H7 cells against inactivation during LP and LA challenge at pH 4.0. This phenomenon is supported by Foster (2000) who suggested that during acid tolerance response, induction of regulative factors that activate protective proteins occurs in a mild acid environment. These proteins subsequently protect the cell during lethal acid exposure.

The FA profile of acid-adapted and non-adapted *E. coli* O157:H7 challenged at pH 4.0 revealed increases in saturation of FAs in acid-adapted cells compared to the non-adapted cells. It has been reported that post-synthetic modification of fatty acids into cyclopropane fatty acids contribute to the resistance of acid-adapted *E. coli* in low pH environments (Chang and Cronan Jr, 1999). In this study, *cfa,* which encodes cyclopropane fatty acid synthase responsible for the synthesis of cyclopropane fatty acids, was down regulated when cells were challenged at pH 4.0. However, the increase in percent saturation of FAs in cell membranes of acid-adapted cells challenged against LA (pH 4.0) correlates with acid resistance in acidified TSB at pH 4.0. In addition to

saturation of FAs, the acid-adapted cells synthesize PUFAs which maintains membrane fluidity, but may not offset restriction of solute movement into and out of the cell as modified by an increase in saturation of the outer membrane lipids. This phenomenon is supported by Russell and Nichols (1999) who explained that the multiple double bonds present in PUFAs acids are important to maintain membrane fluidity, nonetheless, it provides a greater degree of packing order of the phospholipids bilayer as compared to the monounsaturated fatty acids.

Acid resistance was demonstrated in both acid-adapted and non-adapted *E. coli* O157:H7 cells in acidified TSB at pH 5.0. This was expected because *E. coli* can survive and grow within the pH range of 5.0 to 8.5 while maintaining an internal pH of 7.6 to 7.8 (Foster 2000). In a similar study, Deng *et al.* (1999) reported no significant difference between survival of acid-adapted and non-adapted *E. coli* O157:H7 cells plated on TSA acidified with acetic, malic and citric acid to a final pH of 5.1. The growth of both acid-adapted and non-adapted cells after 6 h indicates that pH 5.0 is temporarily bacteriostatic to *E. coli* cells; these cells can inherently adapt and subsequently resume normal growth. The FA profile at pH 5.0 did not differ from that of acid-adapted and non-adapted cells at pH 7.4 indicating that changes in outer membrane FAs that occurred during acid-adaptation remained unchanged during LA challenge at pH 5.0. Nonetheless, acid-adaptation conferred cross-protection against activated LP in acidified TSB at pH 5.0.

In conclusion, acid inducible genes are expressed during mild acid exposure and not during lethal acid challenge. In the absence of *rpoS*, acid-adapted *E. coli* O157:H7 exhibits high acid resistance in lethal acid environment as well as cross-protection against the LP system suggesting that RpoS-independent systems are not only responsible for acid resistance, but also contribute to cross-protection against activated LP in combination with low pH. Acid resistance and cross-protection of *E. coli* O157:H7 can increase its chances of survival in fermented LP-activated products and can limit the potential benefits of LP-activation in low pH foods.

3.5 Acknowledgements

This research was supported by the National Research Fund and the Third World Organization for Women in Science.

Chapter 4:

THE INFLUENCE OF LACTOPEROXIDASE, HEAT AND LOW PH ON SURVIVAL OF ACID-ADAPTED AND NON-ADAPTED ESCHERICHIA COLI *O157:H7 IN GOAT MILK*

Published: *International Dairy Journal (2009, 19, 417-421).*

Abstract

In hot climates where quality of milk is difficult to control, a lactoperoxidase (LP) system can be applied in combination with conventional preservation treatments at sub-lethal levels to inhibit pathogenic microbes. This study investigated the effect of combined heat treatments (55 °C, 60 °C and 72 °C) and milk acidification (pH 5.0) on survival of acid-adapted and non-adapted *Escherichia coli* O157:H7 strains UP10 and 1062 in activated LP goat milk. Heat treatment at 72 °C eliminated *E. coli* O157:H7. Acid-adapted strains UP10 and 1062 cells showed resistance to combined LP and heat at 60 °C in fresh milk. The inhibition of acid-adapted and non-adapted *E. coli* O157:H7 in milk following combined LP-activation, heat (60 °C) and milk acidification (pH 5.0) suggest that these treatments can be applied to reduce *E. coli* O157:H7 cells in milk when they occur at low numbers (< 5 $\log_{10}$ cfu/ml) but does not eliminate *E. coli* O157:H7 to produce a safe product.

Keywords: Lactoperoxidase, acid-adaptation, low pH, heat, *Escherichia coli* O157:H7

4.1 Introduction

Goat milk production in many developing countries is dispersed and diversified among small holder farmers. In some African regions, where ambient temperature rises well above 30 °C, spoilage of milk is rapid and high losses may discourage commercial production of goat milk. *Escherichia coli* O157:H7 has been isolated from fresh and pasteurized milk as well as cheeses made from unpasteurized milk (Wang *et al.,* 1997). *E. coli* O157:H7 has a low infectious dose; therefore its occurrence in goat milk, even at low numbers, is critical.

The use of the lactoperoxidase (LP) system has been recommended as a safe and effective natural intervention technique for the preservation of milk in the absence of refrigeration (IDF, 1988). This system comprises the LP enzyme naturally found in milk, together with exogenous sources of thiocyanate and hydrogen peroxide that are required to activate the LP system (Björck, 1987). This antimicrobial system can be applied alone or in combination with conventional methods in milk preservation (FAO/WHO, 2006). Although there have been several studies that have investigated the effect of pasteurization on LP activity in milk, the efficacy of the LP system on reduction of *E. coli* O157:H7 numbers before pasteurization of milk has only been suggested (FAO/WHO, 2006). In addition, many dairy products rely on acidification processes that characterize their manufacture and preservation to control survival and growth of some pathogens. However, *E. coli* O157:H7 has the ability to adapt to mild acidic pH-values that result in enhanced resistance to lethal acid environments (Lin *et al.,* 1996). There is also evidence that this acid adaptive response can confer cross-protection against other environmental stresses such as heat treatment and salt (Rowe and Kirk, 1999).

Antimicrobial technologies used in preservation of dairy foods have been studied in broth systems and in isolation against *E. coli* O157:H7. However, cross-protection studies of acid-adapted *E. coli* to combined preservation treatments in food systems are scant. To the knowledge of the authors, such a study making use of combined preservation methods

applied in dairy processing has not been conducted in dairy products. In regions where milk quality is difficult to control, and in instances were standard preservation methods such as low salt and low moisture have been applied at reduced intensities, the application of an activated LP in combination with low pH and heat treatment at sub-pasteurization temperatures may be employed to improve the microbiological quality of milk. The goal of this study was therefore to determine the effect of combined LP, heat and low pH treatments on survival of acid-adapted *E. coli* O157:H7 in goat milk.

4.2 Materials and Methods

4.2.1. *E. coli* O157:H7 strains and acid-adaptation

E. coli O157:H7 strains UP10 and 1062 obtained from the Veterinary Institute of the Agricultural Research Council, Pretoria, Republic of South Africa (RSA), were used in this study. Working cultures were stored in Tryptone Soy Broth (TSB; Biolab, Wadeville, RSA) at 2 °C. The cultures were activated by transferring 1 ml of culture into 10 ml of sterile TSB and incubated at 37 °C for 24 h. Acid-adapted and non-adapted cultures were prepared according to the procedure of Buchanan and Edelson (1996). Acid-adaptation was confirmed by challenging acid-adapted and non-adapted cultures in TSB acidified to pH 4.0 with 6 M lactic acid (Saarchem, Krugersdorp, RSA) for 6 h at 37 °C.

4.2.2 Milk source

Fresh Saanen goat milk obtained from the University of Pretoria's Experimental farm was used as the medium for challenge tests. The goats were milked following standard procedures with a milking machine. Milk from individual Saanen goats was pooled together and used within one hour of milking for challenge tests. To evaluate the initial microbiological quality of fresh Saanen goat milk, the mean aerobic plate count of the goat milk was determined to be 5.44 (±0.42) $\log_{10}$ cfu/ml (the standard deviation of the

mean is indicated in parenthesis). The combined treatments were carried out in two batches. The first batch was carried out in fresh goat milk (pH 6.9) and the second batch conducted in goat milk acidified with 6 M lactic acid to pH 5.0. A pH-value of 5.0 was used because indigenous dairy products in Southern Africa such as Madila and Omashikwa are fermented from unpasteurized milk, reaching a final pH between pH 5.0 and 4.5.

4.2.3 Inoculation of milk with *E. coli* O157:H7

Aliquots (10 ml) of fresh and acidified goat milk were aseptically transferred into sterile MacCartney bottles and inoculated with 1 % (v/v) of acid-adapted *E. coli* O157:H7 strain UP10 or strain 1062. To prepare control incubations for acid-adaptation experiments, 1 % (v/v) *E. coli* O157:H7 strain UP10 and strain 1062 that had not been adapted to acid were dispensed into 10 ml aliquots of fresh and acidified goat milk.

4.2.4. Activation of the lactoperoxidase system

Before activation of the LP system, the thiocyanate content of goat milk was determined according to the International Dairy Federation method (IDF, 1988) to be 2.27 ppm. The activity of the LP was measured according to Seifu *et al.* (2004) using one-step ABTS (2,2'-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid, Sigma, St. Louis, MO, USA) solution as a substrate. Absorbance was measured at 412 nm as a function of time for 2 min at 10 s intervals. The LP activity was then calculated according to Kumar and Bhatia (1999) to be 0.21 U/ml in fresh Saanen goat milk. Following thorough mixing, the LP system in both acid-adapted and non-adapted *E. coli* O157:H7 milk cultures were activated by adding sodium thiocyanate (Saarchem, Krugersdorp, RSA) to a final concentration of 14 mg/l together with 30 mg/l of sodium percarbonate (Aldrich Chemical Company Inc., Milwaukee, WI, USA) as a source of hydrogen peroxide (IDF, 1988). The untreated LP control milk cultures had neither sodium thiocyanate nor sodium per carbonate added. All inoculated goat milk samples were mixed thoroughly and

incubated at 25 °C for 6 h.

4.2.5 Heat treatment

After 6 h of incubation, the samples were divided into three lots: lots A, B and C. All three lots were comprised of both acid-adapted and non-adapted *E. coli* O157:H7 cultures in control and activated LP milk. These samples were heat-treated as follows: sample lots A, B and C were heat-treated at 55 °C, 60 °C or 72 °C respectively by submerging samples in a thermostatic water bath at the appropriate treatment temperatures. The temperature of the samples was monitored by inserting a thermocouple probe of a digital thermometer into a control McCartney bottle, containing 10 ml of goat milk, in the water bath. The goat milk samples took an average of 7 min, 8 min and 18 min to reach temperatures of 55 °C, 60 °C and 72 °C respectively. The samples were maintained at the appropriate heating temperature for 15 s each. All samples were cooled on ice for 1 min immediately after heat treatment. This procedure was repeated for acidified goat milk cultures.

4.2.6 Microbial analyses

Samples were taken from inoculated goat milk at time 0 h before any treatments, 6 h after LP activation in fresh and acidified milk, and after heat treatment (7 h after initial LP treatment, as the heating step took an additional 1 h) at 55 °C, 60 °C and 72 °C of activated LP and untreated LP milk cultures at pH levels 6.9 and 5.0. Milk samples were serially diluted in sterile 0.1 % (w/v) buffered peptone water (Oxoid, Basingstoke, UK). Sorbitol-negative *E. coli* O157:H7 cells were enumerated following spread plating on Sorbitol MacConkey Agar (SMAC, Oxoid) and incubation at 37 °C for 24 h. To determine the initial bacterial quality of fresh goat milk, the total aerobic bacteria from fresh milk samples only were enumerated on Plate Count Agar (Biolab) after incubation at 25 °C for 24 h.

4.2.7. Statistical analysis

Three replicate experiments were conducted for the combined treatments in fresh goat milk and in goat milk acidified to pH 5.0. The experimental variables were *E. coli* adaptation (acid-adapted versus non-adapted), treatments (pH, activated LP and temperature), and time (0 and 6 h). For each sample, the mean $\log_{10}$ cfu/ml *E. coli* O157:H7 counts from individual treatments and their interactions in the combined effects were evaluated using Analysis of Variance (ANOVA). The significance level was set at $P < 0.05$. ANOVA was performed using Statistica software for Windows version 7 (Tulsa, OK, USA).

4.3 Results

4.3.1 Lactoperoxidase activity

The mean LP activities of fresh and heat treated Saanen goat milk are shown in Fig. 4.1. The LP activity of goat milk was unaffected by heat treatment at 55 °C or 60 °C for 15 s. Heat treatment at 72 °C caused a 50 % reduction in LP activity.

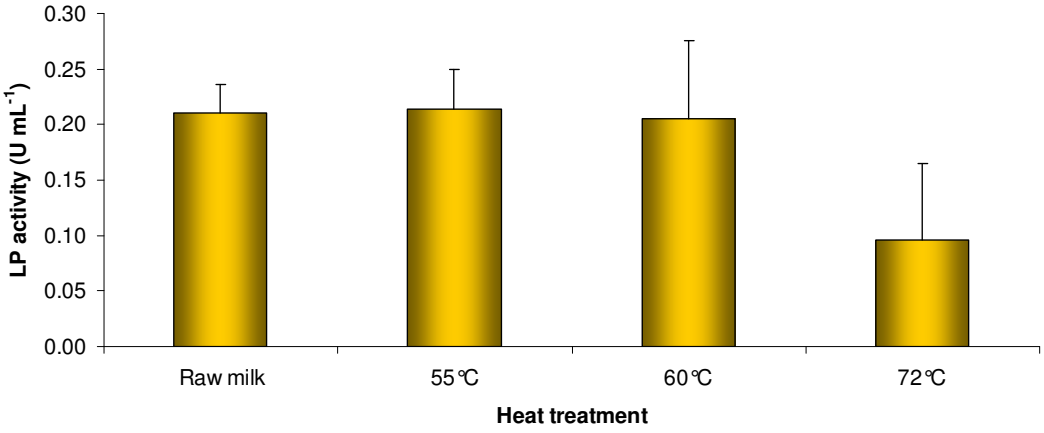


Figure 4.1: The lactoperoxidase activities of fresh goat milk (pH 6.9) before and after heat treatment at 55 °C, 60 °C and 72 °C for 15 s (excluding time taken to reach target temperature). The error bars represent standard error of the mean.

4.3.2 The effect of combined treatments of activated lactoperoxidase and low pH on survival of *E. coli* O157:H7

Survival of acid-adapted and non-adapted *E. coli* O157:H7 strains UP10 and 1062 in activated LP milk at pH levels 6.9 and 5.0 are shown in Fig. 4.2. Both *E. coli* O157:H7 strains UP10 and 1062 showed similar growth patterns for acid-adapted and non-adapted cells in goat milk. In both cases, acid-adapted cells gave poorer growth in fresh milk compared to the non-adapted cells. However, both acid-adapted and non-adapted strain 1062 cells grew significantly better ($P < 0.05$) in fresh goat milk compared to strain UP10 cells. The LP alone did not significantly affect *E. coli* O157:H7 cells. The acid-adapted strain 1062 cells were resistant to the combined low pH and activated LP treatments, which had little impact on growth and survival, whereas LP had a bacteriostatic impact on non-adapted cells under the same conditions (Fig. 4.2). Both acid-adapted and non-adapted strain UP10 cell numbers were not significantly affected by combined LP and low pH treatments.

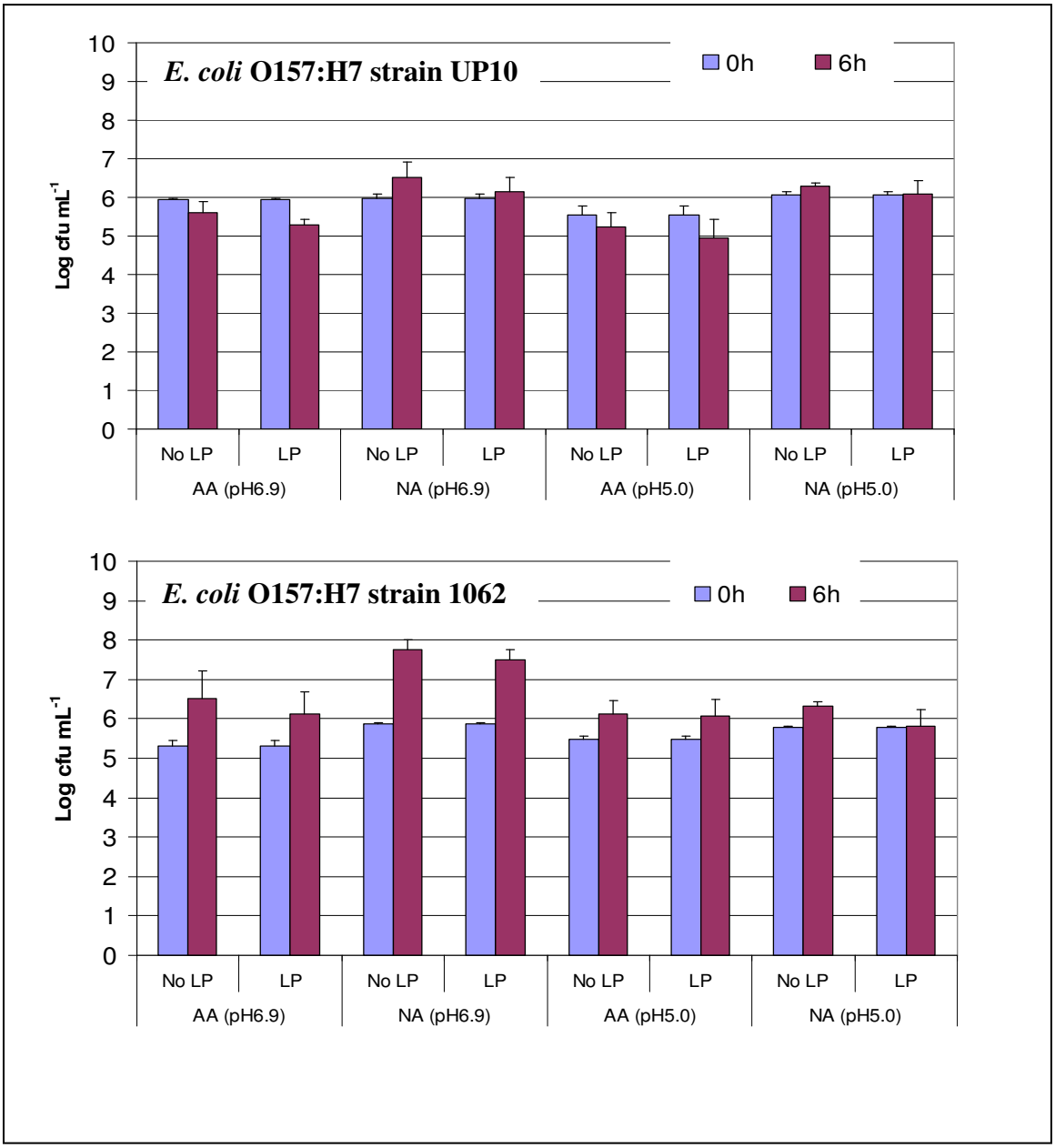


Figure 4.2: The effect of activated lactoperoxidase (LP) on acid-adapted (AA) and non-adapted (NA) *E. coli* O157:H7 strains UP10 and 1062 in fresh (pH 6.9) and acidified (pH 5.0) goat milk incubated for 6 h at 25 °C. Error bars represent standard error of the mean

4.3.3 The effect of combined treatments of activated lactoperoxidase, low pH and heat on survival of *E. coli* O157:H7

In fresh milk (pH 6.9), the non-adapted cells of strain UP10 showed greater sensitivity to heat treatment alone at 55 °C compared to the acid-adapted strain UP10 cells (Fig. 4.3). However, both acid-adapted and non-adapted strain UP10 cells showed identical sensitivity to the combination of heat (55 °C) in activated LP milk. The effect of the combination of activated LP and heat treatments significantly differed ($P < 0.05$) between non-adapted cells of strain UP10 and strain 1062 at 55 °C, where the non-adapted strain 1062 cells showed resistance to heat and LP-activation. Conversely, the non-adapted strain UP10 cells were sensitive to heat at 55 °C and LP treatment. Heat treatment alone at 55 °C caused a 1.28 $\log_{10}$ cfu/ml reduction in non-adapted strain UP10 cells whereas the combination of heat treatment (55 °C) and the LP system caused a 1.58 $\log_{10}$ cfu/ml reduction in non-adapted UP10 population. At 60 °C, acid-adapted and non-adapted cells of both strains showed similar sensitivities to combined heat and LP system treatment (Fig. 4.3). Heat treatment at 72 °C reduced all *E. coli* cells regardless to adaptation and strain in both activated LP and untreated LP goat milk, to undetectable levels (detection limit was 10 cfu/ml).

The survival of acid-adapted and non-adapted *E. coli* O157:H7 strains UP10 and 1062 cells to heat treatments in acidified and LP-activated goat milk are presented in Fig. 4.4. Heat treatments in acidified goat milk (pH 5.0) had a greater inhibitory effect on *E. coli* cells ($P < 0.05$) compared to heat treatment in goat milk at pH 6.9. Activated LP alone did not significantly affect both acid-adapted and non-adapted cells. However, the application of LP in combination with heat treatment at 55 °C and low pH (5.0) significantly ($P < 0.05$) inhibited non-adapted strain UP10 cells. Such inhibition was not observed in strain 1062 cells under the same conditions. Heat treatment at 60 °C inhibited both UP10 and 1062 strains to a larger extent compared to heat treatment at 55 °C.

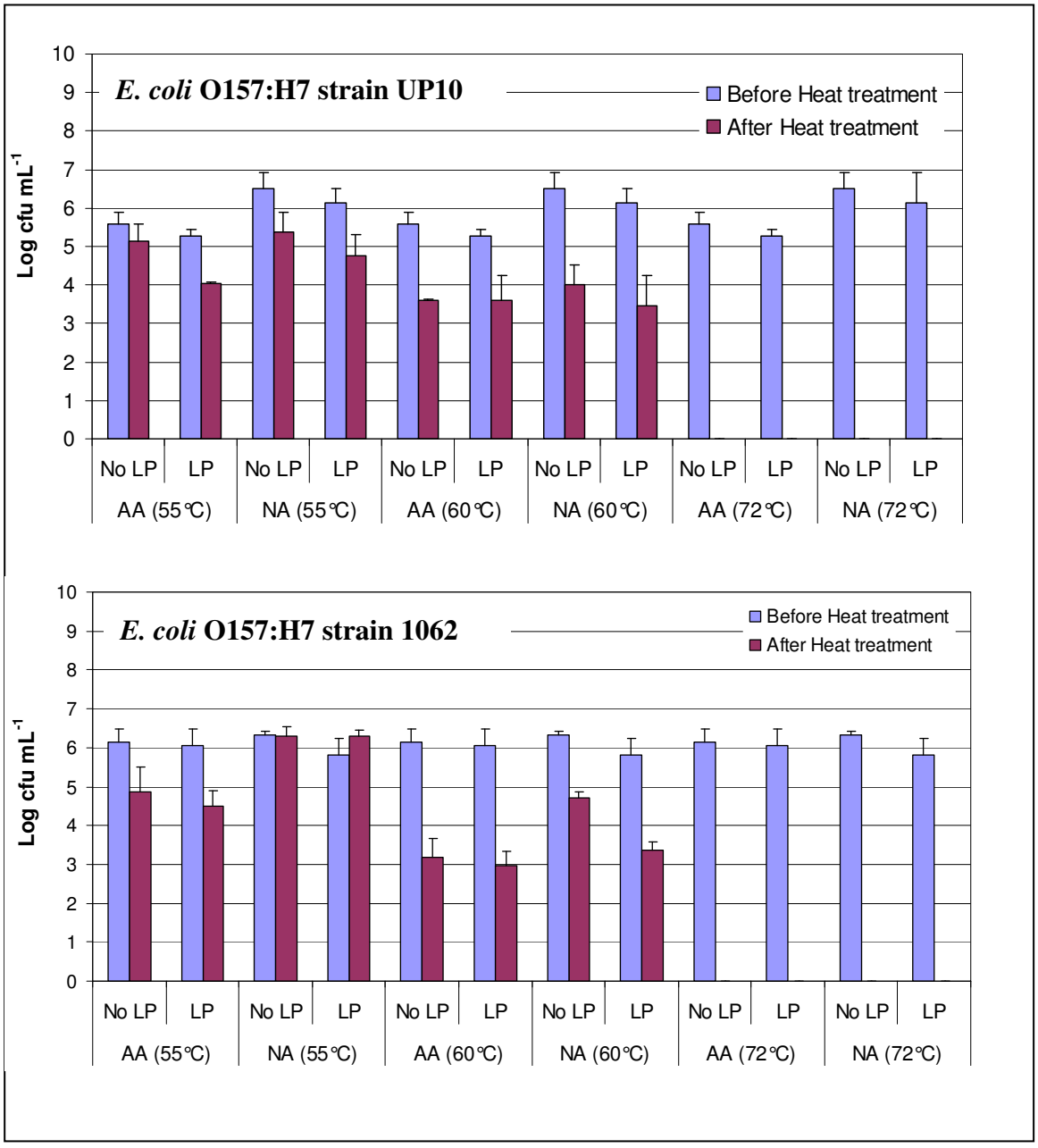


Figure 4.3: The survival of acid-adapted (AA) and non-adapted (NA) *Escherichia coli* O157:H7 strains UP10 and 1062 to the combined effect of heat (55°C or 60°C for 15 s, excluding time taken to reach target temperature) in activated lactoperoxidase (LP) goat milk at pH 6.9. Error bars represent standard error of the mean

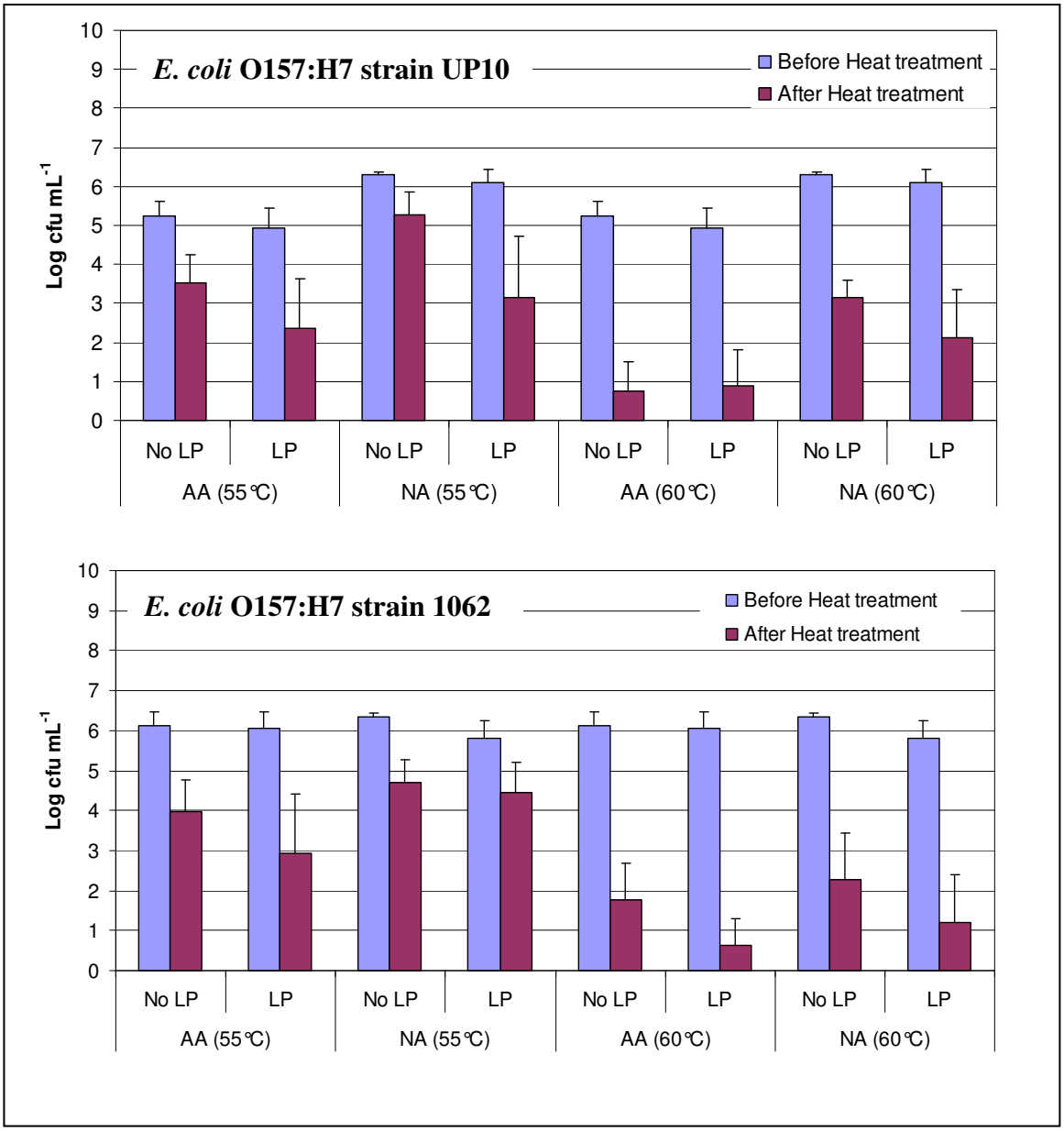


Figure 4.4: The survival of acid-adapted (AA) and non-adapted (NA) *Escherichia coli* O157:H7 strains UP10 and 1062 to the combined effect of heat (55°C or 60°C for 15 s, excluding time taken to reach target temperature), activated lactoperoxidase (LP) and low pH (pH 5.0) treatments in goat milk. Error bars represent standard error of the mean

4.4 Discussion

There have been several reports that acid-adaptation of *E. coli* enhances its survival in acidic foods (Leyer *et al.,* 1995; Ryu and Beuchat, 1998; Sainz *et al*., 2005). In this study, the acid-adapted strain UP10 cells were inhibited in fresh (pH 6.9) and acidified (pH 5.0) goat milk. Though unusual, a similar inhibition of acid-adapted *E. coli* O157:H7 cells compared to cells not adapted to acid (non-adapted) in yoghurt has been reported by Hsin-Yi and Chou (2001). In their study, acid-adapted *E. coli* cells showed greater sensitivity to low pH compared to non-adapted cells in Yakult, a diluted fermented milk drink (pH 3.9) and yoghurt (pH 3.9) throughout 144 h of storage at 7 °C. Riordan *et al.* (2000) also observed a greater decline of acid-adapted *E. coli* O157:H7 cells compared to non-adapted cells during the fermentation of pepperoni (pH 4.5 to 4.8).

The reduced numbers of acid-adapted *E. coli* O157:H7 observed in goat milk in this study suggests that, although acid-adaptation induces protective mechanisms to enhance survival at lethal pH values, the acid-adaptation process may have increased sensitivity of *E. coli* O157:H7 cells to stresses encountered in goat milk. In this study, the oxidative stationary phase acid resistance was induced in acid-adapted *E. coli* O157:H7 according to the method of Buchanan and Edelson (1996). Since the oxidative acid resistance system is glucose repressed, it is likely that this system may have been repressed to some degree in goat milk. Such repression may have been stressful and thus impacted upon growth of acid-adapted cells. According to Glass *et al.* (1992), *E. coli* cells can survive and grow within the pH range of 4.5 to 9.0. For this reason, it is also possible that acid-adaptation was not essential for survival in milk at pH 6.9 and pH 5.0. Given that maintenance of acid-adaptation is an energy demanding process, the acid-adapted cells may have readapted to the pH of milk, a process which may have been stressful and also impacted upon growth. Nonetheless, the susceptibility of acid-adapted *E. coli* O157:H7 in goat milk warrants further investigation.

Heat sensitivity of *E. coli* has been well documented (Kaur *et al*., 1998). The application

of heat remains one of the important technologies used for the control of *E. coli* occurrence in foods. Nevertheless, the heat sensitivity of *E. coli* can be influenced by adaptive responses to sub-lethal heat shock, acid tolerance, starvation and entry into the stationary phase (Jenkins *et al*., 1988; Kaur *et al.,* 1998; Rowe and Kirk, 1999; Arsène *et al.,* 2000). The reduction of cell numbers with an increase in heat observed in this study tallies with earlier findings by D'Aoust *et al.* (1988) who reported an average of 2 $\log_{10}$ cfu/ml reduction of *E. coli* O157:H7 cells heated at 60 °C in fresh milk. In this study, acid-adapted cells were sensitive to combined treatments of activated LP and heat at 55 °C and 60 °C. Heat treatment at 72 °C eliminated *E. coli* O157:H7 cells in goat milk and therefore remains the most effective method to reduce *E. coli* O157:H7 in milk. It should however be noted that the time to reach target temperatures were relatively long (7 min, 8 min and 18 min to reach 55 °C, 60 °C and 72 °C respectively) and may have impacted upon the inhibition of *E. coli* O157:H7 cells in goat milk.

The resistance of non-adapted strain 1062 cells to combined heat (55 °C) and activated LP was noteworthy. It was, however, observed that the non-adapted cells of strain 1062 were generally resistant to activated LP at pH 6.9. This tolerance to activated LP could have induced resistance to heat treatment at 55 °C. The LP-induced resistance to heat treatment was, however, absent at 55 °C in non-adapted cells of strain UP10, suggesting that resistance to LP and mild heat treatment (55 °C) may be an inherent characteristic of *E. coli* O157:H7 strain 1062. In this study, the differences in sensitivities between the non-adapted cells of the two strains tested to combined heat, low pH and activated LP treatments were also observed for acid-adapted *E. coli* O157:H7 cells of both strains tested. Benito *et al*., (1999) reported that *E. coli* O157 strains vary greatly in their resistance against environmental stresses including mild heat treatment. It was thus not surprising that strains 1062 and UP10 reacted differently to combined treatments.

Although both acid-adapted and non-adapted cells were resistant to activated LP alone, the combination of LP and heat treatments inhibited both acid-adapted and non-adapted

E. coli O157:H7 cells at 55 °C, and non-adapted cells at 60 °C. This observation indicates LP sensitization of *E. coli* cells to sub-lethal heat treatment at pH 6.9. The inhibitory effect of the combination of LP and heat treatments was more pronounced at pH 5.0. Tenovuo *et al.* (1991) reported that at low pH (≤ 5.3), the antimicrobial hypothiocyanite compound generated by the LP catalysis of the oxidation of thiocyanate in the presence of hydrogen peroxide, occurs as an uncharged molecule which makes it easier to pass through the bacterial membrane into the cell to inhibit metabolic processes. Such combined treatments can therefore be applied to dairy products to reduce the numbers of contaminating *E. coli* O157:H7 cells that may occur, especially when contaminating *E. coli* O157:H7 occurs in low numbers. Nonetheless, the effect of combined treatments of heat (55 °C or 60 °C), low pH (pH 5.0) and activated LP caused less than 5.0 $\log_{10}$ cfu/ml reduction in both acid-adapted and non-adapted *E. coli* O157:H7 and is therefore insufficient to produce a safe product.

4.5 Conclusion

The sensitivity of both acid-adapted and non-adapted *E. coli* O157:H7 cells in activated LP goat milk treated with heat (60 °C) and low pH (pH 5.0) at sub-lethal levels suggests that these treatments may be applied to reduce the numbers of both acid-adapted and non-adapted *E. coli* O157:H7 cells in milk. However, these treatments may be insufficient to eliminate the presence of *E. coli* O157:H7 in acidified dairy products.

4.6 Acknowledgement

This research was supported by the National Research Fund and the Third World Organization for Women in Science.

Chapter 5:

EFFECT OF LACTOPEROXIDASE SYSTEM AND ESCHERICHIA COLI *O157:H7 GROWTH ON ACID-PRODUCTION BY SINGLE STRAIN AND INDIGENOUS LACTIC ACID BACTERIA IN GOAT MILK*

Submitted to Dairy Science and Technology

Abstract

This study determined the effect of the lactoperoxidase (LP) system on growth and acid production by single strain and indigenous lactic acid bacteria (LAB) and the survival of inoculated *Escherichia coli* O157:H7 during the fermentation of goat milk. LP activated raw and pasteurized goat milk were inoculated with single strain *Lactococcus* spp. and *Bifidobacterium longum* BB 536 and incubated for 24 h at 30 °C to simulate commercial milk fermentation, while the traditional Madila product was fermented with indigenous LAB for 5 days at 30 °C. Goat milk was also inoculated with *E. coli* O157:H7 to determine survival during fermentation of the LP activated milk. The viability of LAB and *E. coli* O157:H7, the pH and the acid production were followed throughout the fermentation periods. None of the LAB cultures tested showed significant susceptibility to the LP system with respect to growth and acid production during milk fermentation. *E. coli* O157:H7 was however inhibited in LP activated milk in the commercial and traditional product. In the traditional product *E. coli* O157:H7 counts were reduced by > 5.0 $\log_{10}$ cfu/ml. The LP system can therefore be applied during the fermentation of traditional and commercial milk processing at ambient temperatures as an additional bacteriological control measure to improve the quality of fermented dairy products.

Keywords: Lactoperoxidase, goat milk, lactic acid, starter culture and *Escherichia coli* O157:H7

5.1 Introduction

Small-scale milk production in developing countries where ambient temperatures are well above 30 °C, suffer high losses, particularly when the market chain lacks adequate infrastructure to preserve the milk. For this reason, most small-scale agro-pastoralists process their left over milk into artisanal fermented dairy products for home consumption; some of which enter the informal market for economic benefit. However, lack of process control results in inconsistent quality of traditional fermented dairy products. Recent implications of dairy and other acidic foods in *Escherichia coli* O157:H7 outbreaks (Besser, Lett, Weber, Doyle, Barett, Wells and Griffin, 1993; Morgan, Newman, Hutchinson, Walker, Rowe and Majid, 1994) have challenged the safety of goat milk products processed under uncontrolled conditions. The persistence of *E. coli* O157:H7 in low pH foods has been attributed to acid-adaptation in the gut, in animal feed or during processing of fermented dairy products (Leyer *et al.,* 1995; Diez-Gonzalez, Callaway, Kizoulis, and Russell, 1998; Dlamini and Buys, 2009). Once acid-adapted, *E. coli* O157:H7 can survive in high acid foods for extended periods of time and can survive lethal pH of the stomach to cause disease in the intestine (Karmali, 1989; Paton and Paton, 1998; Seputiene *et al.,* 2005). Since *E. coli* O157:H7 is commonly found in raw milk, it can also contaminate milk post-pasteurization following poor milk handling. For this reason, proper handling of raw and pasteurized milk and the application of appropriate hygiene and preservation methodologies are important to inhibit *E. coli* O157:H7 since low pH alone is no longer sufficient to eliminate its occurrence in fermented dairy products.

The lactoperoxidase (LP) system can be activated in raw milk and in the pasteurized product as an additional bacteriological control measure in dairy processing. LP is a naturally occurring enzyme in milk that catalyses the oxidation of thiocyanate into hypothiocyanite in the presence of hydrogen peroxide (Reiter and Härnulv, 1984).

Hypothiocyanite has a bacteriostatic effect on *E. coli* in milk (Seifu *et al.,* 2004). In spite of heat sensitivity at temperatures above 70 °C (Kussendrager and Van Hooijdonk, 2003), the LP enzyme is reported to maintain activity at pasteurization temperatures of 63 °C for 30 min and 72 °C for 15 s (Barret *et al.,* 1999).

Studies have shown that the LP system is not only antagonistic against undesirable microbes, but it also affects growth and lactic acid production of some lactic acid bacteria (LAB) at both ambient and refrigeration temperatures (Nakada *et al.,* 1996; Seifu *et al.* 2003). Acid production is critical in dairy fermentation since it is used to assess the activity of the starter cultures (Cogan, Barbosa, Beuvier, Bianchi-Salvadori, Cocconcelli, Fernandes, Gomez, Gomez, Kalantzopoulos, Ledda, Medina, Rea and Rodriguez, 1997) and it serves as an indicator of satisfactory progress in yoghurt and cheese processing (Scott, 1981). Consequently, there are concerns that LP activation in milk will not only affect milk quality, but that inhibition of acid production will prolong formation of the necessary casein gels and enable outgrowth of acid-adapted enteropathogens that may occur in milk (FAO/WHO, 2007).

To our knowledge, there have been no studies on the effect of LP activation on indigenous lactic starter cultures used in artisanal fermented dairy products. Although there have been limited studies on the effect of LP activation on single strain lactic starter cultures, further studies are needed to enable selection of LP resistant lactic cultures that can be developed for fermentation of specialized dairy products. In order to respond to the concerns relating to the application of the LP system in milk intended for processing into fermented dairy, this study was designed to first of all investigate the sensitivity of single *Lactococcus* spp. and *Bifidobacterium longum* to LP activation in pasteurized goat milk. Subsequently, selected susceptible and resistant LAB were used to ferment LP activated goat milk that had been inoculated with *E. coli* O157:H7 as a model system to determine if inhibition of acid production would occur that could affect the growth of *E. coli* O157:H7 in the fermenting product. Finally, the application of the LP system was tested in the fermentation of a traditional dairy product called Madila by using an

indigenous lactic culture. Goat milk with inoculated *E. coli* O157:H7 was used to determine whether the effect of LP system on LAB and *E. coli* O157:H7 in the traditional product would differ from that of the commercial product.

5.2 Materials and Methods

5.2.1 Milk Source

Fresh Saanen goat milk was sourced from the University of Pretoria, experimental farm. The Saanen goats were milked following standard procedures with a milking machine. Milk from individual goats was pooled and delivered within one hour of milking. One hundred ml portions of fresh goat milk were transferred into sterile 150 ml blue capped Schott bottles and pasteurized at 63 °C for 30 min in a thermostatically controlled water bath before inoculation and activation of the LP system. Pasteurized milk was used for the processing of commercial fermented milk, while raw milk used for traditional Madila fermentation.

5.2.2 Cultures

Escherichia coli O157:H7 strains UP10 and 1062 were obtained from the Onderstepoort Veterinary Institute, Agricultural Research Council, (Republic of South Africa (RSA)). Cultures were maintained on MaConkey agar (Oxoid, Hampshire, England) plates stored at 2 °C. Working cultures were prepared by transferring a single colony of each *E. coli* O157:H7 strain from MacConkey agar into sterile Tryptone Soy Broth (TSB; Biolab, Wadeville, RSA) and incubated for 24 h at 37 °C. The activating inoculum was prepared after two successive 24 h transfers of 0.5 ml of each of the *E. coli* O157:H7 strains UP10 and 1062 into 100 ml sterile TSB at 37 °C. This culture was used as inoculum for challenge tests.

The following lactic starter cultures were used in this study: single strain *Lactococcus lactis* subsp. *lactis* 345, *Lc. lactis* subsp. *cremoris* 326, *Lc. lactis* subsp. *cremoris* 328, *Lc. lactis* subsp. *diacetylactis* 339 and *Lc. lactis* subsp. *diacetylactis* 340 in vacuum sealed ampoules were obtained from the Department of Food Bioscience, University of the Free State, RSA; *Lc. lactis* subsp. *lactis* AM1 was isolated from traditional Amasi; and *Bifidobacterium longum* BB536 was obtained from Morigana (South Korea). Active cultures were prepared by growing cultures in 100 g/l sterile skim milk at 22 °C for 16 h.

5.2.3 Inoculation and fermentation

All the 100 ml volumes of pasteurized goat milk were inoculated with 1 ml LAB. Each LAB was inoculated into two separate bottles containing 100 ml pasteurized milk; the LP system was activated in one of the two bottles and the second bottle served at the untreated LP control. Before activation of the LP system, the thiocyanate content of goat milk was determined according to the International Dairy Federation (IDF, 1988). The LP activity was determined by spectroscopic measurement using one-step ABTS (2,2'-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid, Sigma, St. Louis, Missouri, USA) solution as substrate (Seifu *et al*., 2004). The LP system was activated by adding sodium thiocyanate (Saarchem, Krugersdorp, RSA) to a final concentration of 14 mg/l. After thorough mixing, 30 mg/l sodium percarbonate (Aldrich Chemical Company Inc., Milwaukee, USA) was added as a source of hydrogen peroxide (IDF, 1988). The inoculated goat milk was then incubated at 30 °C for 6 h in a thermostatically controlled water bath.

To determine the effect of activated LP system on LAB in commercial fermented milk and its impact on survival of *E. coli* O157:H7, 100 ml pasteurized goat milk samples inoculated with 1 ml selected single strain lactic cultures were also inoculated with a 1 ml *E. coli* O157:H7 cocktail containing strains UP10 and 1062 before activation of the LP system. The initial concentration of LAB and *E. coli* O157:H7 were determined by plate counts before incubation at 30 °C in a thermostatically controlled water bath for 24 h.

To prepare traditional Madila, fresh unpasteurized goat milk was transferred into two plastic buckets in 400 ml volumes. The LP system was activated in one bucket containing 400 ml goat milk while the second milk sample was used as the untreated LP control. The activated LP and control goat milk samples were each inoculated with 10 % (v/v) traditional skim milk culture and 1 % (v/v) *E. coli* O157:H7 strain UP10. Goat milk samples were allowed to ferment at 30 °C for 5 days. After 24 h and on each subsequent day for a total of 5 days, one day old soured milk was added to fermenting Madila in a 4:1 (fermenting Madila: sour milk) ratio (Ohiokpehai and Jagow, 1998). The one day old soured milk was prepared by inoculating unpasteurized goat milk with 1 % (w/v) freeze dried traditional fermented milk and incubating at 25 °C for 24 h. On day 5, the whey from the fermented Madila was drained through a sterile jute bag. The Madila was then mixed with cold unpasteurized goat milk in a ratio of 4:1 (four parts Madila: one part milk).

5.2.4 Acid challenge

The surviving *E. coli* O157:H7 from activated LP and the LP control fermenting Madila samples were tested for acid-adaptation after 24 h. An acid challenge test was conducted by transferring 1 ml of milk sample into 10 ml TSB acidified with 6 mol/l lactic acid (Saarchem, Wadeville, RSA) to pH 4.0 for 4 h at 37 °C. Survival of adapted *E. coli* O157:H7 from activated LP and control Madila were compared to survival of non-adapted *E. coli* O157:H7 challenged in acidified TSB (pH 4.0) for 4 h at 37 °C.

3.3.2.5 Chemical analyses

In order to determine the concentration of thiocyanate to add to milk, the thiocyanate concentration of milk was determined according to the IDF (1988) method. Eight ml of raw milk was thoroughly mixed with 4 ml of 20 % (w/v) trichloroacetic acid (Saarchem,

Gauteng, RSA) and allowed to stand for 30 min. The mixture was then filtered though a Whatman No. 40 filter paper and 1.5 ml of the clear filtrate mixed with 1.5 ml of ferric nitrate reagent (16 g of $Fe(NO_3)_3.9H_2O$ (Saarchem) in 50 ml distilled water). The absorbance was measured at 460 nm wavelength with a Lamda EZ150 UV spectrophotometer (Perkin Elmer, USA) and the thiocyanate concentration determined from a standard curve.

The titratable acidity (TA), used to measure lactic acid production was determined by titrating 9 ml of milk with 0.1 mol/l NaOH (Promark Chemicals, Robertsham, RSA). TA was expressed as percent lactic acid (Bradley *et al.,* 1993).

The pH readings were taken at the time of sampling of thoroughly mixed samples by inserting the pH electrode (Hanna Instruments, Italy) directly into the fermenting milk samples.

5.2.6 Microbiological analyses

Fermenting milk was sampled for viable *E. coli* O157:H7 and LAB counts after 0, 2, 4, 6 and 24 h for commercial Amasi/Maas-type fermented milk and 0, 1, 2, 3, 4, 5 days for traditional Madila. Serials dilutions were prepared with 1 g/l buffered peptone water (Oxoid, Hampshire, UK) and spread plated on M 17 agar (Oxoid) for *Lactococci* spp. counts, MRS agar (Oxoid) for *Lactobacillus* spp. and *Leuconostoc* spp. counts and Sorbitol MaConkey agar (SMAC, Oxoid) for *E. coli* O157:H7 counts. M 17 plates were incubated at 30 °C for 24 to 48 h, MRS plates were incubated at 37 °C for 48 h and SMAC plates were incubated at 37 °C for 24 h preceding enumeration of sorbitol negative *E. coli* O157:H7. Detection limit for microbial counts were 10 cfu/ml.

5.2.7 Statistical analyses

Analysis of Variance (ANOVA) was used to determine whether activated LP had a

significant effect on lactic acid production, and viability of lactic starters and *E. coli* O157:H7 cultures throughout the processing of commercial fermented milk (24 h) and the Madila processing period (5 days). Each sample was analyzed in duplicate and the experiment was repeated three times. The significance level was set at $P \leq 0.05$. ANOVA was performed using Statistica (Tulsa, Oklohama, USA, 2008).

5.3 Results

5.3.1 Quality of raw and pasteurized Saanen goat milk

The LP activity, titratable acidity (TA), pH of raw and pasteurized Saanen goat milk are presented in Table 6. The TA and pH of raw and pasteurized goat milk were within standard values. The average counts for *E. coli*, *Lactococcus* spp. and *Lactobacillus* spp. in fresh goat milk are also presented in Table 6. No bacterial counts were detected in goat milk pasteurized at 60 °C for 30 min (Detection limit was 10 cfu/ml^{-1}).

Table 6: Chemical and microbiological quality of raw and pasteurized Saanen goat milk (N = 6)

Analyses	**Milk**	**Mean**	**Standard Deviation**
Lactoperoxidase activity	Raw	0.09 U/ml	0.02
	Pasteurized	0.04 U/ml	0.02
Titratable acidity	Raw	0.12 %	0.01
	Pasteurized	0.14 %	0.04
pH	Raw	6.52	0.04
	Pasteurized	6.47	0.20
Escherichia coli	Raw	3.07 $\log_{10}$ cfu/ml	0.19
Lactococcus spp.	Raw	4.24 $\log_{10}$ cfu/ml	0.26
Lactobacillus spp.	Raw	3.44 $\log_{10}$ cfu/ml	0.04

5.3.2 The effect of LP activation on single strain LAB in goat milk

All LAB cultures tested grew in pasteurized and LP activated goat milk reaching populations of 9.1 to 9.4 $\log_{10}$ cfu/ml with the exception of *Lc. cremoris* 326 that reached a final concentration of 8.5 $\log_{10}$ cfu/ml after 6 h (Table 8). Although there was a significant strain ($P \leq 0.05$) effect on growth and acid production of the seven individual LAB strains tested (Table 7), they did not show significant susceptibility to the LP system ($P > 0.05$). The highest acid production was observed in *Lc. lactis* AM1 isolated from traditional Amasi while the lowest acid production was observed in *Lc. cremoris* 326 (Table 8). The acid production correlated positively with decrease in pH.

Table 7: The effect of single strain lactic acid bacteria (LAB) on LAB counts, pH and titratable acidity in goat milk fermented at 30 °C for 6 h

LAB strains	LAB counts (Log_{10} cfu/ml)	pH	Titratable acidity (%)
Lc. lactis subsp. *diacetylactis* 339	8.97^{c}	5.46^{a}	0.33^{ac}
Lc. lactis subsp. *diacetylactis* 340	8.64^{a}	5.55^{ab}	0.31^{a}
Lc. lactis subsp. *lactis* 345	6.62^{a}	5.56^{ab}	0.31^{a}
Lc. lactis subsp. *cremoris* 326	7.93^{d}	6.01^{c}	0.20^{b}
Lc. lactis subsp. *cremoris* 328	8.86^{bc}	5.44^{a}	0.34^{c}
B. longum BB536	8.39^{e}	5.66^{b}	0.19^{b}
Lc. lactis subsp. *lactis* AM1	8.71^{ab}	5.53^{a}	0.37^{d}
P value	0.000	0.000	0.000

Different alphabets following mean values in the same column indicate significant differences ($P \leq 0.05$)

Table 8: Changes in the mean values (†standard deviation) of pH, titratable acidity and lactic acid bacteria counts in pasteurized and lactoperoxidase (LP) activated Saanen goat milk fermented at 30 °C

LAB strains	Time (h)	pH		Titratable acidity (%)		LAB counts (Log cfu/ml)	
		No LP	LP	No LP	LP	No LP	LP
Lc. diacetylactis 339	2	5.92 (†0.09)	5.93 (0.09)	0.21 (0.005)	0.20 (0.003)	8.97 (0.04)	8.95 (0.03)
	6	4.37 (0.03)	4.37 (0.01)	0.55 (0.017)	0.54 (0.019)	9.41 (0.06)	9.38 (0.04)
Lc. diacetylactis 340	2	6.02 (0.05)	6.03 (0.04)	0.19 (0.003)	0.17 (0.007)	8.72 (0.07)	8.73 (0.10)
	6	4.46 (0.02)	4.46 (0.02)	0.53 (0.012)	0.53 (0.012)	9.10 (0.11)	9.05 (0.07)
Lc. lactis 345	2	6.01 (0.05)	6.06 (0.06)	0.19 (0.006)	0.17 (0.003)	8.73 (0.12)	8.66 (0.06)
	6	4.46 (0.02)	4.47 (0.03)	0.56 (0.014)	0.53 (0.012)	9.13 (0.12)	9.09 (0.16)
Lc. cremoris 326	2	6.28 (0.01)	6.28 (0.01)	0.16 (0.009)	0.15 (0.007)	8.01 (0.19)	8.03 (0.11)
	6	5.30 (0.25)	5.28 (0.27)	0.34 (0.080)	0.34 (0.077)	8.49 (0.25)	8.41 (0.31)
Lc. cremoris 328	2	5.90 (0.06)	5.94 (0.06)	0.21 (0.007)	0.20 (0.009)	8.65 (0.37)	8.59 (0.43)
	6	4.37 (0.01)	4.39 (0.01)	0.58 (0.019)	0.56 (0.012)	9.32 (0.07)	9.40 (0.05)
Bifidobacterium longum BB536	2	6.06 (0.24)	6.14 (0.22)	0.16 (0.023)	0.16 (0.023)	8.16 (0.36)	8.06 (0.23)
	6	4.70 (0.21)	4.75 (0.22)	0.62 (0.078)	0.59 (0.073)	9.25 (0.01)	9.19 (0.10)
Lc. lactis AM1	2	5.94 (0.03)	5.97 (0.04)	0.16 (0.023)	0.16 (0.023)	8.52 (0.11)	8.60 (0.09)
	6	4.96 (0.02)	4.56 (0.02)	0.66 (0.015)	0.66 (0.021)	9.30 (0.05)	9.25 (0.02)
P value (LAB strain)		0.000		0.000		0.000	
P value (LP system)		0.658		0.257		0.724	
P value (Time)		0.000		0.000		0.000	
P value (LP vs time)		0.986		0.911		0.982	
N		3		3		3	

5.3.3 The effect of LP activation on single strain LAB in goat milk in the presence of *E. coli* O157:H7

There was no significant difference between activated LP and control populations of all LAB strains tested although cell numbers differed significantly ($P \leq 0.05$) for individual cultures (Table 10). There was also a significant ($P \leq 0.05$) overall LAB strain effect on acid production by single lactic cultures (Table 9). All LAB tested in the presence of *E. coli* O157:H7 had significantly higher ($P \leq 0.05$) acid production after 6 h compared to cultures that had no *E. coli* O157:H7. The percentage increase in acid production is presented in Fig. 5.1. In the presence of *E. coli* O157:H7, acid production by *Lc. lactis* subsp. *cremoris* 326 culture was similar to that produced by *Lc. lactis* subsp. *diacetylactis* 340 and *Lc. lactis* subsp. *lactis* 345 after 6 h of fermentation (Table 10). Also, all cultures tested with the exception of *Lc. lactis* AM1 showed a greater increase in acid production in the activated LP milk after 6 h of fermentation compared to the untreated LP controls. *Lc. cremoris* 326 showed the greatest difference in acid production (Fig. 5.1).

Table 9: The effect of single strain lactic acid bacteria (LAB) on LAB counts, *E. coli* O157:H7 counts and titratable acidity in goat milk fermented at 30 °C for 24 h

LAB strains	LAB counts (Log_{10} cfu/ml)	*E. coli* O157:H7 (Log_{10} cfu/ml)	Titratable acidity (%)
Lc. lactis subsp. *diacetylactis* 340	8.65^{a}	6.71^{b}	0.464^{a}
Lc. lactis subsp. *lactis* 345	8.67^{ab}	6.63^{ab}	0.469^{a}
Lc. lactis subsp. *cremoris* 326	8.74^{b}	6.63^{ab}	0.459^{a}
B. longum BB536	8.90^{c}	6.59^{ab}	0.475^{a}
Lc. lactis subsp. *lactis* AM1	8.91^{c}	6.55^{a}	0.498^{b}
P value	0.000	0.151	0.000

Different alphabets following mean values in the same column indicate significant differences ($P \leq 0.05$)

Table 10: Changes in the mean values (†standard deviation) of titratable acidity, lactic acid bacteria (LAB) and *Escherichia coli* O157:H7 counts in pasteurized and lactoperoxidase activated Saanen goat milk fermented by single strain lactic acid bacteria at 30 °C

LAB strains	**Time (h)**	**Titratable acidity (%)**		**LAB (Log cfu/ml)**		***E. coli* O157:H7 (Log cfu/ml)**	
		No LP	**LP**	**No LP**	**LP**	**No LP**	**LP**
Lc. diacetylactis 340	2	0.24 (†0.009)	0.25 (0.032)	8.41 (0.017)	8.43 (0.134)	6.56 (0.031)	6.69 (0.076)
	6	0.66 (0.012)	0.66 (0.009)	9.27 (0.004)	9.21 (0.126)	7.31 (0.25)	7.34 (0.063)
	24	0.70 (0.015)	0.82 (0.028)	8.95 (0.123)	9.01 (0.068)	5.97 (0.229)	5.94 (0.197)
Lc. lactis 345	2	0.22 (0.015)	0.26 (0.022)	8.71 (0.035)	8.47 (0.047)	6.68 (0.015)	6.54 (0.095)
	6	0.66 (0.032)	0.66 (0.017)	9.09 (0.082)	9.21 (0.029)	6.93 (0.135)	7.12 (0.052)
	24	0.82 (0.015)	0.81 (0.009)	8.91 (0.166)	8.83 (0.016)	6.02 (0.170)	5.84 (0.216)
Lc. cremoris 326	2	0.25 (0.009)	0.22 (0.009)	8.50 (0.070)	8.43 (0.016)	6.72 (0.195)	6.89 (0.020)
	6	0.64 (0.025)	0.66 (0.019)	9.11 (0.016)	9.13 (0.013)	6.85 (0.090)	7.07 (0.073)
	24	0.81 (0.015)	0.81 (0.009)	9.22 (0.310)	8.92 (0.134)	6.17 (0.371)	5.42 (0.074)
Bifidobacterium longum BB536	2	0.23 (0.009)	0.21 (0.009)	8.83 (0.097)	8.77 (0.054)	6.88 (0.054)	7.05 (0.121)
	6	0.70 (0.012)	0.67 (0.006)	9.31 (0.026)	9.24 (0.023)	7.07 (0.197)	6.98 (0.041)
	24	0.84 (0.10)	0.81 (0.012)	9.25 (0.111)	9.13 (0.087)	5.41 (0.117)	5.19 (0.147)
Lc. lactis AM1	2	0.29 (0.032)	0.28 (0.032)	8.93 (0.028)	8.86 (0.027)	6.70 (0.103)	6.76 (0.107)
	6	0.70 (0.013)	0.69 (0.015)	9.28 (0.018)	9.26 (0.020)	6.96 (0.204)	7.03 (0.066)
	24	0.83 (0.015)	0.80 (0.009)	9.00 (0.153)	9.21 (0.050)	5.65 (0.164)	5.32 (0.283)
P value (Strain)		0.000		0.000		0.151	
P value (LP system)		0.745		0.822		0.635	
P value (Time)		0.000		0.000		0.000	
P value (LP vs time)		0.959		0.735		0.012	
P value (LAB vs *E. coli* O157:H7)		0.000		0.000		NA	
P value (LP/ LAB/ *E. coli* O157:H7)		0.954		0.960		NA	
N		3		3		3	

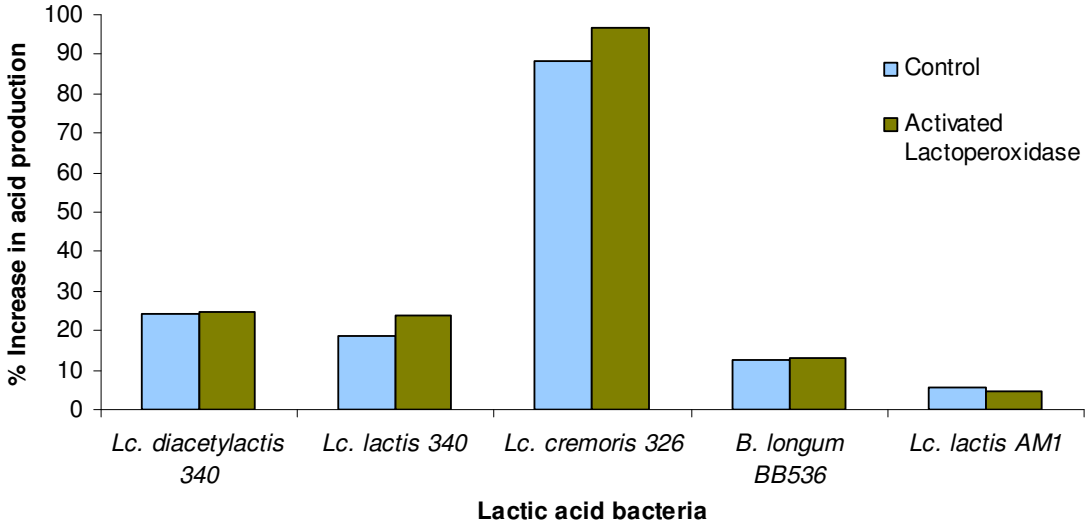


Figure 5.1: The percent increase in acid production by lactic starter cultures after 6 h fermentation of goat milk inoculated with *Escherichia coli* O157:H7 compared to 6 h fermentation of goat milk that had no *E. coli* O157:H7 present

Similar to the single strain LAB cultured in the absence of *E. coli* O157:H7, the activated LP system did not have a significant effect on acid production of single strain LAB in the presence of *E. coli* O157:H7 throughout the fermentation period. Nonetheless, marginal reduction in acid production was observed in the LP activated 24 h culture of *B. longum* BB536 and *Lc. lactis* AM1. *Lc. diacetylactis* 340 on the other hand showed resistance to activated LP system with 17 % increase in acid production compared to the control after 24 h (Table 10).

The *E. coli* O157:H7 counts generally increased in goat milk during the first 6 h of fermentation. Inhibition of *E. coli* O157:H7 was subsequently observed after 24 h in all single strain LAB cultured goat milk (Table 10). Inhibition of *E. coli* O157:H7 was however not uniform for all the LAB strains tested. Here, LP system had a significant effect ($P \leq 0.05$) on *E. coli* O157:H7 over time. Although LP inhibition of *E. coli* O157:H7 was not apparent in the *Lc. diacetylactis* 340 culture (19 % in LP activated culture compared to 18 % in the control), significant reductions were observed in *Lc. lactis* 345 (18 % in LP activated culture compared to 13 % in the control); *B. longum*

BB536 (26 % in activated LP culture compared to 24 % in the control culture) and *Lc. lactis* AM1 (24 % in LP activated culture compared to 19 % in the control). Overall, *Lc. cremoris* 326 showed the greatest difference in *E. coli* O157:H7 inhibition between the LP activated culture (23 %) and the control culture (10 %).

5.3.4 The effect of the activated LP system during processing of a traditional fermented product in the presence of *E. coli* O157:H7

The LP system did not significantly affect growth and acid production of indigenous LAB in Madila fermentation. The numbers of LAB increased reaching an optimum of 9.28 $\log_{10}$ cfu/ml after 24 h fermentation (Table 11). The LAB concentration subsequently declined marginally maintaining a level of approximately 8 $\log_{10}$ cfu/ml throughout the fermentation period until day 5 when LAB numbers declined further. Similarly, pH of fermenting Madila was unaffected by activated LP throughout the fermentation period ($P > 0.05$) (Table 11). The pH of the activated LP Madila declined to pH 4.22 and pH 4.19 in untreated LP Madila after 24 h. The pH did not change significantly during the subsequent fermentation period. The TA of both activated LP and control Madila increased after 24 h followed by constant acid production until day 3 (Table 11). On days 4 and 5, both activated LP and control Madila showed progressive increase in TA, however, the activated LP Madila had higher TA compared to the control. The activated LP system had a significant effect ($P \leq 0.05$) effect on TA over the 5 days fermentation period.

The *E. coli* O157:H7 numbers in both activated LP and control Madila increased marginally after 24 h fermentation. Subsequently, the *E. coli* O157:H7 counts in activated LP Madila declined progressively until it reached < 1.0 $\log_{10}$ cfu/ml at the end of the fermentation period (Table 11). The *E. coli* O157:H7 colony counts in the control Madila also declined until day 3 after which colony counts levelled reaching 4.25 $\log_{10}$ cfu/ml at the end of the fermentation period. The LP effect on *E. coli* O157:H7 survival during fermentation of Madila was statistically significant ($P \leq 0.05$).

Table 11: Changes in pH, titratable acidity and counts of *Escherichia coli* O157:H7 and indigenous lactic acid bacteria during processing of traditional Madila at 30 °C

Lactoperoxidase treatment	**Time (Days)**	***E. coli* O157:H7 (Log cfu/ml))**	***Lactococcus* (Log cfu/ml)**	***Lactobacillus* & *Leuconostoc* (Log cfu/ml)**	**pH**	**Titratable Acidity (%)**
No LP	0	6.95 (†0.08)	6.68 (0.19)	7.30 (0.19)	6.35 (0.08)	0.17 (0.01)
	1	7.23 (0.73)	9.13 (0.16)	9.28 (0.04)	4.19 (0.08)	0.75 (0.00)
	2	4.39 (0.79)	8.55 (0.16)	8.77 (0.40)	4.19 (0.12)	0.77 (0.03)
	3	3.43 (0.33)	8.51 (0.16)	8.90 (0.22)	4.36 (0.04)	0.73 (0.01)
	4	3.89 (0.15)	8.44 (0.10)	8.84 (0.23)	4.09 (0.04)	0.96 (0.04)
	5	4.25 (1.15)	7.35 (0.59)	7.30 (0.52)	3.92 (0.05)	1.17 (0.05)
LP	0	6.95 (0.07)	6.67 (0.11)	7.21 (0.12)	6.44 (0.02)	0.16 (0.01)
	1	7.27 (0.75)	9.03 (0.13)	9.13 (0.15)	4.22 (0.08)	0.77 (0.05)
	2	4.85 (0.38)	8.49 (0.18)	8.45 (0.17)	4.11 (0.04)	0.73 (0.03)
	3	3.75 (0.19)	8.28 (0.17)	8.61 (0.19)	4.33 (0.12)	0.71 (0.05)
	4	2.76 (0.22)	8.33 (0.17)	8.40 (0.24)	3.97 (0.10)	1.09 (0.08)
	5	0.52 (0.52)	7.44 (0.42)	7.48 (0.37)	3.79 (0.08)	1.35 (0.03)
P value (LP)		0.052	0.636	0.243	0.382	0.062
P value (LP vs time)		0.010	0.993	0.884	0.625	0.043
N		3	3	3	3	3

† Standard deviation

After 24 h of fermentation, *E. coli* O157:H7 counts in activated LP and control Madila were challenged to lethal acid treatment at pH 4.0 for 4 h to determine whether *E. coli* O157:H7 in the fermenting medium had become acid-adapted. Acid challenge caused 1.81 $\log_{10}$ cfu/ml and 1.65 $\log_{10}$ cfu/ml reductions in *E. coli* O157:H7 counts in activated LP and control Madila respectively. The non-adapted cell colonies were not detected after 4 h acid challenge at pH 4.0 (Data not shown).

The overall effect of LP activation, single strain lactic acid bacteria (LAB) and fermentation time on goat milk fermentation parameters in the presence and absence of *E. coli* O157:H7 is shown in Table 12.

Table 12: Effect of single lactic acid bacteria (LAB) strains, lactoperoxidase system (LP) and time on pH, titratable acidity and counts of lactic acid bacteria and *E. coli* O157:H7 in commercial and traditional fermented goat milk.

Samples	**Measurements**	**LAB strain**	**LP**	**Time**	**LP *vs* Time**
Commercial fermented milk	pH	S	NS	S	NS
No *E. coli* O157:H7	TA	S	NS	S	NS
	LAB	S	NS	S	NS
Commercial fermented milk	TA	S	NS	S	NS
E. coli O157:H7	LAB	S	NS	S	NS
	E. coli O157:H7	NS	NS	S	S
Madila	pH	NA	NS	S	NS
E. coli O157:H7	TA	NA	NS	S	S
	Lactococcus	NA	NS	S	NS
	Lactobacillus & *Leuconostoc*	NA	NS	S	NS
	E. coli O157:H7	NA	NS	S	S

S = Significant effect ($P \leq 0.05$); NS = Not significant ($P > 0.05$); NA = Not applicable

5.4 Discussion

While lactic acid gives fresh flavour to fermented milk products (Heap and Lawrence, 1988), it is also important for coagulation of milk. Therefore, rapid production of lactic acid is the most important attribute of lactic starter cultures (Cogan *et al.,* 1997). In cheese making, a good starter culture should reduce the pH of milk from approximately 6.6 to 5.3 in 6 h (Cogan *et al.,* 1997). In yoghurt processing, a lower pH of 4.6 to 4.7 is required for coagulation of milk (Tamine and Robinson, 1999). In this study, all the single strain LAB tested with the exception of *Lc. cremoris* 326 were fast acid producers that reduced the pH of pasteurized goat milk to an average of pH 4.5 in 6 h. The relatively low acid production of *Lc. cremoris* 326 correlated positively with colony counts in pasteurized goat milk. Since all conditions were the same, the difference in rate of growth and lactic acid production was characteristic of the strain.

The lack of activated LP inhibition of all the LAB strains tested is supported by other authors. For example, Nakada *et al.,* (1996) observed no significant difference in viability for single strain cultures *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* in yoghurt with or without subjection to the LP system, although acid production was inhibited in activated LP cultures at 41 °C. In this study, acid production by single strain *Lactococcus* spp. and *B. longum* BB536 was not significantly suppressed in activated LP goat milk. This apparent resistance could be due to the low LP activity of Saanen goat milk used in this study. LP activity of milk has been found to be highly variable depending on the type of milk and the period of lactation (Chávarri *et al.*, 1998). However, the level of LP activity in Saanen goat milk recorded in this study falls within the range of 0.04 to 0.16 U/ml reported by Fonteh *et al.* (2002) for raw goat milk during the lactation period. Regardless of the low LP activity of milk, the marginal reduction of acid production by *B. longum* BB536, *Lc. cremoris* 328 and *Lc. lactis* 345, compared to acid production in control milk, suggest that these LAB could potentially be susceptible to the LP system at a higher LP activity.

The presence of *E. coli* O157:H7 did not affect growth of LAB in goat milk. The increased acid production of LAB in *E. coli* O157:H7 inoculated milk compared to the milk that had no *E. coli* O157:H7 was due to the additional lactic acid production by *E. coli* O157:H7 due to metabolism of lactose. The LP effect on acid production in the presence of *E. coli* O157:H7 was variable for the individual LAB tested. This difference lies in the strain to strain variation of lactic cultures (Roginski, Broome, Hungerford and Hickey, 1984), and the interaction between the lactic cultures, *E. coli* O157:H7 and the stresses encountered in the fermenting medium. Although the nature of this interaction was not investigated, the lactic cultures were clearly influenced by the presence of *E. coli* O157:H7 since lactic acid production in activated LP milk differed from cultures that had no *E. coli* O157:H7 present. The greater increase of acid production in LP activated milk was unexpected. Given that *E. coli* O157:H7 cells were significantly inhibited by the LP system, the difference in acid production could not be attributed to acid production by *E. coli* O157:H7 alone. It appears that increased acid production was stimulated by lactic starter cultures in the presence of antimicrobial compounds and an antagonistic pathogen.

Apart from lactic acid inhibition of *E. coli* O157:H7 in fermented milk, other factors such as the production of bacteriocins and ethanol could have contributed to *E. coli* O157:H7 inhibition. Some species of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are known to produce nisin and lactococcin respectively that have broad antimicrobial spectrum (Holo, Nilssen, and Nes, 1991; Rodriguez, Cintas, Casaus, Horn, Dodd, Hernández and Gasson, 1995). Although studies have indicated that these antimicrobial peptides inhibit Gram positives, when coupled with activated LP, these bacteriocins could have an additional inhibitory effect on *E. coli* O157:H7 as observed in the LP activated *Lc. lactis* subsp. *cremoris* 326 24 h fermented milk culture compared to the control culture. The increased inhibition of *E. coli* O157:H7 observed in *Lc. lactis* AM1 and *B. longum* BB536 cultures in both LP activated and control milk could be due to their characteristic antimicrobial properties. Both *Lc. lactis* subsp. *lactis* and *B. longum*, particularly strain *B. longum* BB536, have been classified as probiotics that are antagonistic against pathogenic microbes (Sanders, 1998; Mercenier, Pavan and Pot,

2003).

In the traditional Madila product, the indigenous LAB were resistant to the LP system. The resistance of indigenous LAB from traditional fermented milk to activated LP system has not been reported. Previous studies examining sensitivity of mixed and single strain lactic starter cultures to activated LP system found that acid production and survival of lactic starter cultures in activated LP milk vary from one investigation to another. Seifu *et al.* (2003) reported that activated LP inhibits acid production of commercial mixed lactic starter cultures. In this study, the indigenous LAB were not only insensitive to activated LP, but lactic acid production was not inhibited in activated LP milk. The lack of LP inhibition of lactic cultures could be due to the reversal of antimicrobial hypothiocyanite by the enzyme NADH-OSCN oxidoreductase into thiocyanate (Carlsson *et al*., 1983). This reversal factor exhibited by NADH oxidoreductase together with NADH oxidase and peroxidase enzymes are stimulated during oxidative stress (Sanders *et al.,* 1999). Investigation of the molecular basis for resistance of these indigenous mixed lactic starters could shed more light on the mechanism of resistance against LP activation. These indigenous LAB cultures could be developed for upscaled Madila processing from activated LP milk.

Although inhibition of acid production was not observed in LP activated fermented milk in this study, acid challenge of the 24 h culture during Madila fermentation indicated that the inoculated *E. coli* O157:H7 had become acid-adapted. This finding is consistent with those of other authors who have reported acid resistance of *E. coli* during fermentation of dairy products (Feresu and Nyathi, 1990; Massa *et al.,* 1997; Vernozy-Rozand *et al.,* 2005). Though acid-adapted, the *E. coli* O157:H7 cells were inhibited in LP activated Madila. Previous studies have indicated a limited period of LP efficacy in milk (FAO/WHO, 2006). It was stated in the guideline for raw milk preservation (CAC, 1991) that the activated LP system can extend the keeping quality of raw milk stored at 30 °C for 7 to 8 h. In this study, activated LP inhibition of acid-adapted *E. coli* O157:H7 was evident after day 4 of Madila fermentation. This observation suggests that when the

activated LP system was coupled with low pH, the combined inhibitory effect was extended for at least 5 days at 30 °C. The delayed LP inhibition of *E. coli* O157:H7 in activated LP Madila suggests that low pH sensitized acid-adapted *E. coli* O157:H7 to the antimicrobial effect of the activated LP system. The increased enzymatic production of $HOSCN/OSCN^-$ and the easy passage of uncharged hypothiocyanite into the cell at low pH (Tenovuo, Lumikari and Soukka, 1991, Van Opstal *et al.,* 2005) could have contributed to the inhibition of acid-adapted *E. coli* O157:H7 in activated LP Madila. Since the combination of LP activation and low pH caused > 5.0 $\log_{10}$ cfu/ml reduction in *E. coli* O157:H7, it can be applied in traditional milk processing and storage at ambient temperature to improve the microbiological safety of fermented milk with respect to *E. coli* O157:H7.

5.5 Conclusion

This study has provided evidence that the application of activated LP did not inhibit lactic acid production by single strain and indigenous LAB during the first 6 h of fermentation, which is a crucial period for growth and acid production of LAB in the processing of fermented dairy products. These cultures can therefore be developed for processing of specialized dairy products from activated LP milk. Though *E. coli* O157:H7 cells were inhibited in LP activated milk, the high numbers in fermented milk after 24 h indicate that the application of LP system in industrial processing of milk may not be sufficient to reduce counts of *E. coli* O157:H7 that occur in milk. However, in traditional processing of milk products, like Madila, where milk is slowly fermented at ambient temperatures over long periods, the LP system can be applied to improve the safety of the product.

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Chapter 6: GENERAL DISCUSSION

In South Africa, goat milk is produced in many rural centers mainly for subsistence, but also on a small-scale for supply of goat milk into the formal sector. High ambient temperatures, poor milk handling and inadequate infrastructure result in high milk losses and inconsistent milk quality. It is therefore common practice to process left-over milk on the farm into fermented dairy products for home consumption and for economic benefit. However, recent reports on dairy products and acidic foods implicated in outbreaks of *E. coli* O157:H7 (Besser *et al*., 1993; Morgan *et al.,* 1994) have raised concerns about the safety of goat milk products processed under uncontrolled conditions. Persistence of *E. coli* O157:H7 in fermented dairy products and acidic fruit juices have been attributed to acid-adaptation either in the gut of ruminants or during processing of fermented dairy (Leyer *et al.,* 1995; Diez-Gonzalez *et al.,* 1998; Dlamini and Buys, 2009). Once acid-adapted, *E. coli* can become cross-protected against other environmental stresses applied in food preservation (Riordan *et al.,* 2000). With the low infectious dose (Tuttle, Gomez, Doyle, Wells, Zhao, Tauxe and Griffin, 1999) the mere survival, rather than multiplication of *E. coli* O157:H7 in food could potentially cause disease when contaminated food is consumed. The overall objective of this study was first of all, to determine whether acid-adaptation of *E. coli* O157:H7 confers cross-protection to preservation treatments applied in dairy processing, and to apply the concept of hurdle technology to control the survival of acid-adapted *E. coli* O157:H7 in goat milk and fermented goat milk products.

6.1 Review of Methodology

6.1.1 Acid-resistance assays for *Escherichia coli* O157:H7

Several concepts involving acid resistance (AR), acid tolerance response (ATR) and acid habituation (AH) have been described in *E. coli* systems (Chung *et al.,* 2006). However,

the most dramatic response to lethal acidic pH, and in particular, cross-protection against unrelated environmental stresses, is induced during stationary phase acid-adaptation (Foster, 2000). For this reason, the authors decided to study stationary phase acid-adaptation of *E. coli* O157:H7 in goat milk.

The method widely used for stationary phase acid-adaptation of *E. coli* O157:H7 is one developed by Buchanan and Edelson (1996). This method involves 18 h culturing of 1 % (v/v) *E. coli* inoculum in Tryptone Soy broth without glucose (TSB-G) for non acid-adapted *E. coli,* and in TSB supplemented with 1 % glucose (TSBG) for acid-adapted *E. coli*. The underlying principle for 18 h culturing in TSBG is that *E. coli* naturally ferments glucose into acid, which gradually decreases the pH of the fermenting medium to approximately pH 4.8 after the 18 h period, when it would have reached stationary phase. The gradually depression of the medium pH to mild acid pH triggers biochemical and physiological changes in the cells that enhance resistance to lethal acid conditions; a process that has been dubbed acid-adaptation (Leyer *et al.,* 1995; Bearson *et al.,* 1997). Buchanan and Edelson (1996) determined acid resistance of acid-adapted *E. coli* by assessing their survival in Brain Heart Infusion (BHI) broth acidified with HCl to pH 2.5 and pH 3.0. Aliquots from inoculated acidified BHI were taken at regular intervals for up to 7 h to determine acid resistance of both acid-adapted and non-adapted *E. coli*. Several investigators have adopted the Buchanan and Edelson method of acid-adaptation of *E. coli* with modifications to suit their respective studies.

Other less popular methods of acid adaptation have been published. In the method described by Leyer *et al.* (1995), acid-adapted *E. coli* was prepared by culturing active *E. coli* cells in nutrient broth acidified with HCl to pH 5.0 for 4 to 5 h. The idea was to habituate *E. coli* O157:H7 cells to acid by growing them (for a couple of cell doublings) at mild acidic pH. This process induces acid resistance to lethal acid pH in *E. coli* similar to that induced in stationary phase cells regardless of medium pH (Goodson and Rowbury, 1989). In their study, Leyer *et al.* (1995) assessed acid-adaptation by challenging acid-adapted cell suspension of approximately 7.7 $\log_{10}$ cfu/ml in E buffer

that had been acidified with lactic acid to pH 3.85.

In another study, Yuk and Marshall (2004) acid-adapted *E. coli* O157:H7 by sequential culturing of *E. coli* cells in TSB at pH 7.3, pH 6.0 and finally pH 5.0 for 18 h at each pH level. Acid resistance was assessed by challenging acid-adapted cells in simulated gastric fluid acidified to pH 1.5 with 5.0 N HCl. Chen *et al*. (2003) used the modified Tsai and Ingram (1997) method to prepare acid-adapted *E. coli*. In their study, *E. coli* O157:H7 culture that had been activated in TSB for 18 h was harvested by centrifugation, washed twice with Butterfield's buffer phosphate diluent (BPD) and suspended in 10 ml TSB acidified to pH 5.0 with 6.0 N HCl for up to 6 h. Acid-adapted and non-adapted *E. coli* O157:H7 cultures were subsequently acid challenged in saline solution acidified with 1.0 N HCl to a pH of 3.0, 4.0 or 5.0.

In this study, the method described by Buchanan and Edelson (1996) was used to prepare acid-adapted and non acid-adapted *E. coli* O157:H7. The Buchanan and Edelson method of acid-adaptation was chosen because it is an easy and straight-forward method to use, it has been proven to be a reliable method of preparing stationary phase acid-adapted *E. coli* and it produces *E. coli* cells with high acid resistance at extreme acid pH levels. The only modification to the Buchanan and Edelson method used in our study was the inoculation of non-adapted *E. coli* O157:H7 into TSB buffered with MOPS (pH 6.5 to 7.9) to maintain the pH of TSB at 7.4. The buffering of TSB for non-adapted *E. coli* O157:H7 culture was important because TSB contains 0.25 % glucose. Although glucose is present in minute quantities, *E. coli* O157:H7 can metabolize the glucose in TSB into acid to depress the pH of the medium, which could initiate acid-adaptation in the process. *E. coli* was cultured in TSB-G and TSB+G for 18 h at 37 ºC. The incubation temperature of 37 ºC was chosen because it is the optimum growth temperature of *E. coli* O157:H7. The incubation time of 18 h was chosen to ensure that cells had reached stationary phase and that acid-adaptation was fully activated at the end of the incubation period.

Unlike the method of Buchanan and Edelson (1996), acid challenge was conducted in

TSB instead of BHI. Buchanan and Edelson used BHI because it is a rich medium and it mimics conditions found in food. Other authors have challenged acid-adapted *E. coli* O157:H7 in TSB supplemented with 0.6 % yeast extract (TSBYE) (Conner and Kotrola, 1995; Stopforth, Skandamis, Geornaras and Sofos, 2007) to promote growth of pathogens (Samelis, Ikeda and Sofos, 2003), Tryptic Phosphate broth (TPB) to aid recovery (Jordan *et al.,* 1999), Luria Bertani broth, a medium rich in amino acids and enhances activation of acid resistance systems (Lin *et al.,* 1995), minimal glucose medium as a defined medium that inhibits the oxidative acid resistance system (Lin *et al.,* 1995), saline solution (Chen *et al.,* 2003) and simulated gastric fluid (Yuk and Marshall, 2004). In this study, acid challenge was conducted in TSB because it simulates conditions likely to be encountered in food and it aids in the recovery of injured cells.

The type of acidulant and pH levels affects survival of acid-adapted *E. coli* O157:H7. For example, Ryu and Beuchat (1998) established that acid-adapted *E. coli* O157:H7 was more sensitive to acetic acid compared to lactic acid at the same pH level. Deng *et al.,* (1999) also showed that acetic acid, citric acid and malic acid had variable inhibition intensities on acid-adapted *E. coli* O157:H7 cells in acidified TSA. Several studies have used HCl as the acidulant in acid-challenge studies (Sainz *et al*., 2005; Yuk and Marshall, 2004; Chen *et al*., 2003, Jordan *et al.,* 1999). Furthermore, HCl is used as an acidulant at extreme low pH levels (pH 3.0 to 1.5) to simulate conditions in the stomach. HCl is secreted in the stomach to reduce the pH of gastric fluid, to denature proteins and to kill bacteria that may be present in ingested food (Benjamin and Datta, 1995; Foster, 2004). Since acid-adapted cells that manage to cause human infection have to breach the lethal acidic pH in the stomach, the use of HCl at extremely low pH levels is relevant. While inorganic acids such as HCl may be an appropriate acidulant for acid challenge, its use has practical limitations because it is not commonly added to foods (Deng *et al*., 1999). On the other hand, organic acids such as lactic acid, malic acid, acetic acid and citric acid are natural by-products of fermentable carbohydrates. In this study, lactic acid was the acidulant of choice for acid challenge tests because it is produced by lactic acid bacteria during fermentation of several acidic foods, it has commercial application in acidifying

dairy products and it is used for decontamination of meat (Stopforth *et al.,* 2007). Unlike HCl, organic acids do not only lower the internal pH following dissociation upon entry into the cell, but their anions also accumulate in the cell to increase turgor pressure (Foster, 1999). Furthermore, research conducted on *Listeria monocytogenes* indicated that organic acids including acetic acid, lactic acid and citric acid exerted greater inhibition on *L. monocytogenes* compared to HCl (Farber, Sanders, Dunfield and Prescott, 1989). The greater inhibitory effect was attributed to the easy passage of some organic acids across the cell membrane into the cell to decrease the internal pH which results in cell inactivation (Farber *et al.,* 1989).

The pH level for acid challenge was chosen to be pH 4.0 because the authors were interested in survival of acid-adapted *E. coli* O157:H7 in food, particularly, fermented dairy products. Since the pH of fermented dairy products rarely goes beyond pH 4.0, the pH level of 4.0 was chosen as the pH limit for acid challenge in this study. During acid challenge, the *E. coli* O157:H7 cultures were periodically sampled after every 2 h for a total of 6 h for microbiological analysis.

6.1.2 Choice of hurdles

This study sought to apply the hurdle technology concept to control contaminating *E. coli* O157:H7 that may become acid-adapted during processing of goat milk or that may be acid-adapted before contamination of milk. Since the rationale for this project centers around improving the quality and microbiological safety of goat milk produced in rural goat milk production centers, the processing and preservation hurdles were selected based on availability and simplicity of application. The lactoperoxidase system has been recommended for preservation of raw milk where infrastructure for refrigeration of raw milk is limited or unavailable (IDF, 1988). The FAO and WHO have jointly developed sachets containing SCN^- and H_2O_2 that can readily be added to milk to activate the LP system (FAO, 2000). The LP enzyme has been found to be resilient to heat denaturation at pasteurization temperature of 63 °C for 30 min (Barret *et al.,* 1999). At mild acid pH,

the LP enzyme is less heat stable nonetheless, it retains residual antibacterial activity. Therefore, LP activation, heat treatment at sub-pasteurization temperatures (55 °C and 60 °C for 15 s), and lactic acid treatment at pH levels of 5.0 and 4.0, as applied in fermented foods, were employed in goat milk processing to determine the susceptibility of acid-adapted or non-adapted *E. coli* O157:H7. In order to establish whether acid-adaptation confers cross-protection of *E. coli* O157:H7 to low pH and LP activation, a sensitivity test was conducted in TSB where acid-adapted and non-adapted *E. coli* O157:H7 were challenged to combined lactic acid (pH levels of 4.0, 5.0 and 7.4) and LP activation.

The choice of fermented milk products was influenced by the indigenous fermented milk products processed on milk production farms from excess milk. Madila is a traditional soured milk product prepared by natural fermentation of milk by milk flora or by back-slopping with a fermented product.

6.1.3 Quantitative Real-Time PCR (qRT-PCR)

The objective of this phase was to determine relative gene expression of acid-adapted *E. coli* O157:H7 challenged against activated LP and lactic acid in rich media to shed light on cross-protection against activated LP observed physiologically. Acid resistance in *E. coli* has been extensively studied using defined medium, for example, minimal glucose medium (Richard and Foster, 2007), glucose minimal salts medium M9 (Vijayakumar, Kirchhof, Patten and Schellhorn, 2004) and minimal E with glucose, glutamine or arginine supplements (Richard and Foster, 2004) for growth or acid challenge of *E. coli* cells. Some RNA extraction protocols recommend the use of minimal media because it is defined compared to rich media that may be inconsistent and may produce cell cultures with variable RNA yield or quality (Qiagen, 2005). However, food systems are complex and in order to imitate conditions that *E. coli* O157:H7 encounters in food, the authors used a rich medium (TSB) to adapt and challenge *E. coli* O157:H7 cells. The TSB used for challenge assays was unsupplemented with either glucose or amino acids because the idea was not to differentially activate AR systems but to understand which AR systems

are expressed during the combined LP and lactic acid challenge in the presence of glucose. The genes expressed were *rpoS*, responsible for cross-protection (Hengge-Aronis, 2000b); *gadA*, an isoform of glutamate decarboxylase (Smith *et al.,* 1992); outer membrane proteins *ompC* and *ompF* (Heyde and Portalier, 1987); *cfa*, gene for cyclopropane fatty acid synthase (Brown *et al.,* 1997); and *corA,* gene for magnesium transporter linked to LP resistance (Sermon *et al.,* 2005).

The Qiagen RNeasy mini kit and Quantitect Reverse Transcription kit were used for RNA extraction, purification and reverse transcription of challenged and control *E. coli* O157:H7 cells into cDNA. It would have been ideal to use RNA protect kit to stabilize the *E. coli* O157:H7 RNA before subsequent synthesis of cDNA. However, the RNA protect kit was not used due to the high cost of the kit. For that reason, the quality of RNA extracted from *E. coli* O157:H7 could have been of suboptimal quality and could have influenced the RT-PCR results. The concentration and purity of RNA and cDNA were determined via spectrometric measurements at 260 nm. Before quantitative real-time PCR assay was performed, the qPCR parameters including choice of reference gene, primer concentrations, template concentration, melting and extension temperatures were optimized using standard curves. Primers were designed with perl primer software version 1.1.14. The RT-PCR products should have been run on agarose gels for verification and to determine purity. However, since we do not have agarose gel electrophoresis equipment in our lab, samples were stored at -18 °C for analysis in another laboratory. Unfortunately, break-down of the -18 °C freezer resulted in loss of samples. The relative gene expression ratios were analyzed with the relative expression software tool (REST, 2005) and confirmed with the following equation generated by Pfaffl (2001):

$$\text{Expression ratio} = \frac{(E_{\text{target}})^{\Delta CT \text{ target (Control-Sample)}}}{(E_{\text{reference}})^{\Delta CT \text{ reference (Control-Sample)}}} \qquad (\mathbf{1})$$

***E* target:** Real-time efficiency of target gene transcript

***E* reference**: Real-time efficiency of reference gene transcript

ΔCT_{target} (Control-Sample): the difference in threshold cycle (CT) value of the control (untreated) gene - that of the sample (treated) gene transcript of the target gene

$\Delta CT_{reference}$ (Control-Sample): the difference in threshold cycle (CT) value of the control gene - that of the sample gene of the reference gene transcript

6.1.4 Microbiological analyses

The survival of acid challenged acid-adapted and non-adapted *E. coli* O157:H7 cells was determined by enumeration on Tryptone Soy agar (TSA) and Sorbitol MaConkey agar (SMAC). *E. coli* O157:H7 cells challenged in TSB were surface plated on TSA. TSA is a non-selective rich medium that aids in the recovery of injured cells (Merk, 2005). Tryptose phosphate agar supplemented with 0.1 % (w/v) sodium pyruvate (TPAP) has also been shown to recover injured cells (Leyer and Johnson, 1992). In the current study, *E. coli* O157:H7 cells in milk were enumerated on SMAC. SMAC is a selective and differential medium that distinguishes sorbitol negative *E. coli* O157:H7 colonies (colourless) from non-pathogenic *E. coli* (pink colonies) (Merk, 2005). Although SMAC plates do not promote growth of injured *E. coli* cells as does TSA plates, they were used to differentiate between inoculated *E. coli* O157:H7 and other bacteria present as part of the heterogenous bacterial population in goat milk.

In order to determine the nature of the lactic fermenting microorganisms in traditional fermented milk (from Botswana and Namibia), presumptive isolation of LAB was conducted on the fermented milk products. Since the predominant LAB present in the traditional fermented milk product are *Lactococcus* spp. and *Lactobacillus* spp. (Gadaga *et al.,* 1999), the traditional fermented milk was pour plated on de Mann Rogosa Sharpe (MRS) agar for isolation of *Lactobacillus* spp. (de Mann, Rogosa and Sharpe, 1960) and M17 for isolation of *Lactococcus* spp. (Terzaghi and Sandine, 1975). The MRS plates were incubated at 37 °C for 48 h for mesophilic *Lactobacillus* spp. and *Leuconostoc* spp.,

and at 42 °C for 48 h for thermophillic *Lactobacillus* spp. The M17 plates were incubated at 30 °C for 48 h for the enumeration of *Lactococcus* spp.. Colonies on M17 plates were subsequently streaked on Arginine Tetrazolium agar (ATA) to differentiate between *Lactococcus lactis* subsp. *lactis* (red/pink colonies) and *Lc. lactis* subsp. *cremoris* (white colonies) (Harrigan and McCance, 1976). The ATA contains tetrazolium dyes that differentiate *Lc. lactis* organisms, by their ability to degrade arginine to produce ammonia that turn colonies red, from *Lc. cremoris* which lacks the enzyme for arginine degradation (Turner, Sandine, Elliker and Day, 1963). *Leuconostoc* spp. were enumerated on Meyeux medium (Mayeux, Sandiene and Elliker, 1962). Meyeux medium is a selective medium that contains sodium azide which suppresses the growth of lactic streptococci. The fermented products were surface plated on Meyeux medium and incubated at 25 °C for 72 h. In this study, *Bifidobacterium longum* was cultured in skim milk and plated on M 17. Other more appropriate media that could have been used for *Bifidobacterium* enumeration were Lee's medium (Lee, Vedamuthu, Washam and Reinbold, 1974), Rogosa's modified selective agar (Samona and Robinson, 1994) and MRS containing 5 % (w/v) lactose (Chick, Shin and Ustunol, 2001). However, preliminary studies of enumeration of *B. longum* with M17 media produced good results and was therefore considered appropriate as medium for enumeration.

To determine contaminants in the traditional milk cultures, the traditional fermented milk was examined for the presence of yeasts and moulds. Pre-poured Malt Extract Agar (MEA) plates were used for enumeration of yeasts and moulds by spread plating (Wickerham, 1951). MEA plates were incubated at 25 °C for 5 days before enumeration. Alternative media that could be used for enumeration of yeast and mould are yeast extract dextrose chloramphenicol agar (YDCA) or yeast extract glucose chloramphenicol agar (YGCA) (IDF, 1990).

6.1.5 Biochemical analysis

6.1.5.1 Fatty acid profile

In order to determine the effect of acid and lactoperoxidase challenge on acid-adapted and non-adapted *E. coli* O157:H7 cells, the fatty acids of acid-adapted and non-adapted cells were extracted following challenge. The challenged *E. coli* O157:H7 cells were harvested at 9000 rpm for 10 min and washed twice with 0.85 % (w/v) sterile saline water. Extraction of outer membrane fatty acids was conducted using the modified one phase method described by Bligh and Dyer (1959). The Bligh and Dyer method involves homogenization of the sample with a mixture of chloroform, methanol and water. Since the sample size was much smaller that analyzed in the Bligh and Dyer method, the volumes of reagents were scaled down. The ratio of chloroform, methanol and water used in the extraction was 2:2:1. This method was used for extraction of total fatty acids because it is a simple and rapid method of lipid extraction. It also produces a high yield of fatty acids that are not modified during the extraction process. The Bligh and Dyer method has been adopted by several authors for the extraction of lipids.

All the essential fatty acids were assessed with the exception of cyclopropane fatty acids (CFAs). It would have been interesting to see the effect of acid-adaptation and the combination of acid and activated LP challenge on the concentration of CFAs in the *E. coli* O157:H7 outer membrane, since CFAs contribute significantly to acid resistance in *E. coli* (Chang and Cronan, 1999). However, the method for identification and quantitation of CFAs had not been validated for the Gas chromatograph used for fatty acid analyses; therefore the CFAs were not analyzed. Nonetheless, this flaw was compensated by following the relative expression level of cfa synthase, the enzyme responsible for synthesis of CFAs, in acid-adapted and challenged *E. coli* O157:H7 cells.

6.1.5.2 Activation of the lactoperoxidase system

The activation of LP system in milk is standardized though there are variations to the norm. Conversely, different methods have been reported for LP activation in broth where the concentration of LP system components vary widely and thus makes comparison difficult. For example, Ravishankar, Harrison and Wicker (2000) activated the LP system in TSB by adding 250 mg/l LP enzyme, 800 mg/l glucose and 800 mg/l glucose oxidase to generate hydrogen peroxide, and 800 mg/l NaSCN to determine its inhibitory effect on *L. monocytogenes*. In another study, Kennedy, O'Rourke, McLay and Simmons (1999) used higher concentrations of components of LP system in a ground beef model in which 180.16 g/l glucose, 2260 U/ml glucose oxidase and 10 g/l LP enzyme were included. De Spiegeleer, Sermon, Vanoirbeek, Aertsen and Michiels (2005) activated LP system by addition of components in the following proportions: LP enzyme (5 µg/ml), KSCN (0.25 mM), glucose (0.4 %), and glucose oxidase (0.1 U/ml) in TSB to determine resistance of knockout porin *E. coli* mutants to activated LP system. In this study, 10 µg/ml LP enzyme, 0.25 mM NaSCN and 0.25 mM $Na_2CO_3.1.5H_2O_2$ were added to TSB to activate the LP system.

The method described by the International Dairy Federation (IDF, 1988) is generally used in activation of the LP system in milk. Since the LP enzyme is naturally found in milk, SCN and H_2O_2 are added to fully activate the LP system. According to IDF (1988), SCN level of 14 mg/l and 30 mg/l of H_2O_2 are required to activate the LP system. This method was therefore used in the activation of LP system in Saanen goat milk in this study. Spectroscopic measurements of LP activity was carried out at 412 nm using 2,2'-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid (ABTS) as the chromogenic substrate (Kumar and Bhatia, 1998).

6.1.5.3 Lactic acid determination

The activity of lactic starter cultures is assessed by their rate of acid production within a

specified period of time. Acid production of LAB can be evaluated by measuring the ability of 1 % (v/v) LAB inoculum to coagulate 10 % (w/v) reconstituted skim milk after 16 h of incubation at 22 °C (Huggins and Sandine, 1984). This method uses the pH of the coagulated skim milk as a measure of acid production; although this method only evaluates the overall acid production capacity of single lactic cultures. The method as described by Bradley *et al.* (1993) for determination of percent w/w lactic acid was used in this study to measure lactic acid production by lactic starter cultures during fermentation of goat milk. This method involves titration of 9.0 ml fermented milk with 0.1 N NaOH using phenolphthalein as indicator. The volume of NaOH dispensed is divided by ten to give the titratable acidity as percent lactic acid. This method was selected based on its simplicity and common usage for measurement of lactic acid in several studies (Lin *et al.,* 1995; Haddadin, Ibrahim and Robinson, 1996; Nakada *et al.,* 1996; and Seifu *et al.,* 2003). The high performance liquid chromatography (HPLC) method of determining lactic acid production has recently become the more accepted method for lactic acid determination. Several studies have used HPLC to determine organic acid concentrations in fermented milk (Samona *et al*., 1996; Narvhus, Østeraas, Mutukumira and Abrahansen, 1998; Chick, Shin and Ustunol, 2001; Gadaga *et al.,* 2001). Although HPLC method is expensive, it is specific for organic compounds and it is more accurate compared to the above mentioned methods.

6.2 Comparative acid-resistance of *E. coli* O157:H7 in Tryptone Soy Broth, goat milk and fermented goat milk

6.2.1 Acid resistance of acid-adapted *E. coli* O157:H7 in Tryptone Soy Broth

Since the implication of low pH foods in recent *E. coli* O157:H7 outbreaks, there have been numerous investigations exploring acid-adaptation and subsequent resistance to lethal acid stress. However, the use of different *E. coli* strains, acidulants, media, adaptation methods and resistance assays have made comparison difficult (Chung *et al.,*

2006). The underlying principle of *E. coli* resistance has nonetheless been established. When exposed to mild acid treatments, *E. coli* becomes acid-adapted by modulating physiological and morphological changes that enhance resistance to subsequent lethal acid treatment (Leyer *et al*., 1995; Bearson *et al.,* 1997). In the current study, stationary phase acid-adaptation was induced in *E. coli* O157:H7 strain UP10. Acid-adapted *E. coli* O157:H7 survived lactic acid stress in acidified TSB at pH level 4.0 with only 1 $\log_{10}$ cfu/ml reduction in the colony count after 6 h treatment at 37 °C whereas the non-adapted cells were inhibited beyond detection when given the same treatment as acid-adapted cells (Fig. 6.1). Lactic acid treatment at pH 5.0 had a bacteriostatic effect on both acid-adapted and non-adapted *E. coli* O157:H7 at 37 °C, confirming that *E. coli* strains, particularly pathogenic *E. coli* O157:H7 can survive mild acid conditions (Glass *et al*., 1992). Similar results were obtained for acid-adapted and non-adapted cells at 25 °C at pH levels 7.4 and 5.0 (Fig. 6.2).

Foster (2000) explained that *E. coli* have the ability to grow within the pH range of 5.0 to 8.5 while maintaining internal pH levels of 7.6 to 7.8. Neutral internal pH is maintained by housekeeping pH homeostasis systems including potassium proton antiporters for narrow shifts to low pH and sodium proton antiporters for narrow shifts to alkaline pH (Foster, 2000). It was however not clear whether these systems were activated or induced at an external pH of 5.0.

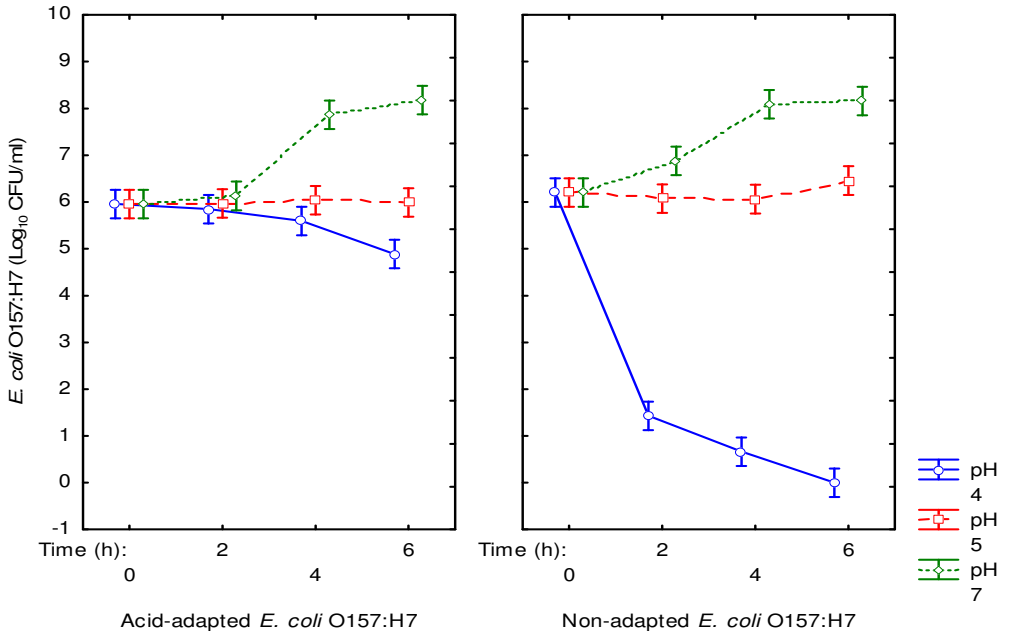

Figure 6.1: The effect of acid challenge at pH levels of 4, 5 and 7 on survival of acid-adapted and non-adapted E. coli O157:H7 in Tryptone Soy Broth incubated for 6 h at 37 °C

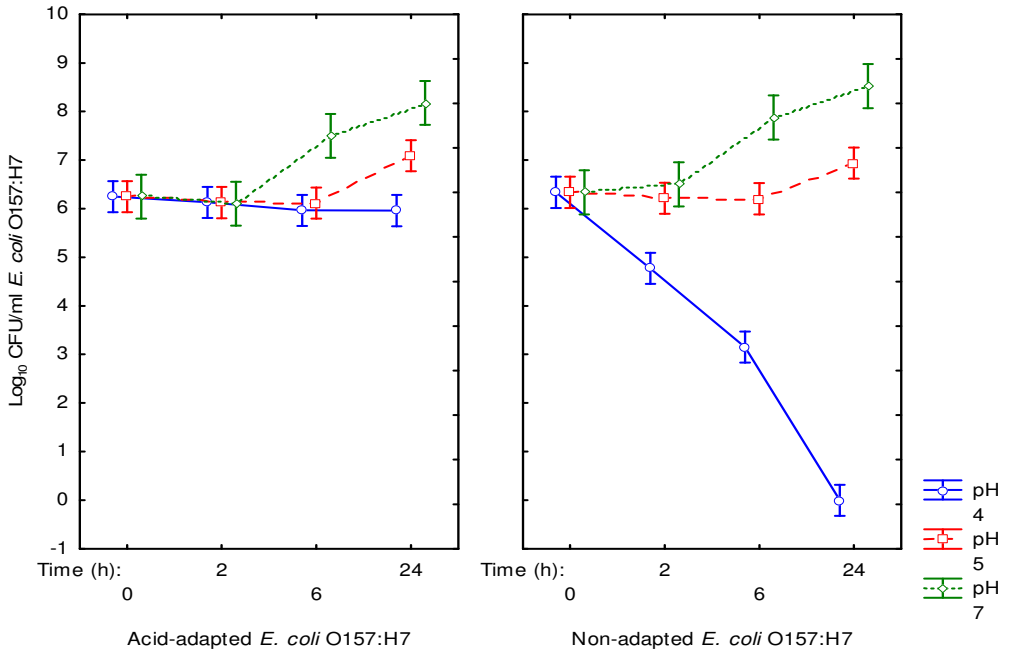

Figure 6.2: The effect of acid challenge at pH levels of 4, 5 and 7 on survival of acid-adapted and non-adapted *E. coli* O157:H7 in Tryptone Soy Broth incubated for 6 h at 25 °C

The similar survival trends observed for acid-adapted and non-adapted *E. coli* O157:H7 UP10 at 25 °C and 37 °C suggests that acid resistance systems induced during acid-adaptation may not contribute to acid resistance at pH 5.0 and may therefore not be necessary for survival at mild acidic pH levels. Growth of both acid-adapted and non-adapted cells after 6 h of incubation at 25 °C indicates that pH 5.0 is only temporarily effective in preventing proliferation of *E. coli* cells; however, these cells can inherently adapt and subsequently resume normal growth. At 25 °C, the lactic acid treatments were less inhibitory on both acid-adapted and non-adapted cells after 6 h at pH 4.0 (Fig. 4.2). Here, pH 4.0 had a bacteriostatic effect on acid-adapted cells while the non-adapted colony counts declined from 6.4 $\log_{10}$ cfu/ml to 3.0 $\log_{10}$ cfu/ml after 6 h. The significant growth difference between acid-adapted and non-adapted *E. coli* O157:H7 cells at pH 4.0 was due to the acid resistance of adapted cells.

Resistance of acid-adapted *E. coli* has been reported in several studies (Leyer *et al.,* 1995; Lin *et al.,* 1996; Deng *et al.,* 1999; Ryu and Beuchat, 1999; Cheng *et al.,* 2003; Sainz *et al.*, 2005). The difference in inhibition of non-adapted *E. coli* cells at both 25 °C and 37 °C was due to the growth rate of *E. coli* at these growth temperatures. At 37 °C, cells grew optimally and were thus most susceptible to environmental stress. This was indicated by the greater inhibitory effect on non-adapted cells at pH 4.0 when incubated at 37^{o}C. At 25 °C, cell growth was less rapid, therefore cells could partially adapt or repair damage caused by external stress.

6.2.2 Effect of acid-adaptation on outer membrane components of *E. coli* O157:H7

One of the first lines of bacterial defence against environmental stress is the cell membrane. Since the cell membrane is an important gateway for entry of substances into the cell, *E. coli* has evolved mechanisms to modulate changes in the cell membrane to control movement of molecules into and out of the cell.

It has previously been reported that outer membrane proteins OmpF and OmpC are

regulated by osmolarity and low pH (Sato, Machida, Arikado, Saito, Kakegawa and Kobayashi, 2000). Studies have shown that at high temperature and osmolarity and a high concentration of antimicrobials, OmpC, the smaller, more specific porin, is up-regulated while OmpF, the larger porin, is repressed (Liu and Ferenci, 1998). In order to investigate whether OmpC and OmpF contribute to acid resistance, relative expression levels of *ompC* and *ompF* from acid-adapted and challenged *E. coli* O157:H7, challenged non-adapted cells and the untreated *E. coli* O157:H7 cells were determined. Our results indicated that *ompF* was up-regulated in both acid-adapted and non-adapted cells. In the acid-adapted cells, the *ompC* gene was also up-regulated but expressed at basal level in non-adapted cells. The increased expression of *ompF* was likely due to the presence of glucose (0.25 %) in TSB or the lack of deregulation due to inhibition of RpoS in TSB.

The fatty acid profile of acid-adapted and non-adapted *E. coli* O157:H7 revealed an increase in saturation of fatty acids of acid-adapted *E. coli* O157:H7 compared to the non-adapted cells. Post-synthetic modification of fatty acids into cyclopropane fatty acids (CFAs) contributes to the resistance of acid-adapted *E. coli* in low pH environments (Chang and Cronan Jr, 1999). Though CFAs were not determined in this study, the increase in percent saturation of fatty acids in cell membranes of acid-adapted *E. coli* O157:H7 correlated positively with acid resistance in acidified TSB at pH levels 4.0 and 5.0. The qRT-PCR results indicated basal expression of cyclopropane fatty acid synthase (*cfa* gene) in acid-adapted cells. This implies that there was no significant difference in the synthesis of CFAs in the acid-adapted cell membranes compared to the control cell membranes. Since *cfa* is regulated by RpoS, the glucose inhibition of *rpoS* could be responsible for the expression of *cfa* at basal level.

Brown *et al.* (1997) reported similar changes in fatty acid profile of acid habituated *E. coli* O157:H7 at pH 5.0. It has been suggested that *E. coli* synthesizes saturated fatty acids (SFA) at the expense of unsaturated fatty acids (Yuk and Marshall, 2004). This corresponds with the fatty acid profile of acid-adapted cells used in the current study where acid-adapted *E. coli* O157:H7 showed increase in palmitic acid and a

corresponding decrease in palmitoleic and oleic fatty acids in their outer membranes. The degree of saturation of cell membranes is important because it directly affects cell functions such as passive transport of solutes and compounds across cell membranes, respiration, protein secretion and other biochemical reactions (Yuk and Marshall, 2004). A high ratio of unsaturated fatty acids to SFA in the cell membrane indicates a low melting point and hence increased membrane fluidity. Hence, increase in SFA in the cell membrane increases the rigidity and plasticity of the cell membrane, which restricts its permeability to toxic compounds such as protons and weak acids (Beales, 2003).

The higher levels of linoleic fatty acids observed in acid-adapted *E. coli* O157:H7 in the current study is however contrary to published data (Brown *et al.,* 1997; Chang and Cronan, 1999; Beales, 2004, Yuk and Marshall, 2004). Nonetheless, the increase in saturated fatty acids in acid-adapted cells observed in this study corresponds with stationary phase type cultures. However, the acid-adapted and non-adapted *E. coli* O157:H7 cells investigated in this study were harvested after adaptation and subsequently introduced into fresh acidified TSB for 6 h. At both pH levels, cells were still in their lag phase after 6 h of incubation. It was therefore imperative to maintain proper functioning of the cell to allow adaptation to environmental conditions. Since membrane fluidity is essential for protein function and respiration of bacterial cells, it is possible that the acid-adapted cells increased the concentration of SFA in the membrane, while increasing synthesis of polyunsaturated fatty acids (PUFA) such as linoleic acid. Increased synthesis of PUFA is important for maintenance of membrane fluidity, but may not offset restriction of solute movement into and out of the cell as modified by increase in saturation of outer membrane lipids. This phenomenon is supported by Russell and Nichols (1999) who explained that the multiple double bonds present in PUFA are important in maintaining membrane fluidity, especially at low temperatures; nonetheless, it provides a greater degree of packing order of the phospholipids bilayer as compared to the monounsaturated fatty acids. This process “seals” the phospholipids bilayer and controls passive diffusion of molecules across the cell membrane. Since higher levels of linoleic acid was present in all acid-adapted cells including control cells grown at pH 7.4,

compared to non-adapted cells, it is hypothesized that selective synthesis of linoleic acid occurs during acid-adaptation which further protects the cell without hindering membrane transport during subsequent acid challenge.

6.2.3 Survival and growth of acid-adapted *E. coli* O157:H7 in TSB versus goat milk

In general, the non-adapted *E. coli* O157:H7 cells showed better growth in both TSB and raw goat milk compared to acid adapted cells at pH levels 5.0, 6.9 and 7.4. The acid-adapted *E. coli* O157:H7 cells in TSB incubated at 37 ºC showed at least a 2 h lag phase before growth at pH 7.4. This lag phase was absent in non-adapted *E. coli* O157:H7. In raw goat milk (pH 6.9), acid-adapted *E. coli* O157:H7 strain UP10 was marginally inhibited during incubation in goat milk for 6 h while non-adapted strain UP10 grew in goat milk. It is possible that since acid-adapted cells are programmed for survival under stressful conditions, they may have to re-adapt to growth at pH 7 leading to a lag phase and a marginally slower growth rate. This process may involve physiological and morphological changes to suit the new environment (Hengge-Aronis, 2000). In goat milk, the process of re-adaptation to growth at neutral pH may have been stressful and impacted on the vulnerability to antimicrobial compounds present in milk. This susceptibility of acid-adapted *E. coli* O157:H7 during growth at neutral pH varies from strain to strain. In the current study, acid-adapted *E. coli* O157:H7 strain 1062 cells were uninhibited in raw goat milk unlike acid-adapted strain UP10 cells. Nonetheless, the acid-adapted strain 1062 cells did not grow as well as the non-adapted strain 1062 cells during the 6 h incubation at 25 ºC. Therefore, while acid-adaptation protects *E. coli* O157:H7 cells to lethal acid damage and other stressful environments, it is also detrimental to cell survival and growth under ideal environmental conditions.

6.2.4 Lactoperoxidase activity in goat milk

The LP activity of Saanen goat milk used in this study was unusually low (0.21 U/ml in September and October, 0.09 U/ml in November and 0.05 U/ml in March). Seifu *et al.*

(2004) reported LP levels of 0.79 U/ml in Saanen goat milk and 0.26 U/ml in indigenous goat milk. Kumar and Bhatia (1999), Chavarri *et al.,* (1998), and Fweja *et al.,* (2007) have reported higher levels of LP in cow and ewe milk. Previous studies have indicated high variability in LP activity among breeds and within individual breeds over different time periods (Fweja *et al.,* 2007). It is likely that the differences in LP concentration could be due to different breeds and pasture composition. Nevertheless, LP concentration was limiting since no exogenous LP was added to improve its antimicrobial efficacy. Consequently, LP did not have a significant effect on lactic acid bacteria and *E. coli* O157:H7 in LP activated fermented goat milk, and acid-adapted and non-adapted *E. coli* O157:H7 cells in LP activated goat milk incubated for 6 h. Lactic acid production was also not significantly affected by LP activation in fermented milk.

Regardless of the low LP activity in fresh Saanen goat milk, residual activity of the LP system was observed in the marginal reduction in growth rate of *E. coli* O157:H7 strains tested. Previous studies have shown that activated LP is bacteriostatic against *E. coli* in Saanen goat milk (Seifu *et al.,* 2004) although a bactericidal effect against *E. coli* has been reported by other authors (Björck, Rosen, Marshall and Reiter, 1975; Van Opstal *et al.,* 2006). The bacteriostatic effect against *E. coli* O157:H7 in the goat milk tested in this study could be attributed to the lower concentration of the lactoperoxidase enzyme in Saanen goat milk. If such low levels of LP enzyme occur in Saanen goat milk, then the question can be asked whether it is beneficial to activate LP system for the preservation of goat milk with low LP activities. This study demonstrated that an average LP activity of 0.29 U/ml may prevent *E. coli* proliferation in Saanen milk depending on the contaminating *E. coli* O157:H7 strain. However, the efficacy of the LP system will be improved when applied concurrently with other preservation treatments such as low pH (pH 5.0) and when lower levels of *E. coli* O157:H7 cells are tested.

6.2.5 Cross-protection of acid-adapted *E. coli* O157:H7 in broth

One stress applied at mild levels can confer cross-protection against another stress in

bacteria if the stress response pathways are shared (Rowe and Kirk, 1999). Cross-protection of adapted enterobacteria to subsequent unrelated stresses applied in food processing was first reported by Leyer and Johnson (1993) for *Salmonella* Typhimurium. Since then cross-protection of acid-adapted *E. coli* O157:H7 against salt (20 % w/v) and heat 56 ºC for up to 80 min has been documented (Rowe and Kirk, 1999). Although acid-adaptation of *E. coli* O157:H7 has been extensively researched, there is a paucity of information on cross-protection of acid-adapted *E. coli* O157:H7 to stresses applied in dairy processing. Cross-protection studies conducted in complex broth media alone may not reflect actual resistance in food systems since complex broth media may provide an ideal environment for growth. Food systems are complex and among others contain nutrients including glucose that promote growth, and chemicals that may inhibit growth or resistance of bacteria to stresses encountered. Cross-protection of stationary phase *E. coli* O157:H7 is mediated by RpoS (Cheville *et al.,* 1996). The RpoS-dependent oxidative acid resistance system is also known to be glucose repressed (Castanie-Cornet *et al*., 1999).

In the current study, stationary phase acid-adaptation was activated in *E. coli* O157:H7. It was expected that the presence of glucose in TSB would inhibit RpoS and therefore limit acid resistance and cross-protection against the combination of activated LP and lactic acid treatments. On the contrary, acid-adapted *E. coli* O157:H7 exhibited cross-protection against activated LP and lactic acid at pH levels 4.0 and 5.0 in TSB. Results from the expression of acid-inducible genes suggested induction of *gadA* which encodes an isoform of glutamate decarboxylase, a component of the GAD acid resistance system. The *rpoS* gene known to mediate cross-protection at both log and stationary phase was however not induced in acid-adapted *E. coli* O157:H7. The GAD system is only partially regulated by RpoS. It can also be induced by the house keeping sigma factor, sigma 70 at a low external pH (Audia *et al.,* 2001). The GAD system however requires at least 0.9 mM glutamate in the medium to be activated (Hersh *et al.,* 1996). Since TSB contains 22.2 mM glutamate, it is likely that the GAD acid resistance system was induced by sigma 70, which recognizes the same promoter sites as the alternate sigma factor, RpoS.

The results from survival studies and qRT-PCR suggest that in a complex medium that has glucose present, the GAD system protects acid-adapted *E. coli* O157:H7, at least in part against cellular damage at pH 4.0. The GAD system may also contribute to cross-protection of *E. coli* O157:H7 against LP-activation in combination with low pH.

Another interesting observation was the increased expression of *corA* in non-adapted *E. coli* O157:H7 challenged to LP activation at pH 7.4. The *corA* gene encodes a magnesium transporter that has been suggested to contribute to *E. coli* resistance against activated LP system (Sermon *et al.,* 2005). The *corA* gene was not induced in acid-adapted *E. coli* O157:H7 challenged to LP activation at pH 7.4 suggesting that induction of acid-adaptation components may repress increased expression of *corA*. It is also likely that increased expression of *corA* did not occur in acid-adapted cells because it was not essential for survival in an activated LP system environment.

6.2.6 Cross-protection of acid-adapted *E. coli* O157:H7 in goat milk

Cross-protection against activated LP in broth has been reported for acid-adapted *Salmonella* Typhimurium in BHI broth (Leyer and Johnson, 1993) and acid-adapted *Listeria monocytogenes* in TSB broth (Ravishankar *et al.,* 2000). There have been several cross-protection studies, mostly starvation induced, in enteric bacteria (Chung *et al*., 2006) but very few cross-protection studies of acid-adapted bacteria have been conducted in food. To the knowledge of the authors, cross-protection of acid-adapted *E. coli* O157:H7 against the LP system in milk has not been reported. LP inhibition of Gram positive and Gram negative bacteria in milk has however been established (Björck *et al.,* 1975; Haddadin *et al.,* 1996; Marks, Grandison and Lewis, 2001; Seifu *et al.,* 2004). In this study, cross-protection of acid-adapted *E. coli* O157:H7 to LP activation, lactic acid challenge and heat treatments was investigated in Saanen goat milk.

In the current study, the LP system alone did not significantly inhibit either acid-adapted or non-adapted cells in fresh goat milk. Also, the acid-adapted *E. coli* O157:H7 did not

show resistance to LP activation vis-à-vis non-adapted *E. coli* O157:H7. There was however a significant strain difference in growth of acid-adapted *E. coli* O157:H7 strains UP10 and 1062 in fresh LP activated goat milk. Acid-adapted *E. coli* O157:H7 strain 1062 demonstrated cross-protection against LP-activation and lactic acid at pH 5.0 in goat milk. The combined LP activation and lactic acid at pH 5.0 had a bacteriostatic effect on the non-adapted strain 1062 cells. The significant difference in survival of the two *E. coli* O157:H7 strains in activated LP goat milk confirm the observation of Benito *et al.,* (1999) that *E. coli* O157 strains are highly variable in their resistance against environmental stresses.

The combination of heat treatment (55 °C) and LP activation inhibited both acid-adapted and non-adapted *E. coli* O157:H7 at pH 6.9. This inhibitory effect was even greater at pH 5.0. Differential influx of hypothiocyanate which occurs as an uncharged molecule at mild pH (pH $\leq$ 5.3) may have contributed to the lethality of the combined treatments. At a higher level of heat treatment (60 °C), the acid-adapted *E. coli* O157:H7 cells exhibited a greater degree of resistance to combined heat, LP and lactic acid at pH 5.0 in comparison to the non-adapted *E. coli* O157:H7, indicating that the harsher the treatments, the greater the resistance. This feature suggests that there is a threshold of environmental stresses that the non-adapted *E. coli* O157:H7 can handle unscathed. Until that threshold is reached, acid-adaptation may not just be redundant but could potentially be detrimental for survival.

In Madila processing, the LP system had a delayed inhibitory effect on inoculated *E. coli* O157:H7. Although *E. coli* O157:H7 cells had become acid-adapted after 24 h, they were inhibited in LP-activated Madila after day 3 at 30 °C. It has been reported that the efficacy of the LP system is limited by temperature and initial bacterial numbers (FAO/WHO, 2006). LP can extend the keeping quality of milk for 7-8 h at 30 °C (CAC, 1991). In the current study, LP inhibition of *E. coli* O157:H7 was observed only after day 3 in Madila processing. This observed inhibition of *E. coli* O157:H7 in LP activated Madila could be due to the synergistic effect of the LP system and decreased pH (pH

4.3). The lack of inhibition of LAB by the LP system throughout traditional Madila fermentation (5 days) suggests that LP system can be applied in the processing of both traditional and modern fermented milk products where slow fermentation processing is employed at ambient temperatures.

Chapter 7: CONCLUSIONS AND RECOMMENDATIONS

This study revealed that in complex media, in particular Tryptone Soy Broth, which contains glucose, the glutamate acid-resistance system protects stationary phase acid-adapted *E. coli* O157:H7 at least in part against inhibition at pH 4.0. The acid-adapted cells are also cross-protected against the activated LP system in combination with low pH in TSB in the absence of RpoS. Increase in expression of the small OmpC porin and increased saturation of outer membrane fatty acids contributed to acid resistance of acid-adapted *E. coli* O157:H7. The magnesium transporter (CorA) may have contributed to survival of non-adapted *E. coli* O157:H7 in LP activated TSB though this protein was not required for resistance of acid-adapted *E. coli* O157:H7 cells to LP activation in TSB. Results from qRT-PCR and outer membrane fatty acids also suggested that changes in gene expression of the LP inducible gene, *corA,* and acid-resistance genes *rpoS*, *gadA*, *cfa*, *ompC* and *ompF* occurs during acid-adaptation and not during the acid and LP challenge. These systems may have contributed to cross-protection of acid-adapted *E. coli* O157:H7 to LP activation and heat treatment at 60 °C in goat milk.

The combination of LP activation, heat treatment at 55 °C and 60 °C, and lactic acid at pH 5.0 inhibited both acid-adapted and non-adapted *E. coli* O157:H7 in fresh Saanen goat milk. These treatments can therefore be applied concurrently to control acid-adapted and non-adapted *E. coli* O157:H7 in milk when they occur in low numbers. Nonetheless, these treatments gave < 5 $\log_{10}$ cfu/ml reduction in *E. coli* O157:H7 counts in milk.

Acid-adaptation inhibited *E. coli* O157:H7 in fresh goat milk at pH 6.9 while the non-adapted *E. coli* O157:H7 cells grew in fresh goat milk at the same pH. This indicates that the process of acid-adaptation could exert an additional stress on *E. coli* O157:H7 cells during growth at optimal environmental conditions where *E. coli* O157:H7 does not require acid-adaptation for survival. In this case, acid-adapted *E. coli* O57:H7 may have to re-adapt to growth at neutral pH.

Though non-adapted *E. coli* O157:H7 were more sensitive to LP activation, heat treatments and low pH, they were able to adapt to mild acid pH during fermentation of milk to become acid-adapted. This processes enhanced prolonged survival during Madila processing.

For the above reasons, contamination of food with acid-adapted *E. coli* O157:H7 could be as dangerous as contamination with the non-adapted *E. coli* O157:H7. While acid-adapted *E. coli* O157:H7 is better suited to harsh conditions, it can be inhibited in food that does not require acid-adaptation for survival, e.g. fresh milk. On the other hand, non-adapted *E. coli* O157:H7 could become acid-adapted in food at mild acid pH which will enhance prolonged survival in such foods. Finally, the variable strain response to combined LP system, heat treatments and low pH suggests that several *E. coli* O157:H7 strains need to be tested in challenge studies for the development of processing and preservation procedures to improve the safety of food products.

Due to the complexity of the *E. coli* O157:H7 stress response, the issue of cross-protection in *E. coli* O157:H7 systems has not been exhausted. Further studies using qRT-PCR and micro-array technologies will shed more light on regulation of acid-inducible cross-protection in complex media and in food systems. In addition, the sensitivity of acid-adapted *E. coli* O157:H7 in milk needs further investigation using several *E. coli* O157:H7 strains in order to draw accurate conclusions. Since *E. coli* O57:H7 normally occurs at low numbers in meat and dairy products, it would be appropriate to conduct such studies with artificially inoculated acid-adapted and non-adapted *E. coli* O157:H7 in low numbers to determine their susceptibility to combined treatments.

Chapter 8: REFERENCES

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Publications and presentations from this work

Scientific paper

Parry-Hanson, A., Jooste, P. J. and Buys, E. M., 2009. The Influence of lactoperoxidase, heat and low pH on survival of acid-adapted and non-adapted *Escherichia coli* O157:H7 in goat milk. *International Dairy Journal* 19, 417-421.

Conference presentations

Oral Presentations

Parry-Hanson, A., Jooste, P. and Buys, E., 2009. Relative gene expression of acid inducible genes in acid-adapted *Escherichia coli* O157:H7 during lactoperoxidase and lactic acid challenge. 4th International qPCR Symposium: Diagnostics and molecular markers. Technical University of Munich, Freising-Weihenstephan, Germany. 9-13th March 2009.

Parry-Hanson, A., Jooste, P. and Buys, E., 2008. Acid-adapted *Escherichia coli* O157:H7: a true survivor. SASDT symposium. Drakensburg, South Africa. 15-16th April 2007.

Poster Presentations

Parry-Hanson, A., Jooste, P. and Buys, E., 2008. Cross-protection of acid-adapted *Escherichia coli* O157:H7. 95th International Association of Food Protection Congress, Columbus, Ohio, USA. 3-6th August 2008.

Parry-Hanson, A., Jooste, P. and Buys, E., 2008. Influence of lactoperoxidase, heat and low pH on acid-adapted *Escherichia coli* O157:H7. Food Micro 2008 – Evolving microbial food quality and safety. Aberdeen, Scotland. 1-4th September 2008.