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Acid Adaptation of *Escherichia coli* O157:H7 in Fermented Goat Milk

by

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Declaration

I declare that the dissertation submitted for the degree of Master of Science (Agriculture) in Food Science and Technology at the University of Pretoria has not been previously submitted by me at any other University or Institution of higher education.

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ABSTRACT

This study was undertaken to determine the effect of acid adaptation on the survival and growth of *Escherichia coli* in broth and fermented goat milk. In the first phase of the study, acid resistance of *E. coli* O157:H7 strain UT 10 and *E. coli* ATCC 25922 was determined in brain heart infusion broth at pH 7.4, 4.5 and 2.5. Variations due to acid stress in the counts of both strains were determined. Alterations in the fatty acid profile due to acid adaptation were also determined. Acid adaptation enhanced the survival of both strains at pH 4.5, but neither strain could survive after 4 h at pH 2.5. At optimum growth conditions (pH 7.4), *E. coli* ATCC 25922 exhibited increased viability over *E. coli* UT 10. At pH 4.5, *E. coli* UT 10 was more tolerant to low pH than *E. coli* ATCC 25922. An increase in saturated fatty acids (SAFAs) of both AA strains was observed, indicating the importance of lipid modification in enhancing survival at low pH.

In the second phase of the study, acid resistance of *E. coli* O157:H7 strains UT 10 and UT 15 were determined in commercial fermented at 30 °C for 24 h and stored at 7 °C for 2 days and traditional Amasi fermented for 3 days at ambient temperature (*ca* 30 °C). *E. coli* O157:H7 counts in traditional Amasi could not be detected after 3 days of challenge while those in commercial Amasi were detected at 2.7 $\log_{10}$ cfu/ml after the same period. The survival of acid adapted (AA) and non-adapted (NA) strains was similar in traditional Amasi while in commercial Amasi, the NA strain survived significantly better than the AA strain.

The results of this study indicate that *E. coli* O157:H7 can survive during fermentation and storage of fermented goat milk Amasi. They also show that survival of the pathogen in a broth model differs from that in a food system. Survival of *E. coli* O157:H7 in fermented milk is affected by fermentation time, pH and storage temperature. The food industry

should therefore adapt their processing/ preservation procedures by taking the most acid tolerant pathogenic *E. coli* strains into consideration in order to ensure the safety of their products.

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CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

Milk has always been an important component in the normal balanced diet of humans since it supplies high quality nutrients (Devendra, 1999). A large amount of milk in South Africa is produced from cows. These are expensive, require more feed and large areas to graze, hence they are not suitable for small-scale farmers who are unable to meet all these requirements (Donkin, 2000). Production of milk from goats has therefore been found to be novel and more appropriate for small-scale farmers. Goats are browsers, they eat less and occupy less grazing space than cows. In some families, the backyard goat produces enough milk for family needs. The government is engaged in an exercise to encourage small-scale rural farmers to be involved in dairy goat farming and it established the Milk Goat Project at the Medical University of South Africa (MEDUNSA) to investigate the feasibility of using crossbred goats for milk production (Donkin, 2000).

Goat milk is believed to be more easily digestible and less allergenic than cow's milk (Haenlein, 2003). In French extensive studies with children allergic to cow milk, the treatment with goat milk produced positive results in 93% of the children and was recommended as a valuable aid in child nutrition because of less allergenicity and better digestibility than cow milk (Fabre, 1997; Reinert & Fabre, 1997). Fat globules are smaller in goat milk and probably one of the reasons for easy digestion (Jabbar, Saddiqi & Iqbal, 2006). Goat milk can thus be a good source of nutrients for immuno-suppressed individuals like those infected by HIV.

Small-scale rural farmers engaged in goat milk production sell raw milk and fermented milk products produced from raw goat milk. Many dairy products from goat milk are made using traditional, flexible manufacturing systems (Fox, 1997). Prompt and effective cooling of goat's milk is not possible for small-scale farmers since milk cooling tanks are expensive. The quality of the milk produced under these conditions is a cause of concern. The milk could have high bacterial counts and could cause diseases to humans if pathogenic strains like those of *Escherichia coli* are present. Acidification is one of the important measures commonly employed to control growth and survival of spoilage and pathogenic microorganisms in foods

(Brown & Booth, 1991). However, various acidic foods such as yoghurt, apple cider and mayonnaise have been implicated in the outbreaks of food-borne diseases caused by *E. coli* O157:H7 (Besser, Lett, Weber, Doyle, Barrett, Wells & Griffin, 1993; Bachrouri, Quinto & Mora, 2002; Cheng, Yu & Chou, 2002). The outbreaks are attributed to adaptation of microorganisms to acidic conditions which increases their resistance to environmental stress.

Previous research has proved that exposure of bacterial cells to a sub-lethal stress can result in subsequent increased resistance to that particular stress or increased resistance to other stresses (Jenkins, Chaisson & Matin, 1990; Farber & Pagotto, 1992; Leenanon & Drake, 2001). Microbial resistance to acid conditions enhances their survival in low pH foods and also enhances the survival of pathogenic microorganisms during the passage through the human stomach, thereby causing disease (Gordon & Small, 1993; Peterson, Mackpwiak, Barnett, Marling-Cason & Haley, 1989). Three acid stress response systems, namely, acid resistance, acid tolerance and acid habituation, have been reported to enhance survival of microorganism at low pH (Leyer & Johnson, 1992; Goodson & Rowbury, 1989; Bearson, Benjamin, Swords & Foster, 1996). Comparing these systems has proved not to be easy due to differences in strategies and media to induce and test acid stress (Bearson *et al*., 1996).

Microbial acid-induced stress results from the combination of the biological effect of low pH and the effect of weak or organic acids which are produced during fermentation or are added to foods as preservatives (Zhao, Doyle & Besser, 1993; Garlant & Kasper, 1994; Abee & Wouters, 1999). In the presence of weak acid preservatives, many organisms have shown to have stress adaptation, which aid microbial survival by inducing a specific pattern of gene expression, which appears to be required for optimal adaptation to weak acid conditions (Beales, 2004). As a result of previous microbial exposure to acid environments, food-borne pathogens and other microorganisms may develop a cross protective response. Duffy, Grau & Vandelinde (2000) observed that *E. coli* O157:H7 grown at pH 5.6 and then stored at pH 4.8 for 96 h, under simulated meat fermentation conditions, had enhanced tolerance to subsequent heat treatments at 55 ^{o}C. In humans, *E. coli* is the most common facultative anaerobe in the gastrointestinal tract and this is also the organism's natural habitat in other mammals and warm-blooded animals (Heidelberg, 1981). Most of the *E. coli* strains present in the gut flora

are harmless symbionts but they are a number of pathogenic forms that are able to cause a wide diversity of human and other animal diseases, ranging from mild diarrhoea to cholera-like diarrhoea and invasive dysentery (Griffiths, 2005).

The effect of microorganisms to alter their physiology during exposure to stress conditions like acid environments and become more resistant to further stresses may counteract the effectiveness of food preservation and compromise food safety (Koutsoumanis, Kendall & Sofos, 2003). Several studies have been done to determine the effect of the survival and growth of acid adapted *E. coli* in cow's milk (Cheng & Chou, 2001). There is paucity of information available on the effect of the survival and growth of acid adapted *E. coli* organisms in goat milk. A greater understanding of the mechanism of acid adaptation may offer an insight into the methods of controlling growth and survival of these organisms in dairy products from goat milk.

This study will determine the effect of acid adaptation on the survival and growth of *E. coli* in goat milk and goat milk products.

CHAPTER 2: LITERATURE REVIEW

2.1 Goat milk

The dairy goat industry is rapidly gaining importance throughout the world (Boscos, Stefanakis, Alexopoulos & Samartzi, 1996). The world goat population was estimated to have increased by 26.4 % between 1993 and 2003 (Table 1). Goat milk production also showed an increase of 8.7 % during the same period (Table 2). Goats rank third in terms of global milk production from different species after cattle and buffaloes (Klinger & Rosenthal, 1997). Goat milk and products thereof are reported to feed more malnourished and hungry people in the world than cow milk (Haenlein, 2003). In developed countries, goat milk and goat milk products, such as yoghurt and cheeses, have gained popularity in meeting the needs of connoisseur consumers who demand high quality foods (Haenlein, 2003).

Table 1. Total world population of goats between 1993 and 2003 (FAO, 2003)

Region/ Country	**Population of Goats (thousands)**		**Change (%)**
	1993	**2003**	
Africa	176996	219736	24.1
Asia	370269	487588	27.1
Europe	18940	18425	-2.7
America	37652	37940	0.7
Oceania	871	817	-6.2
World	604727	764510	26.4

The uniqueness of goat milk and its products, when compared to cow milk, stems from its nutritional and health benefits which are related to a number of medicinal problems, foremost being food allergies and other gastro-intestinal ailments (Haenlein, 2003). Many unreliable reports about the nutritional and medical benefits of goat milk have, however, been reported,

with little technical studies conducted and published in accredited journals and technical books (Beck, 1989; Freund, 2000). Cow milk allergy is a problem that varies with countries and age of people. β-lactoglobulin, which is absent in human milk, has been identified as the protein causing allergies in cow milk. Some authors, however, dispute this (Buergin-Wolff, Signer, Friess, Berger, Birbaumer & Just, 1980; Taylor, 1986). Cow milk allergy has a prevalence of 2.5 % in children during the first 3 years of life (Businco & Bellanti, 1993), 12-30 % in infants less than 3 months old (Lothe, Lindberg & Jacobson, 1982). In Italy, it has been reported in 3 % of children less than 2 years of age (Bevilacqua Martin, Candalh, Fauquant, Piot, Bouvier, Manfredi, Pilla & Heyman, 2000)

Table 2. Goat milk production between 1993 and 2003 (FAO, 2003)

Region/ Country	**Milk Production (thousands of tonnes)**		**Change (%)**
	1993	**2003**	
Africa	2100	2745	30.7
Asia	6241	6291	0.8
Europe	2169.1	2421.4	11.6
America	357	359	0.5
Oceania	25	30	20.0
World	10867	11816	8.7

Goat milk is however not totally free of allergic properties. The allergic properties of goat milk are affected by the presence or absence of α-s-1-casein hence goat milk lacking this casein is less allergic (Haenlein, 2003). This has lead to selection against α-s-1-casein in some countries (Moioli *et al*., 1998). In the processing of goat milk products, less curd yield, longer rennet coagulation time, more heat lability and weaker curd firmness has been achieved with goat milk having less or no α-s-1-casein but with α-s-2-casein (Moioli *et al*., 1998).

Goat milk exceeds cow milk in monounsaturated fatty acids, polyunsaturated fatty acids and medium chain triglycerides, which are beneficial for human health, especially for

cardiovascular conditions (Babayan, 1981; Haenlein, 1992). This superiority of goat milk has not been successfully tapped into the marketing of goat milk products such as cheeses and yoghurt. Manipulating the feeding regime of goats can change the fatty acid composition of goat milk to even more beneficial fatty acids (Sanz Sampelayo *et al*., 2002).

Compared to average cow milk, average goat milk is much higher in butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), Lauric (C12:0), myristic (C14:0), palmitic (16:0), Linoleic (C18:2), but lower in stearic (C18:0) and oleic acid (C18:1) (Jenness, 1980; Haenlein, 2003). Capric, caprylic acids and medium chain triglycerides (MCT) have a unique metabolic ability to provide direct energy instead of being deposited in adipose tissues and lower serum cholesterol (Schwabe, Bennett & Bowman, 1964; Greenberger & Skillman, 1969; Kalser, 1971; Tantibhedhyangkul & Hashim, 1978; Alferez, Barrionuevo, Lopez, Sanz, Sampelayo, Lisbona, Roberts & Campos, 2001). They have thus being widely used to treat a wide variety of clinical disorders such as malabsoption syndromes, steatorrhea, hyperlipoproteinemia, intestinal resection, premature infant malnutrition, epilepsy, cystic fibrosis, coronary by-pass and gallstones. It is not surprising, therefore, that goat milk and its products are popular among health conscious consumers and certain ethnic groups (Park, 1991).

The safety of food worldwide remains challenged by the potential for emergence of new pathogens and re-emergence of known pathogens (Altekruse Cohen & Swerdlow, 1997). Microorganisms have developed mechanisms to evolve, mutate and adapt to environmental stresses, permitting them to withstand otherwise lethal conditions (IFT, 2006). The quality milk is determined by aspects of composition and hygiene. The compositional quality of milk is mainly influenced by feeding, management systems, genetics and breed. Hygienic parameters are decisive for food safety but might also influence the composition of milk (Heeschen, 1987). According to Lowenstein & Speck (1983), challenges in managing the safety of milk derive from the various sources of contamination. Undesirable organisms may get into the milk either through the body (endogenously) or from some external source (exogenously) after milk has been drawn. Milk also serves as an excellent culture and protective medium for certain microorganisms, particularly bacterial pathogens, whose multiplication depends mainly on temperature, competing microorganisms and their metabolic

products (Heeschen, 1994). It has become increasingly clear, internationally, that diseases in dairy animals and the production and handling of milk under poor hygienic conditions can lead to widespread outbreaks of human diseases (Giesecke, Du Preez & Petzer, 1994).

Milk and milk products have been associated with outbreaks of diseases worldwide. The public health problems associated with the consumption of unpasteurized cow's milk have been well documented (Djuretic, Wall & Nichols, 1997) and there is no evidence that the risk from unpasteurized goat milk and goat milk products is any less. Goats and sheep may be a reservoir for verocytotoxin-producing *Escherichia coli* O157:H7 and this is of particular concern because of its extreme virulence and the low dose needed to produce infections (Chapman, Siddons & Harkin, 1996; Bielaszewska, Janda & Blahova, 1997). Raw milk often contains microorganisms which may cause food-borne diseases (Adesiyun, Webb & Rahaman, 1995; Headrick, Korangy, Bean, Angulo, Altekruse, Potter & Klontz, 1998; Steel, McNab, Poppe, Griffiths, Chen, Degrandis, Fruhner, Larkin, Lynch & Odumeru, 1997). It has been implicated as a vehicle of infection in two outbreaks of HUS in infants drinking raw milk from their family's farms (Martin, Shipman, Wells, Potter, Hedberg, Wachsmuth, Tauxe, Davis, Arnoldi & Tilleli, 1986). A study done by Little & Louvois (1999) found that *Escherichia coli* was present in 25 percent of unpasteurized milk in England and Wales. In 1992, a large outbreak of bloody diarrhea caused by *E. coli* O157 infection occurred in Southern Africa. In Swaziland 40, 912 physician visits for diarrhea in children less than five years old were reported to be caused by *E. coli* O157:H7 (Effler, Issaacson, Arntzen, Heenan, Canter, Barret, Lee, Mambo, Levine, Zaidi & Griffin, 2001).

Pasteurization of raw milk was introduced in the 19th century to increase the safety of milk and milk products. Small-scale farmers, however, do not have sufficient capital to purchase pasteurizing equipment hence they sell unpasteurized milk. In South Africa, raw milk and raw milk products are largely consumed in rural areas. During the sale of these products, aspects of food safety and hygiene are not always sufficiently considered.

2.2 Incidence of food-borne disease

At their meeting in 1983, renowned scientists convened by the Food and Agriculture Organization (FAO) and the World Health Organisation (WHO) concluded that illnesses due to contaminated food was perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity (Kaferstein, 2003). Devastating outbreaks of diseases such as Salmonellosis, Cholera, enterohaemorrhagic *E. coli* and hepatitis A have occurred in both developing and developed countries, and the results of epidemiological surveillance are showing an increase in prevalence of food-borne illness in the last three decades (Kaferstein, 2003). In developing countries, biological contaminants, largely bacteria, viruses and parasites, are responsible for a wide range of diseases, including, campylobacteriosis, *E. coli* gastroenteritis, typhoid and paratyphoid fever (Kaferstein, 2003). Recently, there has been an outbreak of acute diarrhoea in 12 districts throughout the northern and eastern parts of Botswana (UN, 2006). The diarrhoeal epidemic affected 18000 children and claimed the lives of 470 children from January to April 2004. According to the tests conducted by National Laboratories and the Centers for Disease Control (CDC), the cause of the diarrhoea was closely linked to water contaminated with *Cryptosporidium* and enteropathogenic *E. coli* (UN, 2006).

Kaferstein (2003) also reported that approximately 1.5 billion episodes of diarrhea causes 1.8 million deaths in children under the age of five years annually. It is estimated that up to 70 % of the diarrheal episodes may be caused by food-borne contaminants. Although many different pathogens have been identified, food contaminants with pathogenic *E. coli* causes up to 25 % of all diarrheal episodes in infants and children, while *Campylobacter jejuni* and *Shigella* species account for 10-15 % and 5-15 %, respectively. It is estimated that up to 10 % of patients with enterohemorrhagic *E. coli* (including *E. coli* O157) infections may develop haemolytic uraemic syndrome (HUS), with a case-fatality ranging from three to five percent (Kaferstein, 2003).

In South Africa, food-borne poisoning became a notifiable medical condition in 1990 (Department of Health, 2006). However, this condition, which is usually clinically mild, is less

likely to be reported, as people are less likely to seek medical attention. Also when people do seek advice, health workers are less likely to report these less severe conditions. Presently in South Africa, food-borne diseases are in many instances underreported (Department of Health, 2006). The major reasons being:

- The lack of efficient and integrated food-borne surveillance and notification system. Although food-borne illnesses can be severe or even fatal, milder cases are often not detected through the notification system.
- South African legislation requires that food poisoning be notifiable only if the same doctor or health facility observes four or more cases. In most cases this is impractical. In addition, because many food-borne illnesses are self-limiting, many patients never see a doctor.
- At present there are no incentives for medical practitioners to follow up suspected cases of food poisoning (through laboratory tests), to confirm the illness and identify the causative agent. As a result, many food poisoning cases and at times deaths, go unnoticed.
- Many pathogens transmitted through food are also spread through water or from person to person, thus obscuring the role of food-borne transmission.
- The present disease notification/ surveillance system of the country is virtually unaware of other emerging food-borne infections, such as *Escherichia coli* O157, Listeriosis, Shigellosis and specific viruses associated with food.

During 1999 – 2004, a total of 136 331 laboratory and clinically confirmed cases of food-borne related illnesses were reported in South Africa (Department of Health, 2006). Food-borne poisoning accounted for 1.4 % (2015 cases). As shown in table 1 below, the specified pathogens caused a total of 530 deaths during the reported years.

Table 3. Reported cases and deaths of food-borne illness in South Africa, 1999-2004 (Department of Health, 2006)

Disease	Total cases (n)	Total deaths (n)	Case Fatality Rate (%)
Cholera	131309	433	0.3
Food poisoning	2015	62	3.1
Hepatitis A	2191	27	1.2
Typhoid Fever	801	29	3.6
Paratyphoid Fever	7	0	N/A
Brucellosis	8	0	N/A
Total	136331	551	0.4

Judging from the fact that most cases are underreported and that the government might be virtually unaware of emerging food-borne infections such as *Escherichia coli* O157 and the revelation by current research, that these organisms have developed an ability to adapt to stresses such as low pH and chilling, more cases of food poisoning are likely to be reported in future.

2.3 *Escherichia coli*

Escherichia coli is a member of the family Enterobacteriaceae. They are gram-negative, short rod-shaped bacteria which are usually motile with peritrichous flagellae, possessing fimbriae, and are facultave anaerobes that are capable of fermentative and respiratory metabolism (Wilshaw, Cheasty, & Smith, 2000). *E. coli*'s natural habitat is in the lower part of the intestine of most warm-blooded animals (Heidelberg, 1981). Most strains of *E. coli* are harmless commensal members of the intestinal flora of mammals in which some strains adhere to the intestinal mucosa while others are only temporary transients in the lumen of the colon. While some strains of *E. coli* live as commensals, many are opportunistic pathogens of humans and other animals (Levine, 1984). *E. coli* is the major cause of neonatal septicemia, neonatal meningitis and urinary tract infections in humans and of a variety of invasive diseases

in mammals. It is also a leading cause of diarrhoeal diseases in humans and other mammals (Neidhardt, 1986)

E. coli can respond to environmental signals such as chemicals, pH, temperature, and osmolarity in a number of very remarkable ways since it is a single-celled organism (Todar, 2002). For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. It can also stop swimming and grow fimbriae that will specifically attach to cells or surface receptor. In response to change in temperature and osmolarity, it can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances. With its complex mechanisms for regulation of metabolism the bacterium can survey the chemical contents of its environment in advance of synthesizing any enzymes necessary to use these compounds. It does not wastefully produce enzymes for degradation of carbon sources unless they are available, and it does not produce enzymes for synthesis of metabolites if they are available as nutrients in the environment (Todar, 2002).

By serological means, it is possible to divide *E. coli* strains into an increasing number of sero-groups and serotypes. The initial serotyping scheme was developed by Kaufman (1947) and it is based on *E. coli* somatic (O), Flagellum (H) and capsular (K) antigens. The O antigen is based on the antigenicity of the O specific polysaccharide of the cell outer membrane lipolysaccharides. The O antigens are the basis of classifying *E. coli* into subgroups (King and Holt, 1984). H antigens are heat-labile flagellar antigens composed of protein. K antigens, identified on the basis of their chemical composition, are acidic polysaccharide capsular antigens. Today, more than 180 O serogroups and more than 60 H serotypes are recognized (King and Holt, 1984). *E. coli* that cause enteric diseases are divided into six different pathotypes that comprise:

- Enteropathogenic *E. coli* (EPEC)
- Enterotoxigenic *E. coli* (ETEC)
- Enteroinvasive *E. coli* (EIEC)
- Enteroaggregative *E. coli* (EAEC)

- Diffusely adhering *E. coli* (DAEC)
- Enterohaemorrhagic *E.coli* (EHEC)

2.3.1 Enteropathogenic *E. coli* (EPEC)

EPEC is a major cause of infantile diarrhea worldwide, mostly in the developing world. A number of serotypes are responsible for the disease and they possess a number of virulence factors although they do not produce any classic toxins (Karper, McDaniel, Jawis & Gomez-Duarte, 1997). EPEC induce a watery diarrhea similar to ETEC, but they do not possess the same colonization factors and do not produce ST (heat-stable) or LT (heat-labile) toxins. They produce a non fimbrial adhesin designated intimin, an outer membrane protein that mediates the final stages of adherence (Todar, 2002). The EPEC cells adhere to the lining of the small intestines causing changes in the electrolyte secretion and structural damage of the small intestines.

The symptoms for young children can include watery diarrhea, fever, vomiting and abdominal pain. In adults, the symptoms include severe diarrhea with nausea, vomiting, abdominal cramps and fever. The onset of the symptoms can range from 17 to 72 hrs (FDA, 2006) EPEC strains have been responsible for many outbreaks of infantile diarrhea, although less frequently in recent years. In adults, outbreaks have occurred due to contaminated water in Europe and North America and in cold meat and pies in the United Kingdom (FDA, 2006).

2.3.2 Enteroinvasive *E. coli* (EIEC)

EIEC are responsible for a bacillary dysentery-like illness and, not surprisingly therefore, share some of the virulence properties associated with *Shigella dysenteriae* (Hart, Batt & Saunders, 1993). EIEC are a significant cause of morbidity and mortality in young children in developed countries although, like most enteropathogens, they are more important in developing countries where sanitation and hygiene levels are of a poor standard. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction (Todar, 2002). This process results in an inflammatory response accompanied by necrosis and

ulceration of the large bowel leading to release of blood and mucous in the stools (Hart *et al*., 1993).

Symptoms of diseases caused by EIEC include chills, fever, headache, muscular pain, abdominal cramps and diarrhoea. These symptoms are very similar to those caused by Shigella. Symptoms usually appear 8 to 24 hrs after ingestion of contaminated food. The infective dose is more than 10^6 *E. coli* per gram of food (FDA, 2006). Many cases of foodborne diseases caused by EIEC strains are seen to occur in warmer months. Outbreaks have occurred due to ingestion of contaminated water and soft cheese.

2.3.3 Enterotoxigenic *E. coli* (ETEC)

ETEC strains are characterized by their production of well defined heat-labile and/ or heat cholera toxin (CT) while heat stable enterotoxin (ST) is non-antigenic and of low molecular weight (Rea & Fleming, 1994). ETEC are an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. The disease requires colonization and elaboration of one or more enterotoxins (Todar, 2002). For the disease to occur, a large number of *E. coli* need to be ingested, survive in the stomach and produce toxins.

Symptoms of ETEC include watery diarrhea with fever, abdominal cramps, malaise and vomiting. Onset of symptoms occurs 8 to 44 hours after ingestion of contaminated food. Infective dose is thought to be between 10^8 and 10^{10} *E. coli* cells ingested for diarrhoea to occur (FDA, 2006). ETEC strains are a major cause of infant diarrhea in developing countries and are often a cause of traveler's diarrhea. They are rarely a problem in developed countries (FDA, 2006).

2.3.4 Enterohaemorrhagic *E. coli* (EHEC)

EHEC are organism that produce a powerful cytotoxin, active against cultured vero cells hence they are often termed Vero cytotoxin (VT) but may be also be known as Shiga-like

toxin. EHEC are therefore also termed verocytotoxigenic *E. coli* (VTEC) and Shiga toxin-producing *E. coli* (STEC). Verotoxigenic *E. coli* produces one or two verotoxins, designated VT-1 and VT-2 which are toxic to Vero and HeLa cells, as first reported by Konowalchuk, Spiers & Satvric (1977). VT-1 is a relatively heat stable high molecular weight Shiga-like toxin, whereas VT-2 is immuno-logically distinct (Doyle, 1991). EHEC also possesses a number of other mechanisms, such as adhesins, which are involved in virulence. EHEC was first identified as a human pathogen in 1982 after two outbreaks occurred in the US (Konowalchuk, *et al*., 1977). Cells of EHEC adhere to the lower intestine, grow and produce toxins. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause Haemolytic uremic syndrome (HUS) (Todar, 2002).

Symptoms include diarrhea, severe abdominal cramps and vomiting. Other more series symptoms include bloody diarrhea, haemolytic uraemic syndrome (HUS), thrombatic thrombocytopenic purpura (TTP) and death. The infective dose is very low. In some outbreaks EHEC were detected in the foods at 2 cells per 25 grams of food (FDA, 2006). An outbreak caused by O111 occurred in Australia in 1995. About 23 people (mainly children) were diagnosed with HUS with one fatality.

2.3.4.1 Enterohemorrhagic *E. coli* O157:H7

E. coli O157:H7 is one of the emerging food-borne infections. It is responsible for 14000 cases reported in the United States in 1994 (Anonymous, 1995), 1600 cases in Canada in 1992 (Lior, 1994) and additional sporadic cases reported worldwide (Griffin & Tauxe, 1991) have raised *E coli* O157:H7 to a food-borne pathogen of international importance (Knight, 1993). It causes severe illnesses such as HUS which is more common in young children and TTP which mainly affects the elderly. The pathogen is exceptional in its severe consequences of infection, low infection dose and acid tolerance (Buchanan and Doyle, 1997). It is more resistant than other *E. coli* and can survive in many acidic foods (Besser *et al*., 1993; McIngrale, Chen, McKillip & Drake, 2000). The acid tolerance of *E. coli* O157:H7 is

believed to play a key role in pathogenesis and food-borne illness (Buchanan & Edelson, 1996).

2.3.5 Enteroaggregative *E. coli* (EaggEC)

Emteroaggregative *E. coli* are self-adherent, tending to auto-agglutinate, giving the appearance under the microscope of a stack bricks (Harrigan, 1998). The distinguishing feature of EaggEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. It is, however, unknown how EaagEC causes persistent diarrhea except that it colonize the intestinal mucosa, mainly that of the colon, followed by secretion of cytotoxins and enterotoxins (Weintraub, 2007; Nataro, Steiner & Guerrant, 1998). EaagEC differs from other diarrheagenic *E. coli* through its ability to adhere to epithelial cells such as HEp-2 in a stacked-brick pattern (Nataro & Kaper, 1998). EaggEC do not induce attaching/effacing lesions characteristic of enteropathogenic *E. coli* or produce cholera-like or Shiga-like toxins characteristic of *Vibrio cholerae* or *Shigella dysenteriae* respectively. Little is known of their pathogenicity except that they produce a toxin known as the enteroaggregative heat-stable enterotoxin (EAST1) (Elliot & Nataro, 1995) and that their aggregative adherence phenotype is mediated by at least two fimbriae called fimbriae I and II (Czeczulin, Balepur, Hicks, Phillips, Hall, Kothary, Navarro-Garcia & Nataro, 1997).

Mayer & Wanke (1995) reported that EaggEC could be recovered from diarrheal stools from AIDS patients. This is a cause of great concern because diarrhea is an important cause of morbidity in immunocompromised people.

2.3.6 Diffusely adhering *E.coli* (DAEC)

Duffusely adherent *E. coli* has also been associated with diarrhea in several studies and its name comes from its ability to adhere to HeLa cells in a diffuse pattern (Niedhardt, 1986). Two classes of the DAEC strains have been identified (Servin, 2005). The first class of DAEC strains includes *E. coli* strains that harbor Afa/Dr adhesins which are associated with urinary

tract infections (Archamband, Courcoux & Labigne-Roussel, 1998; Daigle, Harel, Fairbrother & Lebel, 1994). The second class includes *E. coli* strains that express an adhesin involved in diffuse adherence which is a potentially cause of infantile diarrhea (Benz & Schmidt, 1992). These DAEC strains have been reported to have one or more homologous of the locus of the enterocyte effacement characteristics of EPEC, which adds to their pathogenic potential. DAEC strains bind to the entire existing surface of HeLa or HEP-2 cells as well-separated, distinct bacteria and usually do not belong to the classic EPEC serotypes (Giron, Jones & Millan-Velasco, 1991). DAEC strains that express adhesins of the Afa/Dr family are involved in 25 to 50 % of cases of cystitis in children and 30 % of cases of pyelonephritis in pregnant women (Archamband *et al*., 1998; Servin, 2005).

2.4 Microbial stress adaptation

During food production and processing, foodborne microorganisms may encounter a wide variety of stresses including physical treatments (heat, pressure, electric pulses, ultrasonic waves and osmotic shock), added chemicals (acids, salts, and oxidants) and biological stresses such as competition, bacterial metabolites and antagonism (Abee & Wouters, 1999). Exposing microorganisms to a stress may induce adaptation to subsequent lethal levels of the same type of stress. This phenomenon is referred to as stress adaptation. (Rodriguez-Romo & Yousef, 2005).

The mechanism of bacterial defence against adverse environmental conditions can be divided into two classes namely adapted limited and multiple adapted responses (De Angelis & Gobbetti, 2004). A limited or specific adaptive response results from microbial exposure to a sublethal dose of a physical, chemical or biological stress, which protects cells against subsequent lethal treatments with the same stress (De Angelis & Gobbetti, 2004)

A multiple adapted response also known as cross-protection, occurs when bacterial cells adapt to an inherent physiological condition or an environmental factor, which results in microbial protection against subsequent lethal stresses, including stresses to which the microorganisms had not been previously exposed (Price, 2000). This cross-protection involves induction of

the general stress response, and it is triggered by a variety of stress conditions such as cell starvation, exposure to high or low temperature, high osmolarity and low pH (Price, 2000).

2.4.1 Hurdle concept and stress hardening

The hurdle concept involves the application of mild preservation factors (hurdles) in combinations, either in sequence or most of the time simultaneously, to enhance microbial inactivation by additive or synergistic effects (Leistner & Gould, 2002; Scott, 1989). The use of a combination of sublethal stresses, although potentially synergistically inactivating microorganisms in food, could lead occasionally to stress adaptation and cross-protective responses (Juneja & Novak, 2003). Lou & Yousef (1996) introduced the concept of "stress hardening" as a complement of the hurdle concept.

Stress hardening refers to the exposure of microorganisms to sublethal stress and their adaptive response that leads to protection against lethal stress. Researchers have indicated that in most cases, during food processing, microorganisms are treated with sublethal stresses sequentially rather than simultaneously (Rodriguez-Romo & Yousef, 2005). Therefore, microbial exposure to sublethal stress could harden the microorganism and protect them against subsequent treatment factors or hurdles. Processing of most food should therefore involve a combination of several preservation factors (hurdles) which are carefully selected and tested.

2.4.2 Acid adaptation of microorganisms

The control of microorganism is one of the most important aspects of food preservation. Preservation techniques are designed to prevent microbial growth in processed foods by disrupting the internal environment of the cells such that growth is no longer possible (Beales, 2004). Food acidification is a common food preservative method and microbial acid-induced stress results from the combination of the biological effect of low pH, and the effect of weak or organic acids which are produced during fermentation or are added to foods as preservatives (Abee & Wouters, 1999).

Acid adaptation is a phenomenon by which microorganisms show increased resistance to environmental stress after exposure to a moderate acid environment. Acid adaptation and increased resistance to acid stress has been observed in various organisms including *Listeria* (Kroll & Patchett, 1992; Hill, O'Driscoll & Booth, 1995; O'driscoll, Gaham & Hill, 1996) *Escherichia coli* (Goodson & Rowbury, 1989; Cheng & Chou, 2001) and *Salmonella* (Foster & Hall, 1990). Microbial cells have developed strategies to respond to acid stress by inducing log-phase and stationary-phase protective responses known acid resistance response, acid tolerance response and acid habituation Bearson *et al*., 1996). Pre-exposure of bacterial cells to mild acidic conditions induces acid tolerance to subsequent lethal stress (Paul & Hirshfield, 2003). Figure 1 shows a composite cell with all the known inducible acid response systems. Proton leakage into the cell lowers internal pH, subsequently inducing present amino acid decarboxylases present in the cell. Low pH also increases the accumulation of RpoS and PhoP, which control production of acid shock proteins (Bearson *et al*., 1996). Acid shock proteins are believed to enhance survival at low pH through neutralization of external acid, adjusting cellular catabolism to a new environment, performing DNA repair and membrane biogenesis and contributing to the microbial pathogenesis (Cheng, Yu & Chou, 2002; Goodson & Rowbury, 1989; Gajiwala & Burley, 2000).

Many acid shock proteins are chaperones which can protect proteins against denaturation (Mujacic & Baneyx, 2006). For example, Hsp31, the product of the $\sigma^{S \text{ and }} \sigma^{D}$ –dependent *hchA* gene, which is a heat-inducible chaperone that prevents protein misfolding at high temperatures, has been reported to play an important role in acid resistance of *E. coli* (Mujacic & Baneyx, 2006). It functions as a holdase that stabilizes unfolding intermediates until stress stops. The absence of Hsp31 affects the functioning of all the three acid resistance systems (Mujacic & Baneyx, 2006). Other identified acid stress-specific periplasmic chaperones include the HdeA and HdeB (Gajiwala & Burley, 2000; Jacob, Muse, Eser & Bardwell, 1999). HdeA has been implicated in the AR 1 system and it relies on low pH-mediated dimer-to-monomer activation to suppress the aggregation of acid-denatured proteins in the periplasm Gajiwala & Burley, 2000; Hong, Jiao, Hu, Zhang, Liu, Fu, Shen, Xia & Chang, 2005).

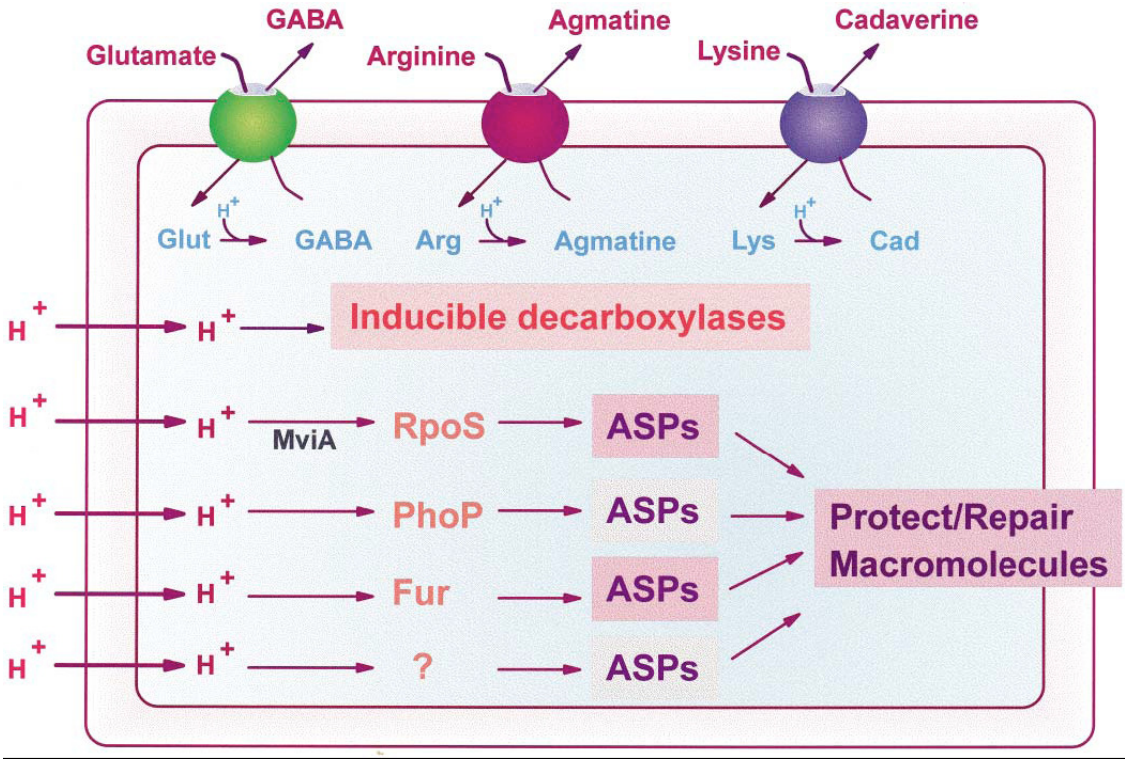


Figure 1. Acid survival responses in enteric microorganism (Bearson *et al*., 1996).

The other defense strategies which protect cells at low pH include changes in membrane composition (Chang & Cronan, 1999; Jordan, Oxford & Ad O'byrne, 1999; Yuk & Marshall, 2004), internal pH homeostasis systems (Park, Bearson, Bang, Bang & Foster, 1996; Richard & Foster, 2004) and systems to repair or protect essential cellular components (Choi. Baumler & Kaspar, 2000; Raja, Goodson, Smith & Rowbury, 1991). Survival of low pH by changing the composition of membranes is discussed in detail in 2.7 below. The ability to regulate internal pH, is a process driven by the controlled movement of cations across the membrane. However this ability to maintain internal pH close to neutrality can be overwhelmed at low extracellular pH values, leading to the death of the cell (Booth & Kroll, 1989). The ability of a microorganism to maintain its internal pH at a value close to neutral is achieved by a combination of passive or active mechanisms (Brown & Booth, 1991).

2.4.2.1 Passive homeostasis

In prevention of large changes in internal pH, as pH of the environment varies, it is thought that the permeability of the cell membrane to protons plays a role, with protons that are present in the environment being prevented from crossing the membrane and reducing the internal pH (Hill, O'Driscoll & Booth, 1995). A cell exposed to low pH environments can also increase the cytoplasmic levels of proteins and glutamates and increase buffering capacity, which may prevent internal pH disruption (Booth & Kroll, 1989).

Recent measurements of internal pH using radiolabelled weak acid distribution assay have shown that the *E. coli* decarboxylase systems are important in pH homeostasis (Foster, 2004). At pH 2.5, cells relying on the glutamate system maintain an internal pH of approximately 4.2 $\pm$ 0.1, whereas cells that are dependent on arginine have an internal pH of 4.7 $\pm$ 0.1 (Richard & Foster, 2004)

2.4.2.2 Active homeostasis

Active homeostasis depends primarily on the potassium ion and proton movement (Hill, O'Driscoll & Booth, 1995). At low external pH a cell must force out ions entering the cell that are associated with weak acids to prevent acidification of the interior of the cell. However, movements of protons across the membrane generates a membrane potential, preventing further proton removal. This membrane potential is, however, broken up by the movement of cations into the cell such as potassium ions, which generates a transmembrane pH gradient and helps maintain internal pH (Booth & Kroll, 1998).

2.5 Mechanisms of action of weak acid preservatives

Microorganisms are in general more sensitive to changes in the internal pH than changes in external pH, although significant changes in either will lead to loss of viability. Weak acids are normally produced by microorganisms themselves through fermentation and they are used in food processing procedures, such as on meat surfaces, to control contaminating pathogens

(Cutter & Siragusa, 1994; Van Netten, Huis, Veld & Mossel, 1994). Benzoic acid is the weak acid commonly used to test the effects of weak acids on cell viability (Lin, Smith, Chapin, Baik, Bennett & Foster, 1996). Strong acids do not affect the pH of cytoplasm to the same extent as weak acids (Beales, 2004). The primary mode of action of weak acid preservatives is to reduce the internal pH below the normal physiological range, which results in an extension to the lag phase and inhibition of growth (Restaino, Lenovich & Bills, 1982). These weak acids in their undissociated form, are capable of diffusing into microbial cells, once inside the cytoplasm, they dissociate and decrease the intracellular pH, which results in disruption of metabolic activities (Rodriguez-Romo & Yousef, 2005).

2.6 Mechanisms of action of strong acid preservatives

Strong acids lower the external pH but are not able to permeate through the cell membrane. This is due to their lower pKa than weak acids and hence dissociate at pH values between 3 and 6, which is the normal pH of most foods (Booth & Kroll, 1989). Strong acids, like hydrochloric acid, exert their antimicrobial effect by denaturing enzymes present on the cell surface and by lowering the cytoplasmic pH due to increased proton permeability when the pH gradient is very large. This can result in reduced growth rate and cause an extension to the lag phase (Cheroutre-Vialette, Lebert, Hebraud, Lebadie & Lebert, 1998)

2.7 Membrane lipids and acid stress

The cell membrane lipid, like that of the phospholipids or the lipid A component of the lipopolysaccharides in gram negative bacteria, is the primary source of fatty acids in microbial cells (Welch, 1991). Biosynthetic pathways of fatty acids vary with species and this determines the fatty acid content of all lipids. Many bacteria synthesize fatty acids with chain lengths of 10 to 19 carbon atoms (Welch, 1991). The 16- carbon saturated cellular fatty acid is abundantly found among prokaryotes.

Bacteria have been reported to alter their membrane lipid composition under stressful conditions or during growth (White, 1994). For example, growth temperature and growth

phase affect the fatty acid composition of *E. coli.* At 37º C, a typical *E. coli* strain contain about 45% palmitic acid, 35% palmitoleic acid, 2% myristic acid and 18% cis-vaccenic acid. During growth, palmitoleic acid and partly cis-vaccenic acid are converted to their cyclopropane derivatives by addition of a methylene group (from S-adenosylmethionine) across the double bond of the phospholipids-bound FA (Law, 1971).

Normal cell function occurs effectively when the membrane bilayer is fluid, but at low pH, the fluidity of bacterial membrane lipids is reduced. Maintaining the right membrane fluidity is of utmost importance in cells since it ensures proper membrane functions such as biochemical reactions, transport systems and protein secretion (Yuk & Marshall, 2004). Several authors have reported that *E. coli* alters its membrane fluidity by increasing the percentage of saturated fatty acids and decreasing the percentage of unsaturated fatty acid during acid stress (Brown, Ross, McMeekin & Nichols, 1997; Yuk & Marshall, 2004; Arneborg, Salskov-Iversen & Mathiasen, 1993). The decrease in membrane fluidity is associated with increased acid resistance since it reduces the flow of protons into the cell (Yuk & Marshall, 2004). At alkali conditions, however, increased fluidity may allow protons to more readily flow out of the cell to decrease environmental pH. The modification of cellular fatty acids in has also been reported in *S. mutans* where acid adaption includes as enrichment in MUFA in the membrane (Quivey, Faustoferri, Monahan & Marquis, 2000)

The formation of cyclopropane fatty acids (CFA) during acid stress has been reported to play an important role in protecting *E. coli* cells against low pH (Brown *et al*., 1997; Wang & Cronan, 1994; Jordan *et al*., 1999). CFA formation is a postsynthetic modification of bacterial membrane lipid bilayer that occurs as cultures of *E. coli* and other bacteria enter the stationary phase (Chang & Cronan, 1999). Production of CFA is governed partly by the RpoS and catalyzed by the soluble enzyme CFA synthase. Chang & Cronan (1999) also reported that CFAs play a fundamental role in decreasing proton permeability of membranes due to conversion of UFAs to CFAs in the phospholipid component. Conversely, some studies have revealed that some strains of *E. coli* produce minimal CFA at low pH. Yuk & Marshall (2004) found that the proportions of CFAs were lowest in cells grown under acidic conditions compared to cells neutral pH (pH 7.3). They attributed this to the failure of rpoS to activate

the CFA synthase. Similarly, Arneborg *et al*. (1993) reported a lower proportion of unsaturated and CFAs in *E. coli* MT102 grown at pH 6.4 than did the same strain grown at pH 8.4.

2.8 Acid adaptation of *E. coli*

E. coli possesses log-phase and stationary-phase acid survival mechanisms with the latter exhibiting more tolerance to acid (Hengge-Aronis, 1993; Arnold & Kasper, 1995). A problem that plagues the acid survival literature is the difficulty experienced when trying to compare various systems studied in different laboratories. Three systems have been named in *E. coli* (Foster, 2000). These are the acid tolerance response (ATR), acid resistance response (AR) and acid habituation (AH). Problems in comparing these systems stem from the different strategies and media used to induce and test acid survival. For example, ATR which has been shown to protect lag-phase cells for several hours at pH 3 appears to be very different from the process of acid habituation that is usually measured as protection for 7 minutes at pH 3 in complex media (Storz & Hengge-Aronis, 2000).

2.8.1 Acid tolerance response

The acid tolerance response (ATR) includes both log-phase and stationary-phase systems (Park *et al*., 1996), however, stationary-phase ATR varies greatly among species (Lin, Lee, Frey, Slonczewski & Foster, 1995) and strains of the same species (Buchanan & Edelson, 1996). Although ATR was first observed in *E. coli* (Goodson & Rowbury, 1989) it was not studied in detail. Paul & Hirshfield (2003) recently demonstrated that pre-exposure of *E. coli* log-phase cells to pH 5.5 and pH 4.3 induces the synthesis of acid shock proteins (ASPs) which most likely are responsible for the induction of ATR. These proteins are proposed to be components of acid survival and resistance systems, functioning in biochemical reactions such as neutralizing of external acid, adjusting cellular catabolism to a new environment, performing DNA repair and membrane biogenesis, aching as chaperones and contributing to the microbial pathogenesis (Adams, Fowler, Kinsella, Howell, Farris, Coote & O'Conor, 2001; Audia, Webb & Foster, 2001; Gajiwala & Burley, 2000).

2.8.2 Acid habituation

Acid habituation studies are generally performed in nutrient broth media and occurs when *E. coli* cells grown at pH 5.0 survive subsequent exposure to lower pH of 3-3.5 better than cells grown at pH 7.0 (Goodson & Rowbury 1989). Acid habituation occurs in log-phase cultures and the repair of DNA damage is the major event in acid habituation. Many compounds have been shown to affect habituation, such as glucose, glutamate, aspartate, $FeCl_3$, KCl and L-proline, which can induce habituation at neutral pH. The control of these systems seem unrelated to Fur, CysB and RelA although acid habituation induced at pH 5 is CysB dependent (Rowbury & Goodson, 1997). Phosphate and cAMP also influence induction of acid habituation (Rowbury & Goodson, 1998). Acid habituation, therefore, occurs in low phosphate media, differentiating it from ATR and AR which occur in high phosphate media. A high phosphate in the medium prevents entry of H^+ into the phosphate specific porin and hence confer acid resistance (Rowbury & Goodson, 1997).

2.8.3 Acid resistance

The most dramatic resistance to acid stress occurs in stationary-phase cells of *E. coli* and *Salmonella flexneri* (Small, and Waterman, 1998; Small, 1998). These systems called Acid Resistance (AR) systems as opposed to log-phase ATR or acid habituation, will protect cells at pH 2 for several hours (Small, Blankenhorn, Welty, & Slonczewski, 1994). Previous work in this area reveals that there are σ^s-dependent and independent systems of acid resistance present in stationary-phase cells. It has also found that three distinct and efficient stationary-phase systems of AR are present in *E. coli*, two of which are also present in *S. Flexneri*.

2.8.3.1 Oxidative system

The oxidative system also known as AR system 1, is RpoS dependent, CRP dependent, and glucose repressed (Castanie-Cornet, Penfound, Smith, Elliott & Foster, 1999). The system protects cells to pH 2.5 in minimal media and was originally thought to be induced by acid pH. It is induced when cells are grown to stationary-phase in Luria Broth and is also called the

oxidative system (Castanie-Cornet *et al*., 1999). At pH 8.0 *E. coli* cultures produce an inhibitor that blocks AR system 1 hence it fails to be induced. AR system 1 also needs activation by a brief exposure to glutamate prior to challenge at pH 2.5 (Castanie-Cornet *et al*., 1999). This is presumed to reflect the need for adequate intracellular glutamate levels for this system to function at extreme low pH. The mechanism by which AR system 1 protects cells requires uncharacterized σ^s and the global regulatory protein CRP- genes (Foster, 2004). The F_0/F_1 proton translocating ATPase is involved but it is unknown whether this complex produces ATP to fuel AR 1 (Richard & Foster, 2003).

2.8.3.2 Glutamate-dependent system

E. coli glutamate-dependent system, also called the acid resistance system 2, requires extracellular glutamate during pH 2 acid challenge (Lin *et al*., 1995; Lin *et al*., 1996). Genetic studies indicate this system requires glutamate decarboxylase, an enzyme that converts intracellular glutamate to γ-amino butyric acid (GABA), and the glutamate/GABA antiporter *gad*C (Castanie-Cornet *et al*., 1999; Hersh, Farooq, Bastard, Blankenshorn & Slonczewski, 1996; Small, & Waterman, 1998). Inducible amino acid decarboxylase systems are thought to provide protection against acid stress through the consumption of intracellular protons. In this model, protons leaking across cell membrane during extreme acid stress can be consumed by amino acid decarboxylation reactions (e.g. glutamate decarboxylase to form GABA). However, to consume protons efficiently there must be a means to transport additional substrate rapidly into the cell and expel the decarboxylation product. This process is carried out by specific substrate/ product antiporters such as the GadC antiporter in the case of the glutamate system. There are two glutamate decarboxylase (GAD) genes in *E. coli* designated *gad*A and *gad*B that map to different chromosomal locations but share 98% identity at the nucleotide level (Smith, Kassam, Singh and Elliott, 1992). The *gad*A gene is monocistronic, but the *gad*B gene forms an operon with *gad*C. Either one of the GAD enzymes will provide pH 2.5 resistance as long as the GadC antiporter is present, but both GAD isoforms are needed to protect the cell at pH 2 (Castanie-Cornet *et al*., 1999). Even though both *gad* transcriptional units respond to stationary-phase and acid pH signals in minimal media, *gad*BC expression is primarily induced by stationary phase while *gad*A responds more to acid pH. This suggests

that GadA is only needed when the cell anticipates, through sensing a progressively acidifying environment, an ultimate encounter with severe acid stress (Castanie-Cornet *et al*., 1999). The glutamate-dependent system was found to provide more protection at pH 2.0 than the other systems and was also reported to be effective in all strains (Lin *et al*., 1996).

2.8.3.3 Arginine-dependent system

The third *E. coli* AR system requires arginine during pH 2 acid challenge (Lin *et al*., 1995). It is an arginine decarboxylase (adiA)-dependent system that appears to function much like the GAD system (Stim & Bennett, 1993). It converts intracellualar arginine to agmatine, consuming a proton in the process. The antiporter for this system has not been identified. Optimum expression of *adi*A occurs at low pH under anaerobic conditions following growth in a complex medium. The positive regulator CysB is required for *adi*A expression and for arginine-dependent acid resistance, although it is probably not the component involved in sensing pH (Lin *et al*., 1996; Shi, X., & Bennett, G.N., 1994). A gene downstream of *adi*A, called *adi*Y, appears to be another positive regulator of *adi*A expression (Stim-Herndon, Flores & Bennett, 1996)

2.8.3.4 *E. coli* reverses membrane potential

Actual measurements of electrical potential have shown that *E. coli* growing at pH 7 in aerobic conditions maintains an electric potential ($\Delta\Psi$) that is negative inside the cell of approximately -90 mV (Richard & Foster, 2004). Under normal conditions, stationary phase cells maintain a $\Delta\Psi$ of about -50 mV but when transferred to pH 2.5, the $\Delta\Psi$ decreases to zero, probably due to a loss of membrane integrity. However, in the presence of glutamate or arginine, *E. coli* flips electrical potential so that the inside of the cell remains positive (Matin, Wilson, Zychlinsky & Matin, 1982). A positive internal charge is assumed to slow proton movement into the cell through repulsion (Matin *et al*., 1982; Zychlinsky & Matin, 1983).

2.9 Alternate sigma factor, RpoS

RpoS is an alterative sigma factor (σ^{s}) which regulates the transcriptional expression of a variety of stress response genes (Hengge-Aronis, 1993; Lange & Hengge-Aronis, 1991; Nguyen, Jensen, Thompson, Gentry & Burgess, 1993), while rpoS is a regulatory gene that is involved in acid resistance. The RpoS protein is the major regulator of the stationary phase or general stress response in *E. coli* and other enteric bacteria (Small *et al.*, 1994; Hengge-Aronis, 1996). Production of RpoS occurs when bacterial cells enter the stationary phase or when subjected to stressful conditions such as weak acids, starvation, high osmolarity and high or low temperature (Lange & Hengge-Aronis, 1994). Exponential phase cells have also been reported to increase levels of σ^{s} when subjected to acidic conditions (Bearson *et al.*, 1996; Lee, Lin, Hall, Beason & Foster, 1995).

Under conditions devoid of any stress, RpoS levels in the cells are low than in stressed conditions. This is attributed to the formation of a stable secondary structure by rpoS mRNA that causes poor translation. Furthermore, RpoS is continuously degraded by clpXP protease under normal conditions in *E. coli* (Schweder, Lomovskaya & Matin, 1996). Cells that produce low levels or defective in rpoS, are acid sensitive since σ^{s} regulates the expression of acid shock proteins (ASP). Lee *et al.,* (1995) and Lin *et al.,* (1995) reported that RpoS is itself an ASP and also controls the expression of eight other ASPs. The induction of RpoS under acidic conditions is controlled by a 38-kDa protein encoded by the mviA gene (Bearson *et al*., 1996; Benjamin, Wu & Swords, 1996).

2.10 Amasi

Amasi is a traditional fermented milk product consumed by the Bantu people of Southern Africa. The quality of Amasi varies with places and season due to differences in fermenting temperatures, fermenting microorganisms and fermenting vessel (Wouters, Ayad, Hugenholtz & Smith, 2001; Kebede, Viljoen, Gadaga, Narvhus & Lourens-Hattingh, 2007). It is consumed by all age groups and has a slightly creamy colour and a sour tangy taste. In South Africa, calabashes were mainly used by the Xhosa and Zulu people to make masi, while the

use of clay pots was popular among the South Sotho People (Fox, 1939). Although fermenting milk in traditional containers is still practiced in rural areas of South Africa, their use has been overtaken by plastic containers and nickel jars which are readily available (Beukes, Bester & Mostert, 2001; Coetzee, Gordeuk, Barnard, Stassen & De Kock, 1996).

Traditional Amasi is produced through fermenting milk spontaneously at ambient temperature for one to four days. According to the method of preparing traditional amasi outlined by Bryant (1949), if Amasi is prepared in a new gourd, milk is poured into the gourd while still warm from the cow. The gourd is placed aside within the hut in winter or outside in the mid-day sun. The fermenting milk develops a watery substance called whey, with the remainder called Amasi. After 24 h, the curdling would have set in and after 48 h, clotting would have commenced although the Amasi is not considered sufficiently ripe for eating at this stage. The whey is allowed to run off through a small hole at the bottom of the gourd after 48 h and the empty space left by the removed whey is filled with new fresh milk from the cow. After 72 h the Amasi is considered ready for eating. The Amasi is mostly poured over the mealie meal (maize flour) porridge called ”*pap”* or drunk straight. To speed the fermentation, backsloping milk with remains of a previous successful fermentation is commonly used in making Amasi (Oliver, 1971). Backsloping is also believed to select for strains that are best for fermentation when continuously used.

Amasi is also produced commercially using Lactococcus lactis subsp. lactis and L. lactis subsp. cremoris, with a shelf life of 21 days at 4 ^{o}C. This permits production of a product with a consistent quality in bigger quantities hence meeting the increasing demand of the product. In South Africa, large-scale production of commercial Amasi is in the form of ‘Maas’ and ‘Inkomasi’ (Keller and Jordaan, 1990). Current research has reported that commercial Amasi may be an ideal vehicle for the delivery of probiotics such as *Bifidobacterium lactis (*Master, Kokott, Slatter, Reid & Abratt, 2008). This is because Amasi is one of the reasonably affordable types of foods that people use to feed their families on a small budget, more especially in the rural areas of South Africa. Amasi is also of great significance for its therapeutic value and it is a source of calcium which is needed by the body for proper development of bones and teeth.

2.11 Survival of *Escherichia coli* O157:H7 in fermented foods

Fermentation, which results in a pH decrease due to the production of organic acids such as lactic acid, has been widely used to ensure safety and keeping quality of fermented foods (Schaack & Marth 1988; Farrag, 1992; Presser, Ratkowsky & Ross, 1997). Other good attributes of fermented milk include pleasant flavours, aroma, texture and improved cooking and processing properties (Holzapfel, 2000). Several studies have demonstrated that *E. coli* O157:H7 can survive in a variety of fermented foods, with acid adapted strains, surviving even better than non-adapted strains. The implication of fermented foods in the outbreaks of food-borne diseases caused by *E. coli* O157:H7 has drawn attention of researchers to intensify their investigation on the acid resistance of this microorganism.

Acid adaptation, which is acquired after exposure of bacterial cells to mild acidic conditions, enhances survival of microorganisms in fermented food. During meat fermentation, adapted cells showed an increased survival compared to non-adapated cells. NA cell populations decreased to 120 cells/g, while adapted cell populations decreased to only 5 x 10^3 cells/g (Leyer *et al.,* 1995). Adapted cells also exhibited better survival in shredded hard salami than NA cells (Leyer *et al.,* 1995).

Tsegaye & Ashenafi (2005) demonstrated that *E. coli* O157:H7 can survive during processing and storage of Ergo, a traditional Ethiopian sour milk. In milk co-inoculated with LAB, initial ($\log_{10}$ 6 cfu/ml) *E. coli* O157:H7 counts grew to $\log_{10}$ 6.5 cfu/ml after 24 h, with levels decreasing to $\log_{10}$ 3.2 cfu/ml after 72 h. Post-fermentation inoculation of *E. coli* O157:H7 resulted in complete elimination of the initial population of $\log_{10}$ 6 cfu/ml after 36 h at ambient temperature while at refrigeration temperatures, 2.2 - 3.3 $\log_{10}$ cfu/ml were detectable after 72 h. Survival of *E. coli* during fermentation was attributed to development of acid resistance during fermentation while the low survival of test strain, at ambient temperature when inoculated after fermentation, was attributed to failure to develop acid resistance and to the high level of antimicrobial substances at inoculation. Lack of activity of both LAB and *E. coli* O157:H7 at refrigeration temperature was thought to be reason for the low reduction in counts under refrigeration. *E. coli* O157:H7 also survived during the fermentation and storage of

yoghurt at 4 ºC for 7 days (Massa, Altieri, Quaranta & De Pace, 1996). *E. coli* counts reduced from 7.4 to 5.4 $\log_{10}$ in yoghurt made with starter cultures plus *Bifidobacterium bifidum* bacteria, whereas in yoghurt made normal starter cultures, *E. coli* counts reduced from 7.1 to 5.3 $\log_{10}$ cfu/ml.

Several studies have demonstrated that low temperature enhances the survival *E. coli* O157:H7 at low pH (Clavero & Beuchat 1996; Cheng & Kaspar, 1998; Faith, Parniere, Larson, Lorang & Kaspar, 1998; Bachrouri *et al*., 2002). This is due to that low pH increases the proportion of undissociated acid present and these traverse the plasma membrane into the higher pH of the cytoplasm, where they dissociate, acidifying the cytoplasm and release acid anions. Accumulation of acid anions in the cell disrupts intracellular processes (Russel, 1992). To survive low temperature, bacteria increases the proportion of usaturated fatty acids in the cell hence preventing formation of gel-like fluids in normally fluid components (Russel, Evans, terSteeg, Hellemons, Verheul & Abee, 1995; Berry and Foegeding, 1997). Gel-like fluids hinder proper functioning of proteins and results in bacterial membrane leakage. Production of cold shock proteins is another mechanism used by bacteria to survive low pH by bacteria (Jones & Inouye, 1994). The exact function of cold shock proteins is not known but it is assumed that they assist to overcome the partial block in protein synthesis by binding to the RNA during trascription and facilitate initiation of translation (Jones & Inouye, 1994).

In contrast, Feseru & Nyati, (1990) reported that *E. coli* survived better at 20 ºC than at 5 ºC in Lacto, a Zimbabwean fermented milk. *E. coli* counts could not be detected after 24 h at 5 ºC storage whereas only one or two $\log_{10}$ cfu/ml reduction in counts was observed in *E. coli* stored at 20 ºC.

2.12 Hypotheses

The hypotheses of the study are as follows:

2.12.1 If non- adapted strains of *E. coli* are subjected to acidic conditions, they will adapt to the acidic conditions because low pH induces a decrease in the percentage of unsaturated fatty acid and an increase in the percentage of saturated fatty acids, thereby increasing membrane rigidity and reducing proton permeability into the cell (Brown *et al*., 1997).

2.12.2 If non- adapted strains and acid adapted strains of *Escherichia coli* are exposed to acidic conditions, the acid adapted strains will show increased acid tolerance in fermented goat milk products because they activate acid resistance systems. Therefore, acid adapted *Escherichia coli* strains will survive and grow better in acidic conditions such as fermented goat milk products than non-adapted strains.

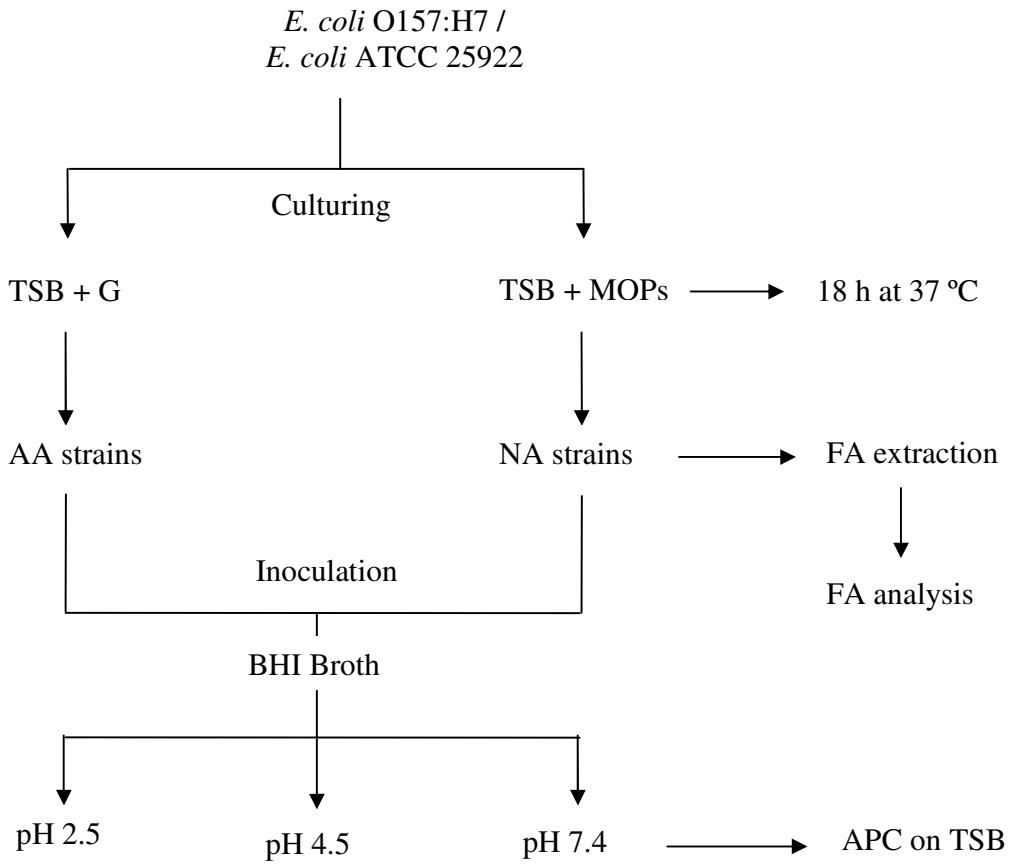
2.13 Objectives

The objectives of the study are:

2.13.1 To determine the effect of acid adaptation on the survival and growth of *E. coli* O157:H7 and *E. coli* ATCC 25922 in Brain Heart Infusion broth and on the fatty acid profile of acid adapted *E. coli* strains.

2.13.2 To determine the survival and growth of AA *E. coli* O157:H7 in traditional and commercial Amasi

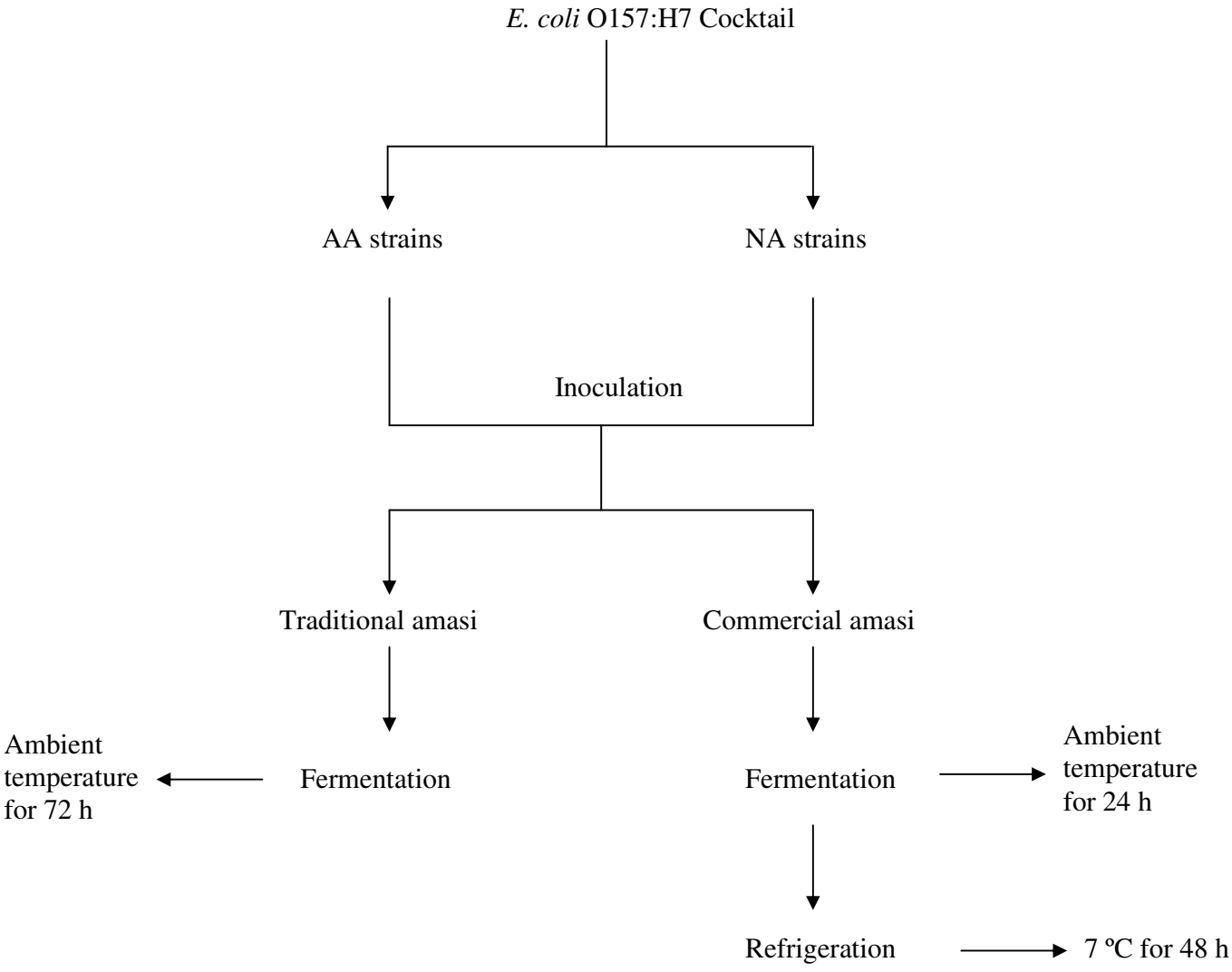
PHASE 1: ACID ADAPTED *ESCHERICHIA COLI* STRAINS IN BROTH



Legend

TSB: Tripticase Soy Broth
G: Glucose
AA: Acid adapted
NA: Non-adapted
FA: Fatty acid
BHI: Brain Heart Infusion
APC: Aerobic plate count

PHASE II: ACID ADAPTED *ESCHERICHIA COLI* IN TRADITIONAL AND COMMERCIAL AMASI



CHAPTER 3: RESEARCH

3.1 SURVIVAL AND GROWTH OF ACID ADAPTED *ESCHERICHIA COLI* STRAINS IN BROTH AT DIFFERENT pH LEVELS

ABSTRACT

Acid resistance of *Escherichia coli* O157:H7 strain UT 10 and *Escherichia coli* ATCC 25922 was determined in brain heart infusion broth at pH 7.4, 4.5 and 2.5. Variations due to acid stress in the counts of both strains were also determined. Acid adaptation enhanced the survival of both strains at pH 4.5, but neither strain could survive after 4 h at pH 2.5. At optimum growth conditions (pH 7.4), *E. coli* ATCC 25922 exhibited increased viability over *E. coli* UT 10. At pH 4.5, *E. coli* UT 10 was more tolerant to low pH than *E. coli* ATCC 25922. An increase in saturated fatty acids (SAFAs) of both AA strains was observed, indicating the importance of lipid modification in enhancing survival at low pH. The results of this study indicated that the food industry should therefore adapt their processing/preservation procedures by taking the most acid tolerant pathogenic *E. coli* strains into consideration in order to ensure the safety of their products.

Keywords: Acid adaptation, *E. coli* O157:H7, Broth

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3.1.1 Introduction

The preservation of food by acidification has always been regarded as a "safe" procedure, since low pH and organic acids prevent the growth of contaminating organisms (Glass, Loeffelholz, Ford & Doyle, 1992). However, current research has revealed that microorganisms have developed strategies to adapt to and tolerate acid stress (Gordon & Small, 1993; Lin *et al*., 1996). Acid adaptation does not only prolong the survival of pathogenic microorganisms such as *E. coli* O157:H7 in food, but also renders questionable, the effectiveness of fermentation as a processing procedure currently employed to preserve food. The implication of low pH foods, such as yoghurt, in food-borne disease outbreaks is attributed to this phenomenon (Morgan, Newman, Hutchinson, Walker, Rowe & Majid, 1993). Prior exposure of bacterial cells to sub-lethal pH has been reported to induce resistance to subsequent low pH conditions (Goodson & Rowbury, 1989; Buchanan & Edelson, 1996). Acid adapted *E. coli* can survive even lower pH values such as pH 2.5 for 2h, which represents an average situation in the human stomach (Gordon & Small, 1993). Extensive studies on acid adaptation have been done on *E. coli, Salmonella* and *Shigella*, but differences in the type of medium, experimental conditions, bacterial strain as well as the stage of growth, makes it difficult to compare results between laboratories (Bearson, Bearson & Foster,1997).

E. coli O157:H7 has received more attention of researchers, not only because of its low infectious dose, but also due to its outstanding ability to survive at low pH (Tuttle, Gomez, Doyle, Wells, Zhao, Tauxe & Griffin, 1999; Peterson *et al*., 1989). Acid tolerance in all *E. coli* strains is pH dependent and can be induced in log-phase or stationary-phase cells, with the latter providing more acid tolerance (Buchanan & Doyle, 1997). Three distinct stationary-phase, low-pH induced acid resistance systems have been identified in *E. coli* (Audia *et al*., 2001; Castanie-Cornet *et al*., 1999; Bearson *et al*., 1997; Lin *et al*., 1995). Two of these systems involve inducible amino acid decarboxylases, hence they are aptly termed the arginine and glutamate acid resistance systems. The other system, known as the oxidative system, is repressed by glucose and once induced, protects cells at low pH in the absence of amino acids in the medium. The amino acid decarboxylase systems maintain internal pH by consuming protons that leak into the cell during decarboxylation, in exchange for a new substrate via a

membrane bound antiporter, while the manner in which the oxidative system coffers acid resistance is unknown (Bearson *et al*., 1997; Audia *et al*., 2001).

In conjunction with the afore-mentioned systems, cells are also reported to alter their membrane fatty acid profile during acid perturbations (Jordan *et al*., 1999; Russel *et al*., 1995). They convert monounsaturated fatty acids to saturated fatty acids and this increases membrane rigidity and reduces inflow of protons into the cell (Brown *et al*., 1997 and Yuk & Marshall 2004).

The aim of this study was to determine the effect of acid adaptation on the survival and growth of a strain of *E. coli* O157:H7 and *E. coli* ATCC 25922 after 16 h in Brain Heart Infusion Broth at pH 7.4, 4.5 and 2.5.

3.1.2 Materials and methods

3.1.2.1 Survey of goat milk for *E. coli* strains

Raw goat milk was collected from six farms around Pretoria, RSA and each farm was sampled twice for the isolation of *E. coli* serotypes

3.1.2.1.1 Isolation of *Escherichia coli* for serotyping

Raw goat milk samples (25 ml) were enriched for 18 hours at 37 ^{0}C in 225 ml MacConkey broth (Oxoid) (Restaino, Frampton and Spitz, 2001; Flores and Stewart, 2004). Serial dilutions (1:10) were made in 0.1% Buffered Peptone Water (BPW) (Oxoid) followed by selective and differential spread plating in duplicate on chromocult coliform agar (Merck Chemicals, Darmstadt, Germany). The plates were incubated at 37 ^{0}C for 24 hours.

3.1.2.1.2 Serotyping

Serotyping is the method of determining the presence of specific antigens in a microorganism. It consists of adding a suspension of the organisms to anti-serum, which contains antibodies that are specific for the known antigens. If the antigens are present, the antibodies in the antiserum will combine with the antigens, causing agglutination or clumping, of the bacterial cells (Benson, 1985).

Typical *E. coli* colonies (5 from each plate) were selected for determination of serological groups. Each of the presumptive *E. coli* colonies were streaked on MacConkey agar (Oxoid) and incubated for 18 hours at 37 ^{0}C. The isolates were further streaked on nutrient agar (Biolab Diagnostics, Midrand, South Africa) and incubated for 18 hours at 37^{0}C. Resultant colonies were serotyped according to the slide agglutination test for pathogenic *E. coli* with Mast Assure™ pathogenic *E. coli* 'O' antisera (Mast Diagnostics, Bootle, UK). The antisera used are classified according to their virotypes as shown in Table 2 below.

Table 4. Classification of the antisera used for serotyping according to their serotypes

EPEC	ETEC	EIEC	VTEC	EAggEC
O111	-	O112	O111	O86
O142		O124	O157	O111
O114				
O119				
O125				
O126				
O26				
O44				
O55				

The morphological results from all farms were positive, however, when these were identified serologically, they were all negative. This meant that the *E. coli* identified morphologically could be commensal strains or other serogroups not tested for. Other *E. coli* strains were then used for the subsequent studies as mentioned below.

3.1.2.2 Microorganisms for Acid Resistance

E. coli O157:H7 UT 10 obtained from the Agricultural Research Council, Onderstepoort Veterinary Institute, South Africa and *E. coli* ATCC 25922 obtained from Agricultural Research Council, Irene, South Africa were used in the study. All strains were cultured in Tryptone Soy Broth (TSB) from Oxoid for 24 hours at 37 ^{0}C and then stored at 4 ^{0}C. These working stock cultures were subcultured on a bimonthly basis.

3.1.2.3 Preparation of inoculum

Acid adapted and non-adapted *E. coli* were obtained in the stationary-phase by following the procedure outlined by Buchanan and Edelson (1996). The working stock cultures (1 ml) were inoculated into 100 ml of either TSB supplemented with glucose (Associated Chemical Enterprises, Glenvista, South Africa) to a concentration of 10 g/l (1%) (TSB+G) and to pH 4.6 or TSB without glucose (TSB-G) buffered with 100 mM MOPS, pH 6.5-7.9 (Sigma-Aldrich Chemie, Steinheim, Germany) and incubated for 18 hours at 37 ^{0}C. The former served as the acid adapted strain whereas the latter served as the non-adapted strain.

3.1.2.4 Acid Challenge

The ability of *E. coli* strains to survive acid stress was determined by a method similar to that described Buchanan and Edelson (1996). The Brain Heat Infusion (BHI) broth (Oxoid) was prepared, adjusted to either pH 2.5 or 4.5 with 2M lactic acid (Merck) and distributed in 10 ml portions to approximately 48 McCartney bottles. BHI at pH 7.4 buffered with 100 mM MOPS was also prepared, distributed into bottles to be used as control. The pH of BHI from

representative tubes was tested after sterilization to verify that any pH changes resulting from autoclaving were $\leq$0.1 pH unit.

Sets of eight bottles containing pH-adjusted BHI were inoculated with 0.1 ml of starter culture of one of the two strains. This resulted in the initial level of the *E. coli* strains of approximately 10^6 CFU/ml. Two bottles were immediately assayed for viable counts as described below, while the remaining bottles were incubated at 37 ^{0}C. At intervals up to 16 h, six bottles per strain were removed and assayed. The pH remained constant throughout the acid challenge.

3.1.2.5 Enumeration of *E. coli*

The acid tolerance of individual cultures (acid adapted and non-adapted) was assessed by the examination of population viability during the acid challenge. The contents of the BHI were serially diluted by transferring 1 ml portions to sterile 9 ml dilution blanks of 0.1% BPW at various stages throughout the acid challenge. Samples (0.1ml) were surface plated on duplicate Tryptone Soy Agar (TSA) from Biolab (Biolab Diagnostics, Wadeville, South Africa) and incubated at 37 ^{0}C for 24 hours after which bacteria were enumerated.

3.1.2.6 Fatty acid extraction

The modified one-phase $CHCl_3$MeOH-H_2O Bligh and Dyer method (Bligh and Dyer, 1959) was used for quantitative lipid extraction. Volumes (100 ml) of acid adapted and non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922 cultures were prepared as described above. The cells were harvested by centrifuging with a Medifringer 7000600 centrifuge (P-Selecta, Barcelona, Spain) at 1 166 g for 45 minutes. The cells were then homogenized for 2 minutes in a stomacher Lab-Blender 400 with a mixture of 10 ml chloroform (Saarchem, Midrand, South Africa) and 20 ml methanol (Saarchem, Midrand, South Africa). To the mixture, 10 ml of chloroform was added followed by blending in a stomacher for 30 seconds, after which 10 ml of distilled water was also added and blending was continued for another 30 seconds. The homogenate was then filtered through Whatman No. 1 filter paper (Whatman International,

Maidstone, UK) in a funnel. To ensure maximum recovery of solvent, pressure was applied with the bottom of a beaker. The filtrate was transferred to a graduated cylinder (50 ml) and a few minutes were allowed for complete separation and clarification. The volume of the chloroform layer, containing the purified lipid, was recorded and the alcoholic layer was removed by aspiration.

3.1.2.6.1 Gas Chromatography analysis of fatty acid methyl esters

Gas chromatography was performed at the Wildlife Sciences (University of Pretoria, South Africa) on a Varian 3300 gas chromatograph equipment (Varian Associates Inc. 1985, United States of America). Samples were injected at 140ºC in the splitless mode with a 0.60 minute relay time. A final temperature of 240ºC was attained at 1.5ºC per minute and maintained for 15 minutes. Helium was used as a carrier gas and the injector and detector were both maintained at 240ºC. Peak areas were quantified using Empower build 1154 program 2002.

3.1.2.7 Statistical analysis

Analysis of variance (ANOVA) was used to determine whether the following factors namely, acid adaptation (AA, NA), strain (*E. coli* UT 10, *E. coli* ATCC 25922), pH (pH 7.4, 4.5, 2.5) and time (16 hours), affected the survival and growth of the *E. coli* strains significantly (95% confidence interval). All samples were analyzed in duplicate and each experiment was repeated three times (n=6). ANOVA was performed using Statistica software for windows version 7 (Tulsa, Oklohama, USA, 2003)

3.1.3 Results

3.1.3.1 Acid Resistance of Acid Adapted and Non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922

There was a significant strain difference ($p \leq 0.05$) between *E. coli* UT 10 and *E. coli* ATCC 25922 when grown at pH 7.4 for 16 h (Table 5). *E. coli* UT 10 counts increased with 1.8 $\log_{10}$ after 16 h, while *E. coli* ATCC 25922 counts increased with 4.2 $\log_{10}$ (Fig. 2). A significant effect ($p \leq 0.05$) was also observed between acid adapted (AA) and non-adapted (NA) *E. coli* UT 10 and *E. coli* ATCC 25922 when grown for 16 h at pH 7.4 (Table 5). However, this difference was only 0.25 $\log_{10}$ cfu/ml and microbiologically not of practical significance, because it was less than 1 $\log_{10}$.

AA *E. coli* UT 10 counts increased from 5.9 to 7.7 $\log_{10}$ cfu/ml after 4 h of exposure at pH 7.4 and then remained unchanged until 16 h, whereas those of the NA strain increased from 6.4 to 7.9 $\log_{10}$ cfu/ml after 4 h of exposure and then remained unchanged (Fig. 2). The 16 h time of exposure had a significant effect ($p \leq 0.05$) on the growth of both strains at pH 7.4. The counts of AA *E. coli* ATCC 25922 increased from 6.1 to 10.4 $\log_{10}$ cfu/ml after 8 h of exposure at pH 7.4 and remained unchanged until 16 h, with the NA strain increasing from 6.7 to 10.1 $\log_{10}$ cfu/ml after 8 h of exposure and remaining unchanged thereafter (Fig. 2).

Table 5. Statistical analyses of the survival and growth of acid adapted and non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922 after 16 h in brain heart infusion broth at pH 7.4.

TREATMENT	***Degrees of freedom***	***P value***
Strain (*E. coli* UT 10 , *E. coli* ATCC 25922)	1	0.000
Acid adaptation (acid adapted, non-adapted)	1	0.029
Time (16 hours)	3	0.000
Strain*Acid adaptation	1	0.722
Strain*Time	3	0.000
Acid adaptation*Time	3	0.315
Strain*Acid adaptation*Time	3	0.922

Similar to pH 7.4, when challenged at pH 4.5 for 16 h, a significant strain difference ($p \leq 0.05$) was also observed between *E. coli* UT 10 and *E. coli* ATCC 25922, as well as a significant difference ($p \leq 0.05$) between AA and NA *E. coli* UT 10 and *E. coli* ATCC 25922 when challenged for 16 h at pH 4.5 (Table 6). *E. coli* UT 10 counts decreased with 0.6 $\log_{10}$, while *E. coli* ATCC 25922 counts decreased by 3.9 $\log_{10}$ after 16 h (Fig. 2).

Table 6. Statistical analyses of the survival and growth of acid adapted and non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922 after 16 h in brain heart infusion broth acidified to pH 4.5 with lactic acid.

TREATMENT	***Degrees of freedom***	***P Value***
Strain (*E. coli* UT 10, *E. coli* ATCC 25922)	1	0.000
Acid adaptation (acid adapted, non-adapted)	1	0.000
Time (16 hours)	3	0.000
Strain*Acid adaptation	1	0.000
Strain*Time	3	0.000
Acid adaptation*Time	3	0.000
Strain*Acid adaptation*Time	3	0.000

AA counts of *E. coli* UT 10 increased from 5.8 to 6.1 $\log_{10}$ cfu/ml after 16 h and NA counts remained unchanged from the initial count of 6.2 $\log_{10}$ cfu/ml after 8 h of exposure, followed by a decrease to 4.9 $\log_{10}$ cfu/ml after 16 h of acid challenge (Fig. 2). The counts of AA *E. coli* ATCC 25922 decreased from 6.1 to 5.1 $\log_{10}$ cfu/ml and NA counts were not detected after 16 h of exposure (Fig.2). Both AA and NA *E. coli* UT 10 counts were stable for 8 h after inoculation, followed by a decrease of 1.4 $\log_{10}$ cfu/ml on the NA strain after 16 h. AA counts of *E. coli* ATCC 25922 decreased by 0.9 $\log_{10}$ cfu/ml after 8 h of acid challenge and remained unchanged until 16 h, while its NA strains depicted a continuous decline in counts until 16 h (Fig. 2).

Table 7. Statistical analyses of the survival and growth of acid adapted and non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922 after 16 h in brain heart infusion broth acidified to pH 2.5 with lactic acid.

TREATMENT	***Degrees of freedom***	***P Value***
Strain (*E. coli* UT 10 , *E. coli* ATCC 25922)	1	0.272
Acid adaptation (acid adapted, non-adapted)	1	0.000
Time (16 hours)	3	0.000
Strain*Acid adaptation	1	0.161
Strain*Time	3	0.299
Acid adaptation*Time	3	0.000
Strain*Acid adaptation*Time	3	0.119

There was no significant strain difference ($p \leq 0.05$) in count between *E. coli* UT 10 and *E. coli* ATCC 25922 when challenged for 16 h at pH 2.5 (Table 7). *E. coli* UT 10 decreased with 6.3 $\log_{10}$ after 4 h at pH 2.5, while *E. coli* ATCC 25922 decreased by 6.2 $\log_{10}$ (Fig. 2). A significant difference ($p \leq 0.05$) was observed between AA and NA *E. coli* UT 10 and *E. coli* ATCC 25922 with time of exposure at pH 2.5 (Table 7). However, this difference was only 0.2 $\log_{10}$ cfu/ml hence it was considered microbiologically not significant. The counts of both strains fell to undetectable levels after 4 h of exposure. The initial counts of AA and NA *E.*

coli UT 10 were 5.8 and 6.8 $\log_{10}$ cfu/ml, while those of *E. coli* ATCC 25922 were 5.8 and 6.5 $\log_{10}$ cfu/ml, respectively.

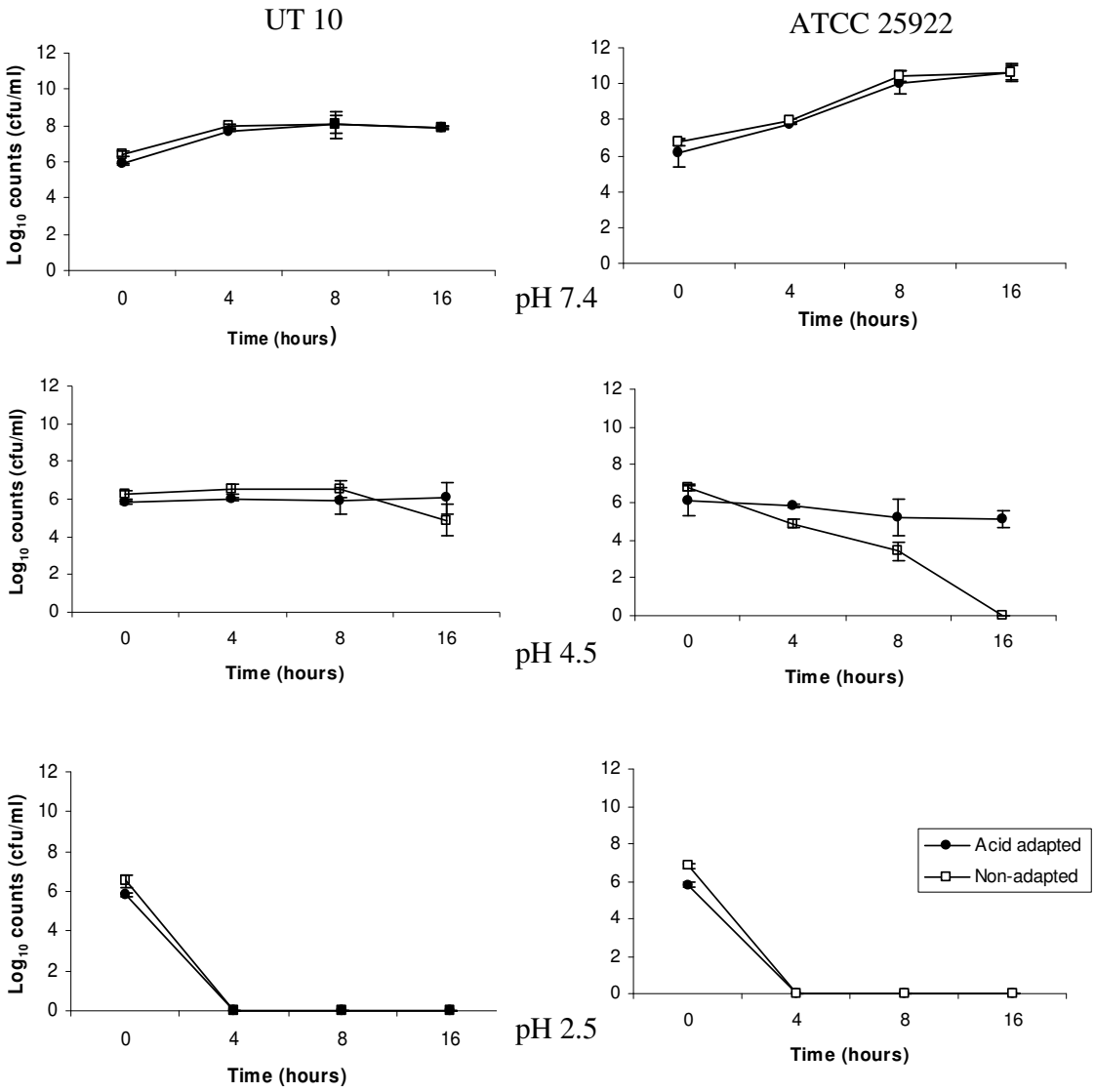

Figure 2. Survival of acid adapted and non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922 after 16 h in brain heart infusion broth at pH 7.4, 4.5 and 2.5.

3.1.3.2 Membrane Fatty Acid Composition of Acid Adapted and Non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922

Regardless of bacterial strain, acid challenge at pH 4.5 for 2 h resulted in an increase in the level of saturated fatty acids (SAFAs), coupled with a decrease in monounsaturated fatty acids (MUFAs) (Table 8). Both *E. coli* UT 10 and *E. coli* ATCC 25922 showed an increase of 13.1% in SAFAs after being challenged at pH 4.5 for 2 h. MUFAs decreased by 19.9 and 13.9% for *E. coli* UT 10 and *E. coli* ATCC 25922, respectively, after the same period. The same trend was observed with AA and NA strains of *E. coli* UT 10 and *E. coli* ATCC 25922 after growth at pH 7.4 and 4.5 for 6 h and 2 h, respectively (Table 8). Palmitic acid was the most abundant fatty acid at 60.6 and 60.2 % for *E. coli* UT 10 and *E. coli* ATCC 25922, respectively, after 2 h at pH 4.5, while its levels at pH 7.4 were 54.8 and 47.12 % after 6 h for *E. coli* UT 10 and *E. coli* ATCC 25922, respectively. Palmitoleic acid was the most sensitive fatty acid at pH 4.5 as it decreased to 18.9 and 14.5% in both *E. coli* UT 10 and *E. coli* ATCC 25922 after 2 h. *E. coli* ATCC 25922 showed high levels of stearic acid at pH 4.5 after 2 h while it was not detected in *E. coli* UT 10 after the same period (Table 8).

Table 8. Changes in the fatty acid profile of acid adapted and non-adapted *E. coli* UT 10 and *E. coli* ATCC 25922 after 2 h and 6 h in brain heart infusion broth at pH 4.5 and 7.4, respectively.

Fatty Acid	pH 4.5				pH 7.4			
	E. coli UT 10		*E. coli* ATC25922		*E. coli* UT 10		*E. coli* ATC25922	
	AA	NA	AA	NA	AA	NA	AA	NA
8:0	ND	ND	ND	ND	ND	ND	21.52	ND
10:0	1.63	ND	ND	ND	ND	ND	6.34	ND
12:0	2.74	2.58	2.55	3.09	3.85	2.15	1.80	4.41
14:0	16.55	13.63	13.23	14.02	17.22	14.12	9.74	15.26
15:0	ND	ND	ND	0.81	ND	ND	ND	ND
16:0	62.93	60.31	54.36	66.02	55.67	53.88	41.04	53.20
17:0	0.09	0.62	0.2	0.25	0.23	ND	0.52	0.44
18:0	3.59	6.12	20.32	2.90	6.13	4.12	3.55	4.26
20:0	0.07	0.55	0.11	0.04	0.23	ND	0.64	0.24
24:0	0.15	0.23	0.16	0.12	ND	0.46	ND	ND
Σ SATFAs	**87.75**	**84.04**	**90.93**	**87.25**	**83.33**	**74.73**	**85.15**	**77.81**
14:1	0.27	ND	0.31	0.45	0.25	ND	0.15	0.21
16:1	2.72	3.33	1.54	4.48	2.06	21.62	12.00	16.02
18:1n9t	0.06	ND	0.06	ND	0.13	1.39	0.58	0.14
18:1n9c	ND	1.66	1.5	0.78	2.28	ND	ND	0.75
20:1	0.07	ND	0.06	ND	0.13	ND	0.09	0.28
Σ MUFAs	**3.12**	**4.99**	**3.47**	**5.71**	**4.85**	**23.01**	**12.82**	**17.4**
18:2n6t	0.03	ND	ND	ND	ND	ND	ND	ND
18:2n6c	8.08	8.88	4.76	6.39	10.24	1.62	0.89	3.12
trans 10,cis12-18:2 CLA	ND	0.34	0.05	0.08	0.14	ND	ND	0.13
20:2	ND	0.28	0.03		0.10	ND	0.20	0.08
20:3n6	1.01	1.46	0.75	0.51	1.36	0.63	0.84	1.47
Other FAs	**9.12**	**10.96**	**5.59**	**6.98**	**11.84**	**2.25**	**1.93**	**4.8**
Total FAME	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

NB: ND – Not detected

3.1.4 Discussion

Acid adaptation enhanced the survival of *E. coli* UT 10 and *E. coli* ATCC 25922 at pH 4.5, but neither strain could survive a 4 h challenge at pH 2.5. At pH 7.4, there was no significant difference ($p \leq 0.05$) between AA and NA *E. coli* UT 10 or *E. coli* ATCC 25922 after 16h. Adaptation to acidic conditions was accompanied by a change in fatty acid profile for both strains, with AA strains showing a greater increase in the percentage of SAFAs than MUFAs.

There was a significant ($p \leq 0.05$) strain difference between *E. coli* UT 10 and *E. coli* ATCC 25922 at pH 7.4. *E. coli* UT 10 reached the stationary-phase earlier than *E. coli* ATCC 25922. Hence a significant ($p \leq 0.05$) interaction between time and strain was observed. This difference can be attributed to the differences in growth rates between the two *E. coli* strains. Similar to pH 7.4, a significant ($p \leq 0.05$) strain effect was also observed at pH 4.5, with *E. coli* UT 10 exhibiting a better survival rate than *E. coli* ATCC 25922. The observed difference in acid stress response of the two strains was in line with results reported by Benjamin & Datta (1995); Duffy *et al*. (2000) and Mckellar & Knight (1999). They found that the behavior of *E. coli* cells under acidic conditions varied among the strains of pathogenic *E. coli* tested and among pathogenic and non-pathogenic *E. coli*. Furthermore, Besser *et al*. (1993), Arnold & Kasper (1995) and McIngrale *et al*. (2000) demonstrated that *E. coli* O157:H7 had a high level of acid tolerance compared to other *E. coli* strains. Although *E. coli* UT 10 was more acid tolerant than *E. coli* ATCC 25922, this was not reflected in the fatty acid profile. *E. coli* ATCC 25922 had a high amount of SAFAs, which is usually associated with increased acid resistance, both under optimal growth conditions and also after acid challenge. This indicates that lipid modification is not the only mechanism that enhances survival of *E. coli* under acidic conditions. The activation of other acid resistance systems such as the amino acid decarboxylase systems and the accumulation of RpoS protein (Bearson *et al*., 1996), in addition to lipid modification, are important for *E. coli* survival at low pH.

When challenged at pH 2.5, the strain effect was not significant ($p \leq 0.05$) in either *E. coli* UT 10 or *E. coli* ATCC 25922 since neither strain could survive after 4 h of exposure. Similar to the results of this study, Sainz, Perez, Villaseca, Hernandez, Eslava, endoza & Wacher

(2005) discovered that *E. coli* UT 10 could not survive after one hour of exposure in Luria-Bertani broth acidified to pH 2.5 with HCl. De Jonge, Takumi, Ritmeester & Van Leusden (2003) also reported that stationary-phase AA cultures of *E. coli* O157 only survived for 2 h in Luria-Bertani broth acidified to pH 2.5, compared to log-phase cultures which could not survive after half an hour of exposure at the same pH. The failure of the strains to survive at pH 2.5 suggested that although they could be moderately acid tolerant they would not survive the pH levels in the human stomach. Contrary to the findings in the present study, Buchanan & Edelson (1996) reported that the *E. coli* O157:H7 strains and AA *E. coli* ATCC 25922 used in their study could survive for 7 h in BHI broth acidified to pH 2.5 with HCl. Benjamin & Datta (1995) also noted that some of the *E. coli* O157:H7 strains tested by them, showed no loss of viability for at least 5 h at pH 2.5.

At pH 4.5, acid tolerance levels of AA and NA strains of *E. coli* UT 10 were found to be similar for 8 h, after which the NA strain showed a decline of 1.7 $\log_{10}$ cfu/ml after 16 h. The observed similarity in the level of survival during the early stages could be attributed to the use of stationary-phase cells for acid challenge. Stationary-phase cells are associated with the expression of acid resistance systems, such as the accumulation of RpoS which regulates the expression of acid tolerance genes in *E. coli* and other enteric bacteria (Fang, Libby, Buchmeier, Loewen, Switala, Harwood & Guincy, 1992; Bearson *et al*. 1997). The reduction in counts of the NA strain after 16 h show an increased level of acid resistance of AA strains, which is acquired through exposure of bacterial cells to mild acidic conditions prior to acid challenge, as elucidated by Goodson & Rowbury (1989). The increased level of acid tolerance following acid adaptation has been reported in both exponential-phase cells as well as in stationary-phase cells, with the latter showing a higher level of acid resistance than the former (Arnold & Kasper, 1995; Benjamin & Datta, 1995). Similar to *E. coli* UT 10, acid adaptation also had a significant effect ($p \leq 0.05$) on *E. coli* ATCC 25922 at pH 4.5.

Underscoring that acid adaptation enhanced survival at pH 4.5, an increase in SAFAs of acid adapted strains at the expense of MUFAs was observed. According to Brown *et al*. (1997), *E. coli* cells convert unsaturated fatty acids to either cyclopropane fatty acids or SAFAs under acid stress. The augmentation of SAFAs in acid challenged cells is associated with increased

membrane rigidity, which in turn, reduces proton permeability into the cell. The correlation between acid tolerance and decreased permeability of the cell envelope has also been reported by Jordan *et al*. (1999). Brown *et al*. (1997) and Yuk & Marshall (2004) reported a similar change in the fatty acid profile of *E. coli* when subjected to low pH. The observed reduction in the percentage of palmitoleic acid in both strains at pH 4.5 suggests that it might be this specific MUFA that is mostly converted to SAFAs under acidic conditions.

The counts of AA *E. coli* ATCC 25922 decreased after inoculation and then remained unchanged from 8 h to 16 h at pH 4.5. This could be due to the presence of a subpopulation of bacteria that was more acid resistant. Sainz *et al*. (2005) also observed a similar effect on AA *E. coli* O157:H7 after a 6 h acid challenge at pH 4.0.

When inoculated into BHI broth at pH 7.4, both AA and NA strains of *E. coli* UT 10 and *E. coli* ATCC 25922 demonstrated a similar pattern of growth. As a result acid adaptation did not have a significant effect ($p \leq 0.05$) in terms of strain and time of exposure. Firstly, this could be due to the fact that pH 7.4 is the optimum growth for *E. coli* hence both AA and NA strains exhibited optimal growth. Secondly, the acid adaptative ability could have been lost when the cells were subjected to optimum growth conditions. Similar results on the loss of acid tolerance upon resumption of growth under optimum conditions were reported by Jordan *et al.* (1999). They indicated that the physiological changes that occur during growth could be responsible for the loss of acid tolerance in acid adapted cells. Schweder e*t al*. (1996) reported that the RpoS is continuously degraded by the ClpXP protease in *E. coli* under normal non-stress conditions.

3.1.5 Conclusion

Acid adaptation enhanced survival of both *E. coli* strains at pH 4.5, with *E. coli* UT 10 exhibiting a better survival rate than *E. coli* ATCC 25922, indicating a significant strain effect. Both AA and NA *E. coli* UT 10 survived to a greater extent than their *E. coli* ATCC 25922 counterparts at pH 4.5. At pH 7.4, *E. coli* ATCC 25922 showed increased viability than *E. coli* UT 10, but there was no significant difference between AA and NA counts for either strain.

The observed increase in SAFAs in both AA strains indicates that lipid modification is important in enhancing survival at low pH. These results confirm that AA and NA *E. coli* UT 10 as well as AA *E. coli* ATCC 25922 could survive in fermented foods contaminated with these organisms. The results from this study indicate that the food industry should therefore adapt their processing/preservation procedures by taking the most acid tolerant pathogenic *E. coli* strains into consideration in order to ensure the safety of their products.

3.2 ADAPTATION OF *ESCHERICHIA COLI* O157:H7 TO ACID IN TRADITIONAL AND COMMERCIAL GOAT MILK AMASI

Abstract

Acid resistance of *E. coli* O157:H7 strains UT 10 and UT 15 were determined in traditional Amasi fermented for 3 days at ambient temperature (*ca* 30 ºC) and commercial Amasi fermented at 30 ºC for 24 h and stored at 7 ºC for 2 days. *E. coli* O157:H7 counts in commercial Amasi were detected at 2.7 $\log_{10}$ cfu/ml after 3 days while those in traditional Amasi could not be detected after the same period. There was no significant difference ($p \leq 0.05$) in the survival of acid adapted (AA) and non-adapted (NA) *E. coli* O157:H7 in traditional Amasi, while in commercial Amasi, the NA strain survived significantly ($p \leq 0.05$) better than its AA counterpart. Regardless of prior adaptation to acid, *E. coli* O157:H7 can survive during fermentation and storage of fermented goat milk Amasi. Also, the fermentation time, pH and storage temperature affects the survival of *E. coli* O157:H7 in the fermented milk.

Keywords: Acid adaptation, *E. coli* O157:H7, Goat milk, Amasi, fermented milk

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3.2.1 Introduction

Amasi is a traditional fermented milk product consumed in all parts of South Africa. Wide variations in taste have been reported to be caused by the use of different fermenting pots such as clay pots, calabashes and gouards (Kebede *et al*., 2007). Fermentation temperatures and fermenting microflora also contribute to different tastes. Traditionally, Amasi is produced from raw milk that ferments naturally at ambient temperature (Bryant, 1949). However, due to the ever escalating population size, and the advent of commercial starter cultures, commercial Amasi is now produced from pasteurized milk, under a controlled processing conditions. Large-scale commercial production of traditional fermented milk in South Africa is in the form of Amasi also known as 'Maas' and 'Inkomasi' (Keller and Jordaan, 1990). Numerous types of lactic acid bacteria (LAB) and other microorganisms such as yeasts have been cited to be responsible for fermentation in Amasi (Beukes *et al*., 2001; Kebede *et al*., 2007). Among the fermenting pots used to make fermented milk traditional Amasi in South Africa, Kebede *et al*. (2007) reported that those made of clay provided a greater diversity of yeasts. This is believed to influence the characteristics of the end product, hence the focus on their use as starter cultures (Fleet, 1990; Jakobsen & Narvhus, 1996; Loretan *et al*., 1998).

The inhibitory effect of fermented foods on contaminating organisms is attributed to the production of antimicrobial compounds and the reduction of pH (Gulmez & Guven, 2003). Lactic acid bacteria are the major producers of inhibitory metabolites in fermented foods. Fermented milk products were found to be more effective in reducing the growth of acid adapted (AA) *E. coli* O157:H7 than acidic fruit juices (Hsin-Yi & Chou, 2001). Of concern, however, is the implication of fermented foods such as yoghurt in food-borne outbreaks caused by *E. coli* O157:H7. Several studies have also demonstrated that AA *E. coli* O157:H7 may survive during processing and storage of fermented dairy foods such as lactic cheese and Ergo (Vernozy-Rozand *et al*., 2005; Tsegaye & Ashenafi, 2005). Refrigeration has also been reported to increase the survival of AA *E. coli* O157:H7 in acidic foods (Cheng & Kaspar, 1998; Clavero & Beuchat, 1996; Faith *et al*., 1998). While the behaviour of most food-borne pathogens has been investigated in other fermented foods, no study has been undertaken to

study the adaptation of *E. coli* O157:H7 in Amasi. The aim of the study was to assess the survival and growth of AA *E. coli* O157:H7 in traditional and commercial Amasi.

3.2.2 Materials and methods

3.2.2.1. *E. coli* serotypes

A cocktail of *E. coli* O157:H7 strains, UT 10 and UT 15, obtained from the Agricultural Research Council, Onderstepoort Veterinary Institute, Pretoria, South Africa, were used in this study. The strains were cultured in Tryptone Soy Broth (TSB) from Oxoid for 24 hours at 37 ^{0}C and then stored at 4 ^{0}C. The stock cultures were sub-cultured through at least two 24 h incubation cycles in fresh broth medium before use in experiments.

3.2.2.2. Preparation of Acid Adapted *E. coli* strains

Acid adapted and non-adapted *E. coli* were prepared to the stationary-phase by following the procedure outlined by Buchanan & Edelson (1996). The working stock cultures (1 ml) were inoculated into 100 ml of either TSB supplemented with glucose (Associated Chemical Enterprises, Glenvista, South Africa) to a concentration of 10 g/l (1%) and to pH 4.6 (TSB+G) or TSB without glucose (TSB-G) buffered with 100 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), pH 7.4 (Sigma-Aldrich Chemie, Steinheim, Germany) and incubated for 18 hours at 37 ^{0}C. The former served as the acid adapted strain whereas the latter served as the non-adapted strain.

3.2.2.3 Processing of commercial Amasi and enumeration of lactic acid bacteria and *E. coli* test strains

Raw goat milk was sourced from the University of Pretoria (UP) experimental farm (Pretoria, South Africa). Commercial Amasi was prepared using a method that purely simulates the processing of commercial Amasi (Figure 4). Pasteurized goat milk was inoculated with a commercial LAB starter culture of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis subsp. cremoris* (MO 0.30) (Sacco, Cadorago, Italy) to give an initial inoculum level of 10^6

cfu/ml. Skim milk (3 %) and gelatine (0.5 %) (Davis, Gauteng, South Africa) were added before pasteurization to stabilize the product. *E. coli* test strains (AA and NA) were inoculated at a level of 10^6 cfu/ml into separate screw cap bottles when the pH of the Amasi reached 5.6. Inoculation was done at pH 5.6 to prevent the non-adapted strain from adapting. After 24 h of incubation at 30 ºC, the samples were kept at 7 ºC for 48 h. Enumeration of *E. coli* and LAB was done after inoculation at days 0, 1, 2, and 3. Samples (1 ml) were serially diluted in 9 ml of 0.1 % Buffered Peptone Water (BPW) and appropriate dilutions were surface plated onto Sorbitol MacConkey agar (SMAC) (Oxoid) for *E. coli* and de Man, Rogosa and Sharp (MRS) agar (Merck) for LAB. SMAC plates were incubated at 37 ºC for 24 h while MRS agar plates were incubated anaerobically at 37 ºC for 48 h.

3.2.2.4 Preparation of starter culture for traditional Amasi production

The inoculum used for making traditional Amasi was isolated from traditional fermented milk sourced in Botswana. The traditional fermented milk inoculum was chilled and transported to the UP. Upon arrival at the laboratory, the total plate count, *E. coli* counts, yeasts and moulds count, titratable acidity and pH of the fermented milk was determined. The traditional fermented milk inoculum was further tested for the presence of pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* and coliforms, following the methods outlined in 3.2.2.5 below. The fermented milk was then put in 90 mm Petri dishes (Merck), frozen to – 20 ºC, freeze-dried (Instruvac lyophilizer, model 13KL) and then stored at – 20 ºC. Before inoculation into being used for the manufacture of the traditional Amasi, the traditional fermented milk inoculum was activated by placing 10 g of freeze dried fermented milk in 90 ml sterile skim milk and incubating at 30 ºC for 24 h.

3.2.2.5 Detection of pathogens in the original traditional fermented milk inoculum

Coliforms and faecal *E. coli* were detected by using the Most Probable Number (MPN) method (Canadian Health Products and Food Branch, MFHPB-19) by inoculating 10 ml of the sample into 5 tubes of double strength Lauryl Sulfate Tryptose (LST) broth (Oxoid) with Durham tubes and by inoculating 1 ml or 0.1 ml of the sample into 5 tubes of single strength

LST broth (10 ml) with Durham tubes. The tubes were incubated for 24 h at 35 ºC. On LST positive tubes, a loopful from the LST positive tubes was transferred to Brilliant Green Lactose 2 % Bile Broth (BGLB) (Oxoid) tubes (10 ml) which were incubated at 35 ºC for 24 h. A loopful from the BGLB positive tubes was transferred into Enterococcosel (EC) broth (Oxoid) tubes (10 ml) which were incubated in a water-bath at 45 ºC for 24 h. A loopful from EC positive tubes was streaked onto Eosins methylene Blue agar (EMB) (Oxoid) plates which were subsequently incubated at 35 ºC for 18-24 h and typical colonies counted. Gas production from BGLB broth confirmed the presence of coliforms while gas production from EC broth confirmed the presence of faecal *E. coli*. *Listeria monocytogenes* was detected using the International Standard Organization (ISO) 11290-1 method, by inoculating 1 ml of the sample into 9 ml half strength Frazer *Listeria* selective enrichment (Oxoid) and incubating the suspension at 30 ºC for 24 h. After incubation, 0.1 ml of the culture was transferred into a 10 ml tube with full strength Frazer and incubated at 35 ºC for 48 h. From the half Frazer or full Frazer culture, a loop of the culture was plated on Oxford agar (Oxoid) or PALCAM agar (Oxoid) and incubated micro-aerobically at 35 ºC for 24 h. Typical colonies were further streaked on Tryptone Soy Agar plates, incubated at 35 ºC for 18-24 h and confirmed with Gram strain (Harrigan & McCance, 1966) and catalase test (Harrigan & McCance, 1966).

Salmonella was detected using the South African Bureau of Standards (SABS) ISO 6579:1993 method, by transferring 0.1 ml of pre-enriched (BPW) sample to a tube containing 10 ml *Salmonella* enrichment broth, Rappaport and Vassiliadis (RVS) (Merck) and by transferring 10 ml of the pre-enriched broth to a bottle containing 100 ml Selenite cystine medium (Oxoid). The tubes containing enrichment broth and Selenite cystine medium were incubated at 42 ºC for 24 h and 35 ºC for 24 h, respectively. A loopful from the *Salmonella* enrichment broth and Selenite cystine medium was streaked onto pre-dried Phenol Red/Brilliant Green Agar (Oxoid) or XLD agar (Oxoid) and incubated at 35 ºC for 20-24 h.

Staphylococcus aureus was detected using the ISO 6888-1 method, by transferring 0.1 ml of the sample to Baird-Parker medium (Merck) plates supplemented with Egg-yolk Tellurite Emulsion (Oxoid) solution. The plates were allowed to dry for 15 minutes and incubated for

24 h at 35 ºC. Typical colonies which are black or grey, were enumerated and confirmed with coagulase test (Harrigan & McCance, 1966).

Faecal enterococci was detected using the South African National Standard (SANS) 7899-2:2004 method, by plating 0.1 ml of the sample on Slanetz and Bartley medium (Oxoid) supplement with triphenyltetrazolium chloride solution. The plates were then incubated at 35 ºC for 44 h and typical colonies (red or maroon) after incubation were further streaked on Bile Esculin Azide (Oxoid) plates and incubated at 44 ºC for 2 h. All colonies showing a tan to black colour after the incubation for 2 h were regarded positive and counted.

3.2.2.6 Processing of traditional Amasi and enumeration of LAB and *E. coli* test strains

Amasi was prepared in hand-made pots that are traditionally used to make Amasi (Figure 3). Two clay pots were purchased from an informal market at Bosman station, Pretoria, South Africa. The clay pots were conditioned by fermenting 3 successive batches of raw goat milk sourced from the University of Pretoria experimental farm (Pretoria, South Africa) in the pots. This was done by allowing raw goat milk to ferment using the freeze dried fermented milk described in section 2.4, as an inoculum, at ambient temperatures for three days, after which, the fermented milk was discarded and fresh milk added for the next fermentation.

Traditional Amasi was prepared following modification of the method outlined by Bryant (1949) (Figure 4). It was prepared in two hand-made clay pots at the dairy factory of the University of Pretoria, South Africa. The clay pots were filled raw goat milk sourced from the University of Pretoria experimental farm (Pretoria, South Africa) and inoculated with a fermented milk inoculum with an initial level of 10^6 cfu/ml LAB. The milk was allowed to ferment naturally at ambient temperature (*ca* 30 ºC) for 3 days. Inoculation of the product with *E. coli* test strains was done after 6 h, when the pH reached 5.6.

Enumeration of *E. coli* and LAB was done at days 0, 1, 2, and 3 using the same method detailed in 3.2.2.3. SMAC was used for *E. coli* while MRS agar and M17 (Oxoid) agar were used for enumeration of LAB. MRS plates were incubated anaerobically using the anaerocult

system (Merck, Darmstadt, Germany) at 37 ºC for 48 h for the enumeration of mesophilic lactobacilli and leuconostocs and at 42 ºC for the enumeration of thermophilic lactobacilli and streptococci while SMAC plates were incubated aerobically for 24 h at 37 ºC. The M17 agar plates were incubated aerobically for 48 h at 30 ºC for the enumeration of lactococci. Total viable counts were performed using Plate Count Agar (Merck) incubated at 25 ºC for 24 h. Yeast and moulds were enumerated using Yeasts and Moulds Petrifilm (Microbiology products, St. Lous, USA). The petrifilms were inoculated with 1 ml of serially diluted sample and incubated at 25 ºC for 24 h. At each sampling time, the pH (Sentron, Gig Harbor, USA) and titratable acidity was also determined by the titration method using NaOH (0.1 mol/l) in the presence of phenolphthalein.

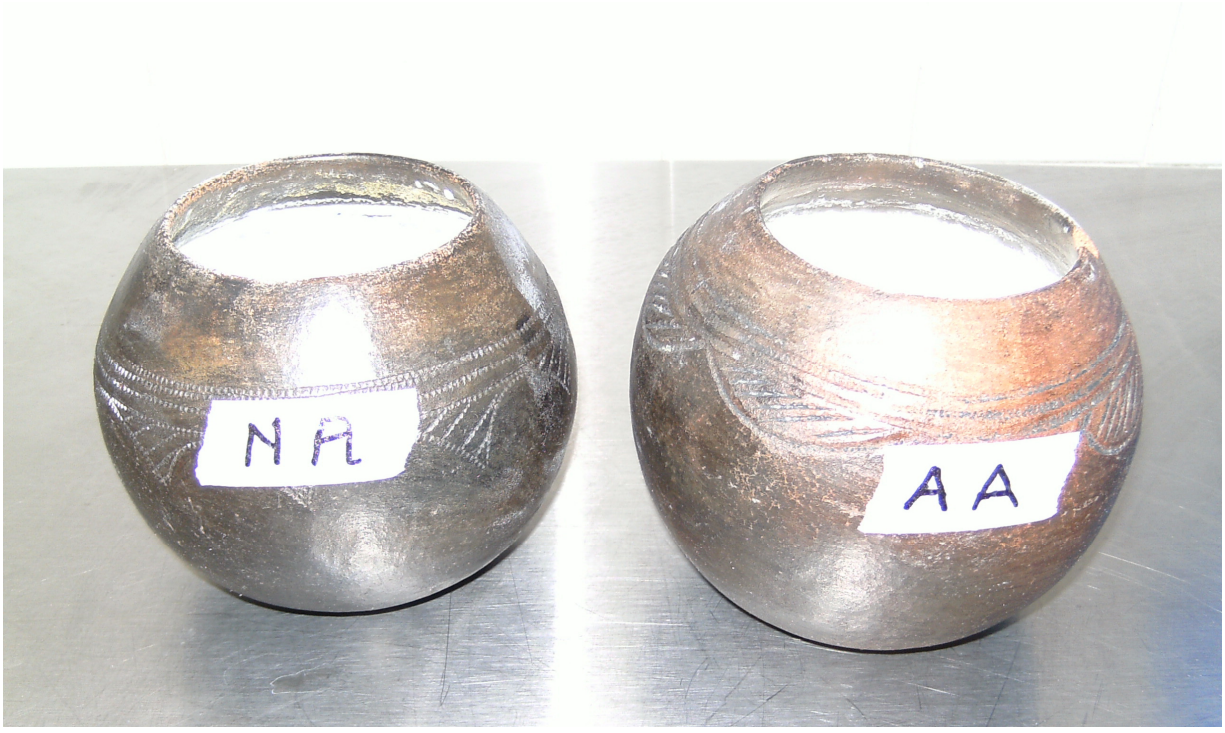

Figure 3. Traditional clay pots with traditional Amasi inoculated with acid adapted (AA) or non-adapted (NA) *Escherichia coli* O157:H7

3.2.2.7 Statistical analysis

Analysis of variance (ANOVA) was used to determine whether factors such as acid adaptation (AA, NA) and time (3 days), affected the survival and growth of the bacteria significantly (95% confidence levels). All samples were analyzed in duplicate and each experiment was repeated three times (n=6). ANOVA was performed using Statistica software for windows (Tulsa, Oklohama, USA, 2003).

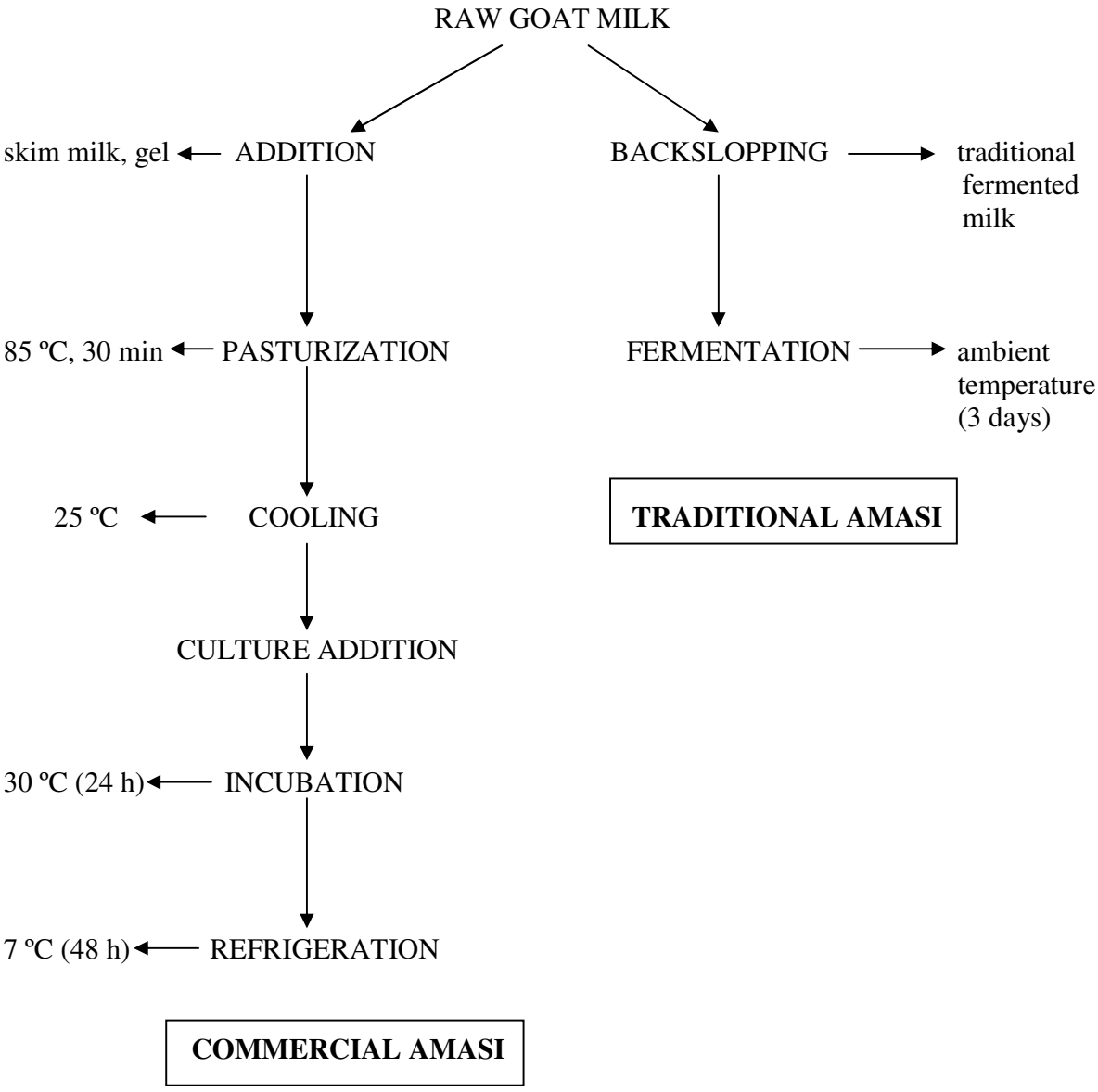

Figure 4. Methodology for the production of commercial and traditional Amasi products

3.2.3 Results

3.2.3.1 Enumeration of the microbial population and detection of potential pathogens in the traditional fermented milk

The fermented milk had total aerobic counts of 5.8 $\log_{10}$ cfu/ml, LAB counts of 6.4 $\log_{10}$ cfu/ml as well as a yeast and mould count of 4.1 $\log_{10}$ cfu/ml (data not shown). Presumptive *Listeria monocytogenes* was detected in the traditionally fermented milk at 3.2 $\log_{10}$ cfu/ml. *Salmonella*, coliforms, *Staphylococcus aureus, E. coli* and faecal enterococci were not detected. The original fermented milk had a pH of 4.0 while its titratable acidity (T.A) was 1.4 %.

3.2.3.2 Enumeration and characterization of the microbial population and detection of the AA and NA *E. coli* in traditional Amasi

The presence of adapted bacteria had a significant effect ($p \leq 0.00$) on the total aerobic counts (TAC) in traditional Amasi over 3 days (Table 9). TAC in the clay pot inoculated with AA *E. coli* O157:H7 decreased with 2.3 $\log_{10}$ cfu/ml while counts in the clay pot inoculated with NA *E. coli* O157:H7 decreased with 1.4 $\log_{10}$ cfu/ml after 3 days (Fig. 5). TAC increased from 7.5 $\log_{10}$ cfu/ml to 8 $\log_{10}$ cfu/ml after 1 day followed by a decrease to 5.2 $\log_{10}$ cfu/ml in the clay pot inoculated with AA *E. coli* O157:H7, while a count of 7.9 $\log_{10}$ cfu/ml was maintained after one day followed by a decline to 6.4 $\log_{10}$ cfu/ml in the clay pot inoculated with NA *E. coli* O157:H7 (Fig. 5).

Yeast and mould counts in the traditional Amasi clay pot inoculated with AA *E. coli* O157:H7 were not significantly different ($p \leq 0.73$) from those in the NA *E. coli* O157:H7 inoculated clay pot (Table 9). Yeast and mould counts in the AA *E. coli* O157:H7 inoculated clay pot increased from 4.5 $\log_{10}$ cfu/ml to 6.2 $\log_{10}$ cfu/ml after 3 days, while those in the NA *E. coli* O157:H7 inoculated clay pot increased from 4.5 $\log_{10}$ cfu/ml at day 0 to 6.4 $\log_{10}$ cfu/ml after 1 day followed by a decrease to 5.4 $\log_{10}$ cfu/ml after 3 days (Fig 5).

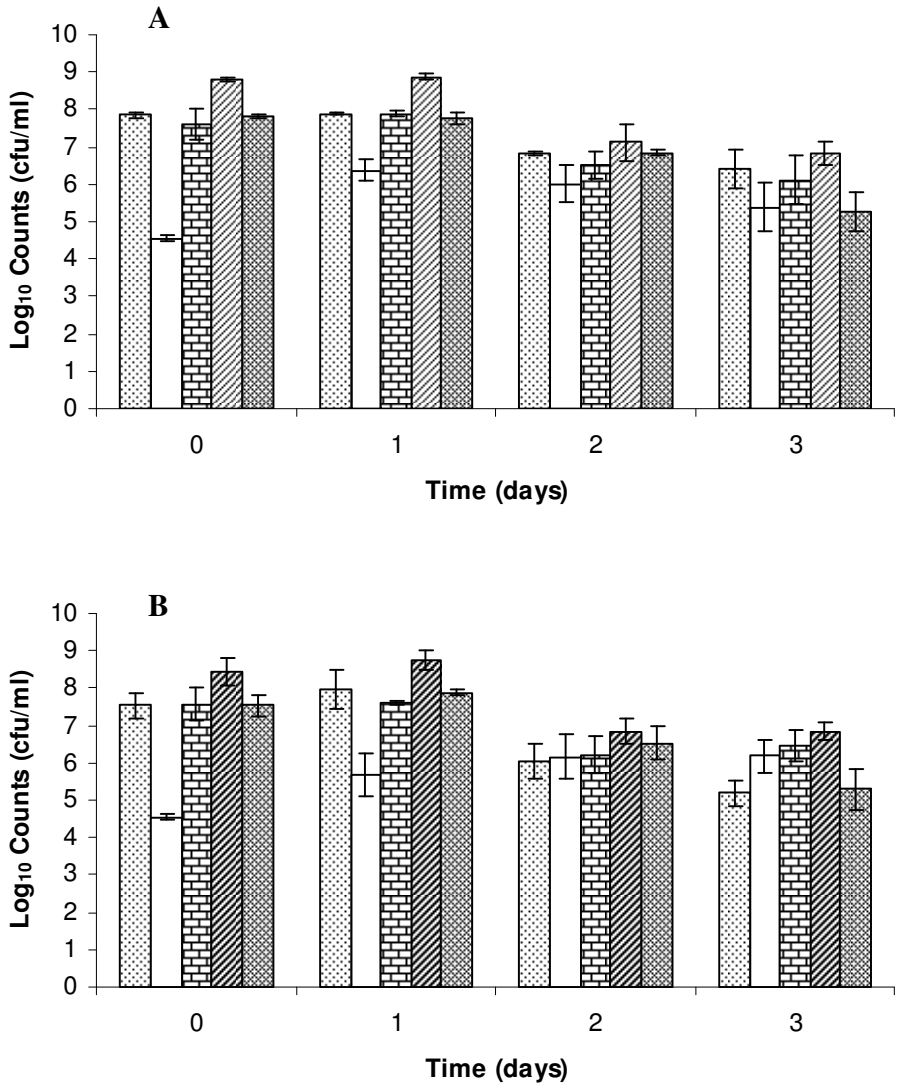

Figure 5. Total aerobic count, ⬚ yeasts and moulds counts □ , Mesophilic LAB counts on MRS agar ▨, lactococci counts ⊞ and thermophilic LAB ▩ in traditional Amasi clay pots inoculated with acid adapted *E. coli* O157:H7 (A) and non-adapted *E. coli* O157:H7 (B) (n=3).

Similar to the yeasts and moulds, survival of mesophilic or thermophilic LAB in the clay pot inoculated with AA *E. coli* O157:H7 did not differ significantly ($p \leq 0.15$) from that in the clay pot inoculated with NA *E. coli* O157:H7 (Table 9). Mesophilic counts in the clay pot inoculated with AA *E. coli* O157:H7 increased from 8.4 $\log_{10}$ cfu/ml to 8.8 $\log_{10}$ cfu/ml after 1 day followed by a decrease to 6.8 $\log_{10}$ cfu/ml after 3 days, while those from the NA *E. coli* O157:H7 inoculated clay pot increased from 8.8 $\log_{10}$ cfu/ml at day 0 to 8.9 $\log_{10}$ cfu/ml after

1 day followed by a decrease to 6.8 $\log_{10}$ cfu/ml after 3 days (Fig. 5). Thermophilic LAB counts in the clay pot inoculated with AA *E. coli* O157:H7 increased from 7.5 at day 0 to 7.9 $\log_{10}$ cfu/ml after 1 day followed by a decrease to 5.3 $\log_{10}$ cfu/ml after 3 days. Those in the NA *E. coli* O157:H7 clay pot increased from 7.6 at day 0 to 7.8 $\log_{10}$ cfu/ml after 1 day followed by a decrease to 5.3 $\log_{10}$ cfu/ml after 3 days (Fig. 5).

Lactoccoccus counts decreased from 7.6 $\log_{10}$ cfu/ml to 6.5 $\log_{10}$ cfu/ml and from 7.6 to 6.1 $\log_{10}$ cfu/ml after 3 days in the clay pots inoculated with AA or NA *E. coli* O157:H7, respectively (Fig. 1). The survival of lactoccocci in the AA *E. coli* inoculated clay pot did not differ significantly ($p \leq 0.05$) from that in the NA *E. coli* O157:H7 inoculated clay pot (Table 9).

Table 9. Statistical analysis of total aerobic counts, yeasts and moulds, lactococci, mesophilic and thermophilic lactic acid bacteria (LAB) from traditional Amasi after 3 days at ambient temperature (n=3).

		P value				
TREATMENT	***Degrees of freedom***	***Total aerobic count***	***Yeasts and Moulds***	***Lactococci***	***Mesophilic LAB***	***Thermophilic LAB***
Acid-adaptation (AA, NA)	1	0.001	0.731	0.723	0.149	0.441
Time (3 d)	3	0.000	0.000	0.000	0.000	0.000
Time*Acid-adaptation	3	0.029	0.074	0.509	0.696	0.621

In general, the survival of yeasts and moulds, lactococci, mesophilic and thermophilic LAB in the clay pot inoculated with AA *E. coli* O157:H7 was not significantly ($p \leq 0.05$) different from that in the clay pot inoculated with NA *E. coli* O157:H7 (Table 9). However, the 3 days time of challenge had a significant effect ($p \leq 0.00$) on the survival of the counts of all microorganisms.

Reduction in pH was similar in both traditional Amasi clay pots inoculated with AA or NA *E. coli* O157:H7 (Table 10). The pH dropped from 5.6 to 4.1 and from 5.6 to 4.0 in the clay pots inoculated with AA and NA *E. coli* O157:H7, respectively over the three day period (Table 10).

Table 10. Changes in pH of traditional Amasi at ambient temperature and commercial Amasi at ambient temperature for 24 h and refrigeration at 7 ºC until 3 days, both inoculated with acid adapted or non-adapted *E. coli* O157:H7. Values are means and standard deviations (n=3).

Time	Traditional Amasi		Commercial Amasi	
	AA (±SD)	NA (±SD)	AA (±SD)	NA (±SD)
0	5.6 (0.0)	5.6 (0.0)	5.6 (0.0)	5.6 (0.0)
1	4.4 (0.0)	4.4 (0.1)	4.7 (0.1)	4.7 (0.1)
2	4.2 (0.0)	4.2 (0.0)	4.6 (0.1)	4.4 (0.0)
3	4.1 (0.1)	4.0 (0.0)	4.4 (0.0)	4.4 (0.0)

3.2.3.3 Enumeration of LAB in commercial Amasi

There was no significant difference ($p \leq 0.52$) between LAB counts in both the AA and NA *E. coli* O157:H7 inoculated in commercial Amasi. LAB counts in commercial Amasi inoculated with AA and NA *E. coli* O157:H7 increased from 7.0 $\log_{10}$ cfu/ml to 8.1 $\log_{10}$ cfu/ml and from 7.7 $\log_{10}$ cfu/ml to 8.4 $\log_{10}$ cfu/ml after 2 days and decreased to 7.9 $\log_{10}$ cfu/ml and 7.3 $\log_{10}$ cfu/ml, respectively, after 3 days (data not shown). Reduction in pH was similar in both AA and NA *E. coli* O157:H7 (Table 10). The initial pH of 5.6 dropped to pH 4.7 after 24 h and was further reduced to pH 4.4 after 3 days (Table 10).

3.2.3.4 Acid resistance of acid adapted and non-adapted *E. coli* O157:H7 in traditional and commercial Amasi products

Acid adaptation had a significant effect ($p \leq 0.04$) on *E. coli* O157:H7 counts in traditional and commercial Amasi (Table 11). However, the observed overall significant difference ($p \leq 0.04$) in traditional Amasi was only 0.4 $\log_{10}$ cfu/ml. AA and NA *E. coli* O157:H7 counts in traditional Amasi decreased from 6.8 $\log_{10}$ cfu/ml to 3.5 $\log_{10}$ cfu/ml and from 6.4 $\log_{10}$ cfu/ml to 3.2 $\log_{10}$ cfu/ml, respectively, after 2 days, and were not detected after the third day (Fig. 6). A 1 $\log_{10}$ cfu/ml and 0.3 $\log_{10}$ cfu/ml difference was observed in traditional Amasi after 1 and 2 days, between AA and NA *E. coli* O157:H7, respectively, with the AA strain showing better survival. Neither strains could however be detected after 3 days, indicating that there was no significant difference ($p \leq 0.09$) in their survival over the full period of 3 days.

Similar to what was observed in traditional Amasi, acid adaptation had a significant effect ($p \leq 0.04$) on *E. coli* O157:H7 counts in commercial Amasi (Table 11). AA *E. coli* O157:H7 counts in commercial Amasi decreased from 6.6 $\log_{10}$ cfu/ml to 2.2 $\log_{10}$ cfu/ml after over 2 days, followed by an increase to 2.4 $\log_{10}$ cfu/ml after 3 days (Fig. 6). A continuous decline, from 6.7 $\log_{10}$ cfu/ml to 3.0 $\log_{10}$ cfu/ml, was observed for NA *E. coli* O157:H7 counts in commercial Amasi over 3 days (Fig. 6). There was also a significant ($p \leq 0.04$) interaction between Amasi, time and acid adaptation (Table 11).

A significant difference ($p \leq 0.00$) existed between *E. coli* O157:H7 counts in commercial Amasi and traditional Amasi after 3 days (Table 11). *E. coli* O157:H7 counts in traditional Amasi decreased significantly by 6.6 $\log_{10}$ cycles after 3 days, while only a 2.5 $\log_{10}$ reduction in *E. coli* O157:H7 counts was observed in commercial Amasi after the same period (Fig. 6). A significant difference ($p \leq 0.00$) was also observed between Amasi and acid adaptation as well as with time (Table 11).

Table 11. Statistical analyses of the survival and growth of acid adapted and non-adapted *E. coli* O157:H7 inoculated at pH 5.6 after 3 days in traditional and commercial Amasi products (n=3).

TREATMENT	*Degrees freedom*	*P value*
Amasi (traditional, commercial)	1	0.003
Acid-adaptation (AA, NA)	1	0.044
Time (3 d)	3	0.000
Amasi*Acid-adaptation	1	0.000
Amasi*Time	3	0.000
Acid-adaptation*Time	3	0.092
Amasi*Acid-adaptation*Time	3	0.022

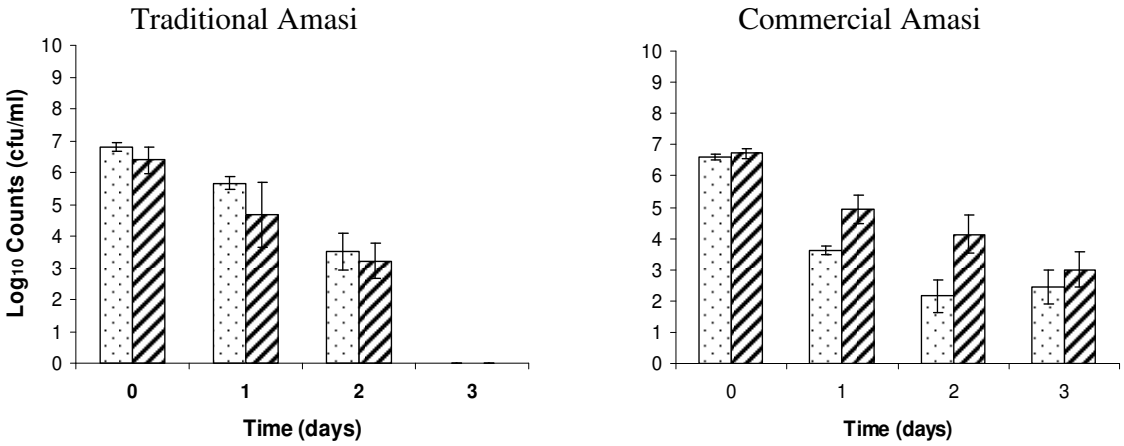

Figure 6. Survival of acid adapted ⬚ and non-adapted ▨ *E. coli* O157:H7 in traditional Amasi at ambient temperature for 3 days and commercial Amasi incubated at 30 ºC for 24 h and then refrigerated at 7 ºC until 3 days (n=3).

3.2.4 Discussion

3.2.4.1 Enumeration and characterisation of microorganisms in the traditional fermented milk

LAB in the traditional fermented milk had the highest counts (6.4 $\log_{10}$ cfu/ml) when compared to total aerobic counts (5.8 $\log_{10}$ cfu/ml) and yeast and mould counts (4.1 $\log_{10}$ cfu/ml). Other traditional fermented milks have, however, been reported to have even higher LAB counts (8 $\log_{10}$ cfu/ml) and total aerobic counts that range from 8 -9 $\log_{10}$ cfu/ml (Beukes *et al*., 2001; Gran *et al*., 2003). In the study conducted by Beukes *et al.* (2001), fermented milk samples were collected in the cooler months (May – August) which presumably favoured the growth of LAB while in the current study, the traditional fermented milk inoculum was collected in November when the temperature was warmer. The difference in LAB counts in the fermented products could also be due to the differences in growth of LAB strains. The presence of presumptive *Listeria* species in the traditional fermented milk inoculum indicates that *Listeria* may survive in traditional fermented milk. *Listeria monocytogenes*, in particular, has been reported to develop acid resistance and survives at pH 3.5, when pre-exposed to mild pH values of pH 5 to 6 (Foster, 2000). Gulmez and Guven (2003) also reported that *Listeria monocytogenes* may survive in different yoghurt and kefir combinations when contamination occurs after pasteurization. In South Africa, the sale of dairy products containing pathogenic organisms is not allowed (South Africa, 1997). However, the traditional fermented milk is made for home consumption. Since *Listeria monocytogenes* is a pathogen that causes disease in pregnant woman, the very young or elderly and in people with immunocompromised systems, the presence of *Listeria* in the traditional fermented milk inoculum warrants further investigation (Adams & Moss, 2000).

3.2.4.2 Enumeration and characterization of microorganisms in traditional Amasi

Yeasts and moulds, which were initially regarded as a sign of poor hygiene, have received more attention in the dairy industry, more so, because of their potential use as starter cultures (Jakobsen & Narvhus, 1996; Loretan *et al*., 1998). Yeast and mould counts in traditional

Amasi increased from 4.5 $\log_{10}$ cfu/ml to 5.8 $\log_{10}$ cfu/ml over 3 days. The high initial yeast and mould count was attributed to the traditional fermented milk inoculum that had yeast and mould count of 4.1 $\log_{10}$ cfu/ml. Initial yeast counts of 3.8 $\log_{10}$ cfu/ml were reported in all traditional containers used to ferment milk in South Africa, with clay pots having a wide diversity of yeasts (Kebede *et al*., 2007). Similar to these results, Gadaga *et al*. (2000) reported that traditional fermented milk from Zimbabwe had yeast and mould counts ranging from <3 to 5.7 $\log_{10}$ cfu/g with average yeast counts of 5 $\log_{10}$ cfu/ml. Yeast counts of 6.0 $\log_{10}$ cfu/ml were also reported by Isono *et al*. (1994) in Tanzanian fermented milk.

Mesophilic LAB in the present study had the highest counts in traditional Amasi reaching 8.9 $\log_{10}$ cfu/ml after 1 day when compared to thermophilic and lactococci LAB which had counts of 7.9 $\log_{10}$ cfu/ml and 7.8 $\log_{10}$ cfu/ml, respectively, after the same period. Similar to with the results of this study, Beukes *et al*. (2001) reported mean LAB counts of 8 $\log_{10}$ cfu/ml in South African traditional fermented milks. In their findings, mesophilic LAB as well as lactococci bacteria had the highest counts, while in the present study, only mesophilic LAB dominated. Differences in experimental conditions such as different fermentation temperatures could have resulted in this difference. A similar decline, with regard to microbial population, was observed after 2 days in all the microorganisms except for yeasts and moulds. This could be due to the interaction of microorganism with each other and depletion of nutrients. Gadaga *et al.* (2000) also noted a decrease in the final population of yeasts in Zimbabwean fermented milk. The reduction in counts after 2 days also corresponded with reduction in pH which reached pH 4.2 in both clay pots inoculated with AA and NA *E. coli* O157:H7. Low pH increases the proportion of undissociated acid which traverses through plasma membrane, dissociates and acidifies the cytoplasm (Russel, 1992). This decreases cellular energy available to support growth functions hence disrupting intracellular processes.

3.2.4.3 Acid resistance of acid adapted and non-adapted *E. coli* O157:H7 in traditional and commercial Amasi products

E. coli O157:H7 in commercial Amasi survived to a greater extent than in traditional Amasi, as *E. coli* O157:H7 counts in the latter could not be detected after three days of challenge. This could firstly be attributed to the lower pH of traditional Amasi (pH 4.0) after 3 days compared to that of commercial Amasi (pH 4.4). Low external pH in bacteria leads to reduction in internal pH, which subsequently reduces the activity of acid sensitive enzymes, damages proteins and DNA in cells (Adams & Moss, 2000). Secondly, the higher initial level (8.4 $\log_{10}$ cfu/ml) of LAB in traditional Amasi compared to that in commercial Amasi (7.4 $\log_{10}$ cfu/ml) could have had more detrimental effects on *E. coli* O157:H7 in traditional Amasi. LAB are widely known for producing secondary metabolites such as bacteriocins or other antimicrobials that act against the growth of several spoilage and pathogenic bacteria (Bankole & Okagbue, 1992; Adams & Nicolaides, 1997; Borregaard and Arneborg, 1998). The traditional fermented milk inoculum could have also contained antimicrobial properties that aided reduction of *E. coli* O157:H7 in the product. The presence of yeasts (4.1 $\log_{10}$ cfu/ml) in the traditional fermented milk inoculum used for making traditional Amasi could have also contributed to the production of more secondary metabolites. Some yeasts have been reported to produce killer toxins that can kill sensitive bacteria (Polonelli & Morace, 1986). The encouragement of growth of starter cultures by yeasts producing essential growth metabolites such as amino acids and vitamins, has also been observed (Kaminarides & Laskos, 1992; Seiler, 1991; Jakobsen & Narvhus, 1996).

Furthermore, several studies have indicated that low temperature enhances the survival of *E. coli* O157:H7 (Cheng & Kaspar, 1998; Clavero & Beuchat, 1996; Faith *et al*., 1998). Bachrouri *et al*. (2002) observed that *E. coli* O157:H7 counts decreased by only 0.8 $\log_{10}$ cfu/g in yoghurt stored at 4 ºC for 72 h, while in yoghurt stored at 22 ºC, they could not detect any bacteria after 16 h of challenge. Since commercial Amasi was refrigerated at 7 ºC for 24 h, survival of *E. coli* O157:H7 could have been enhanced by low temperature, compared to that in traditional Amasi, which was kept at ambient temperature throughout the three days of challenge. Low temperature triggers alteration in the cell membrane fatty acid composition

and also results in the production of cold shock proteins (Jones *et al*, 1987; Berry & Foegeding, 1997). The proportion of unsaturated fatty acids increases, preventing fluid components from forming a gel. Gel-like fluids hinder proper functioning of proteins and cause bacterial membrane leakage (Russel *et al*, 1995). Contrary to this finding, Gran *et al.* (2003) reported that fermented milk made from starter cultures was more effective in reducing *E. coli* than fermented milk from back-slopping. In their study, *E. coli* counts were 5.7 $\log_{10}$ cfu/ml after 48 h fermentation at 25 ºC in Zimbabwean Amasi fermented by back-slopping while the counts were 0.8 – 2.8 $\log_{10}$ cfu/ml in Amasi fermented with starter cultures after the same period. The study was however, done with non-adapted cells and inoculation of *E. coli* was done immediately after milk collection, while in the present study, it was done at pH 5.6 when the level of LAB had increased. Since yeasts were not determined in their study, it remains unknown whether their presence or absence had an impact on the growth of bacteria. Slow pH decrease and lactic acid production, together with the possible existence of AA *E. coli* in the fermented milk product used for back-slopping, were given as reasons for the survival of *E. coli* in the product made with back-slopping.

Acid adaptation enhanced the survival of *E. coli* O157:H7 in traditional Amasi while NA *E. coli* O157:H7 survived significantly ($p \leq 0.04$) better than its AA counterpart in commercial Amasi. Concurring with the latter, Hsin-Yi and Chou (2001) reported that acid adaptation reduced survival of *E. coli* O157:H7 (ATCC 43889 and 43895) in Yakult and low-fat yoghurt stored at 7 ºC, but enhanced survival of both strains in commercial fruit juice. Although *E. coli* O157:H7 possesses a higher level of acid tolerance compared to other *E. coli* serotypes, strain variations in acid tolerance have been reported (Arnold and Kasper, 1995; Miller & Kasper, 1994**;** Benjamin & Datta, 1995). The strains used in the present study could, therefore, be less acid tolerant, hence explaining the failure of acid adaptation to boost survival at low pH.

Furthermore, the better survival of the NA *E. coli* O157:H7 strain compared to the AA *E. coli* O157:H7 strain in commercial Amasi could be due to the possibility that the NA strains simply adapts with changing pH while the AA strain, which had previously adapted to lower pH, had to adapt again to the new environment. The sudden shift of the AA strain to normal

optimum growth conditions followed by the subsequent demand to re-adapt could have resulted in its failure to acquire maximum adaptation. It is also worth mentioning that the pH of the AA strain inoculum was 4.6 at the time of inoculation. The loss of adaptation when growing at optimum conditions was reported by Jordan *et al.* (1999) and Schweder *et al.* (1996).

In traditional Amasi acid adaptation of *E. coli* had a significant effect ($p \leq 0.00$) on the total aerobic counts. This was due to the difference in survival of AA and NA *E. coli* O157:H7 in traditional Amasi. The survival of yeasts and moulds, lactococci, mesophilic and thermophilic LAB in the clay pot inoculated with AA *E. coli* O157:H7 did not differ from that in the NA *E. coli* O157:H7 inoculated clay pot. This indicates that the survival of neither AA nor NA *E. coli* O157:H7 in the clay pots negatively affected the growth of other bacteria.

3.2.5 Conclusion

E. coli O157:H7 was detectable in commercial Amasi after 3 days at 7 ºC but not in traditional Amasi processed at ambient temperature over the same period. Both AA and NA *E. coli* O157:H7 showed a similar survival over 2 days in traditional Amasi, while in commercial Amasi, the NA strain survived significantly better than its AA counterpart. Regardless of prior adaptation to acid, *E. coli* O157:H7 can survive during fermentation and storage of fermented goat milk Amasi. The fermentation time, pH and storage temperature affects the survival of *E. coli* O157:H7 in the fermented milk.

CHAPTER 4: GENERAL DISCUSSION

4.1 Methodology

The first phase of the project was initially planned to be done using *E. coli* O157:H7 strains isolated from raw goat milk. However, we could not isolate *E. coli* O157:H7 from the farms surveyed in the study hence strains from other sources were used. The reason for not been successful in isolating *E. coli* O157:H7 from raw goat milk could possibly have been due to that the pathogen exists in very small quantities in the milk or the level of hygiene at the farms sampled for the study was high. For the isolation, raw goat milk was enriched for 18 h in MacConkey broth incubated at 37 °C to increase the number of organisms in the milk to detectable levels. Incubation at 37 °C was used because it is the optimum growth temperature for *E. coli*. After making serial dilutions, the samples were plated on Chromocult TBX (Tryptone Bile X-glucuronide) agar, which is a selective agar for the detection and enumeration of *E. coli* in foodstuff and water. It contains an enzyme ß-D-glucuronidase, which differentiates most *E. coli* species from other species and bile salts to inhibit the growth of gram-positive bacteria. Presumptive colonies were serotyped according to the slide agglutination test for pathogenic *E. coli* (Mast Assure™; Mast Diagnostics).

For the acid challenge study, Brain Heart Infusion broth (BHI) and Tryptone Soy Agar (TSA) were selected over selective or minimal media because the former more closely resemble the composition of foods associated with the transmission of enterohamorrhagic *E. coli* (Buchanan & Edelson, 1996) while TSA allows resuscitation of injured cells. Selective media and even minimal selective media do not support the growth of acid injured cells hence they underestimate the concentration of viable *E. coli* cells (Weaver *et al*., 1996; Blackburn & McCarthy, 2000).

AA cells were prepared to the stationary phase using the method by Buchanan & Edelson, (1996). The method was selected because of its ability to foster maximal acid tolerance in *E. coli*. The gradual reduction in pH observed when cells are cultured in Tryptone Soy Broth (TSB) supplemented with 1 % glucose confers acid adaptation similar to fermentation in foods

(Buchanan & Edelson, 1996). The bacteria ferment the available glucose and produce lactic acid, acetic acid, pyruvic acid, which subsequently causes a depression in pH. NA cells were not supplemented with glucose but were buffered with MOPS to prevent any change in pH. The use of MOPS was incorporated after preliminary studies done without the buffer showed no difference between the survival of AA and NA strains. This was attributed to that the NA strain could be partially adapting to acid due to presence of a minute amount glucose (2.5 g/l) in TSB. The acid challenge study was done at different pH levels. Challenging the strains at pH 7.4 was done to determine the survival acid adapted strains under optimum growth conditions while pH 4.5 is the average pH of most fermented foods. Challenging the cells at pH 2.5 was done to access whether the strains could survive in the human stomach. For a pathogen to cause disease, it must survive the acidic conditions of the stomach before it reaches the intestine (Benjamin & Datta, 1995). Cells were prepared to the stationary phase because several studies have demonstrated that stationary phase cells are more tolerant to low pH than log phase cells (Buchanan & Doyle, 1997; Hengge-Aronis, 1993; Jenkins *et al*., 1990). The high level of acid tolerance of stationary phase cells is attributed to several morphological and physical changes that occur in cells upon entry to stationary phase. These include reduction in cell size, cell becoming rounder in shape and accumulation of storage compounds such as glycogen and polyphosphate (Huisman, Siegele, Zambrano & Kolter, 1996).

Several acid challenge studies have been done using inorganic acids to lower the pH of the laboratory media (Arnold & Kasper, 1995; Lin *et al*., 1996; Jordan *et al*., 1999; Leyer *et al*., 1995). Because inorganic acids, such as HCl, are rarely used in foods, their use to access the response of enteric bacteria to low pH has practical limitations (Deng, Ryu & Beuchat, 1999). In the current study, lactic acid was used to acidify BHI broth. Lactic acid was favoured because in food fermentations, it is produced by naturally present microflora or added lactic acid producing bacteria. Inorganic acids such as hydrochloric acid are, however, of tremendous importance in the stomach where they are naturally secreted to denature proteins and inactivate pathogenic microorganism (Deng, Ryu & Beuchat, 1999).

Lipid extraction was done using the Bligh and Dryer (1959) method. The method was chosen because it is rapid, simple and efficient for the extraction and purification of lipids from biological materials. It is also reproducible and free from deleterious manipulations. Other methods for lipid extraction are too time-consuming while others involve heating and evaporation, which are considered unsuitable for lipid composition studies. In the Bligh and Dryer method, lipids are extracted and purified in a single operation. The extraction is achieved through using a mixture of chloroform and methanol. Dilution with chloroform and water separates the homogenate into two layers, namely the chloroform and methanol layers. The chloroform layer contains the lipids while the methanol layer contains all the non-lipids. Since the volume of bacteria harvested was small, the volumes in the method were scaled down but the proportions of chloroform, methanol and water, before and after dilution were 1: 2: 0.8 and 2: 2: 1.8 as stipulated in the method.

Before the extraction of lipids, the cells were challenged at pH 2.5 or 4.5 or 7.4. This was done by inoculating of AA or NA *E. coli* UT 10 or *E. coli* ATCC 25922 strains into BHI broth adjusted to the respective pH levels. Since both *E. coli* UT 10 and *E. coli* ATCC 25922 could not survive at pH 2.5, the amount of harvested cells could not be enough to warrant further extraction of lipids and so fatty acid analysis could not be done on strains challenged at pH 2.5.

Commercial Amasi was prepared by inoculating pasteurized milk with Amasi starter culture (MO 0.30). The milk was pasteurized to inactivate pathogenic microorganisms and other competing microorganism that it could contain. Commercial starter cultures consist of single or multiple strains depending on the product to be made (Holzapfel, 2000). The major activity of the starter culture in food fermentation is to convert carbohydrates to desired metabolites such as alcohol and organic acids (Holzapfel, 2000). These are good natural preservatives but also contribute positively to the quality of the product. Traditional Amasi was prepared by allowing raw goat milk to ferment spontaneously at ambient temperature. Spontaneous fermentation is widely practiced in producing Amasi in rural areas of South Africa. It results from competitive activities of a variety of microorganisms present in the milk with those best adapted to the food substrate dominating the process. To reduce the fermentation time,

backsloping was used in making traditional Amasi. Backsloping involved inoculating raw milk with a sample from a previous successful fermentation. The traditional inoculum used in the study was pre-screened for the presence of pathogens. This was done to ensure that it did not contain other pathogens such as *Salmonella tryphimurium, Listeria monocytogens* and *Shigella flexneri* which could compete with the target microorganism (Bearson *et al*., 1996; Dykes & Moorhead, 1999). These pathogens also evolve complex, inducible acid survival mechanisms under acid stress.

Enumeration of test strains from commercial and traditional Amasi was done using MacConkey sorbitol agar. This is a selective and differential media for detection of *E. coli* O157:H7. Its selectivity comes from the fact that *E. coli* O157:H7 does not ferment sorbitol and therefore produces colourless colonies while most *E. coli* ferment sorbitol and form pink colonies. Incubation of plates was done at 37 °C because it is the optimal temperature for growth of *E. coli* O157:H7. The test strains were inoculated when the pH of the product reached 5.6 to prevent the non-adapted strain from adapting. Adaptation of bacteria with gradual decline in pH during fermentation of food has been reported in previous studies.

4.2 Acid resistance of *E. coli* O157:H7 in broth at different pH levels

E. coli O157:H7 is one of the important emerging pathogens that has been implicated in the outbreak of foodborne diseases. It has received more attention due to its ability to cause disease at low infection doses and through its outstanding ability to survive at low pH. When challenged alongside a non-pathogenic *E. coli* strain (ATCC 25922), *E. coli* UT 10 exhibited a higher level of acid resistance at pH 4.5. Alnold & Kaspar (1995) suggested that the increased acid resistance exhibited by *E. coli* O157:H7 could be due to the activation of multiple protective systems simultaneous or to a high level of protective proteins than other *E. coli*. The high level of acid tolerance of *E. coli* UT 10, compared to other *E. coli*, has also been reported by other authors (Besser *et al*., 1993; McIngrale *et al*., 2000). Furthermore, the level of acid resistance of *E. coli* O157:H7 varies between strains of pathogenic *E. coli* and between pathogenic and non-pathogenic strains.

Acid adaptation has been reported to enhance survival of *E. coli* at low pH by maintaining internal pH close to neutrality. Acid adaptation is acquired through exposure of cells to a sublethal stress (Foster, 1999; Foster & Hall, 1990). Such exposure can occur during preservation of foods using the hurdle technology and during fermentation of foods. As observed in phase 1 of the current study, prior exposure of bacterial cells to mild acidic conditions enhanced the survival of both *E. coli* UT 10 and *E. coli* ATCC 25922 when challenged in BHI broth at pH 4.5. The study was done in broth to examine the survival of *E. coli* at ideal growth conditions and also to allow for easy regulation of the growth conditions. However, at pH 7.4, acid adaptation did not enhance the survival of acid adapted strain. This confirms that acid adaptation is lost upon growth at normal conditions. At pH 2.5, both strains could not be detected after 4 h of exposure. This suggest that the strains were moderately acid tolerant and hence they may not survive in the stomach of humans. Survival of *E. coli* O157:H7 at pH 2.5 has been reported by Benjamin & Datta (1995) and Buchanan & Edelson (1996). Differences in the test environments such as media and preparation of cells could be the reason for the differences in acid survival levels of *E. coli* O157:H7. Another factor could be the differences in the level of acid resistance which varies between strains of pathogenic *E. coli* and between pathogenic and non-pathogenic strains (Duffy *et al*., 2000; Mckellar & Knight, 1999). These variations complicate adaptation studies by making it difficult to determine acid resistance levels of individual organisms and subsequently making it tough for the industry to develop effective mechanisms of inactivating acid resistant microorganism.

4.3 Lipid modification and acid resistance

In order to survive low pH, *E. coli* convert MUFAs to SAFAs. Although *E. coli* UT 10 survived better than *E. coli* ATCC 25922 at pH 4.5, this was not reflected in the fatty acid profile as *E. coli* ATCC 25922 had more SAFA than *E. coli* UT 10. The increase in the level of SAFAs at the expense of MUFAs increases membrane rigidity which subsequently reduces the inflow of protons into the cell. Unsaturated fatty acids are not only converted to SAFAs during acid acid stress but also to cyclopropane fatty acid (CFA). The GC column used in the identification of the fatty acids, however, could not identify CFA. CFA formation provides protection of bacterial cells at low pH by decreasing permeability of protons into the cell and

possibly through interaction of CFA-containing phospholipids with membrane proteins, which also passively decrease proton permeability. Several researchers have shown that cellular CFA levels increase during acid adaptation (Brown *et al*., 1997, Dufoure *et al*., 1984; Dunkley *et al*., 1991). They attribute this to accumulation of RpoS protein that increase cfa expression by activating the RpoS-dependent P2 promoter (Wang & Cronan, 1994). Contrary to this, Yuk & Marshall (2004) reported that cells grown in pH 9.0 and pH 5.0 had the lowest CFA than cells grown at neutral pH. Arneborg *et al*. (1993) also found that *E. coli* MT 102 grown at pH 6.4 had lower proportions of USAFAs and CFAs than the same strain grown at pH 8.4. They noted that *E. coli* increases the synthesis of saturated fatty acyl chains at the expense of both unsaturated and cyclopropane fatty acyl chains as a response to lower pH.

The observed high amount of SAFAs in *E. coli* ATCC 25922 compared to *E. coli* UT 10 which survived better in the growth survival study, indicates that lipid modification is not the only mechanism that enhances survival at low pH. In addition to lipid modification, other mechanisms such as the amino acid decarboxylase systems and the accumulation of RpoS protein are also activated. The amino acid decarboxylases systems (Arginine and glutamate-dependent systems) enhance survival by consuming protons that leak into the cell during acid stress in exchange of their decarboxylated end products. RpoS is a regulator of the stationary-phase stress response in *E. coli*. It is itself an acid shock protein (ASP) and controls the expression of other ASPs (Lee *et al*., 1995). ASPs are presumed to prevent and repair acid-damage to macromolecules.

4.4 Acid resistance in broth model versus food systems

Acid resistance studies have normally been done in broth models. Broth provides an ideal environment for the growth of bacteria and further allows for easy alteration of growth conditions. Different types of broth such as Luria broth, TSB and BHI broth (Buchanan & Edelson, 1996; Alnold & Kaspar, 1995; De Jonge *et al.,* 2003) have been used in acid tolerance studies and this has lead to difficulties in comparing results. The pH of the broth is normally adjusted with inorganic acids such as HCl. Broth systems do not however, fully mimic the conditions that bacteria has to survive in food. The lack of other microorganisms in

the broth, which could interact positively or negatively with the target microorganism, could lead to inaccurate estimates of what happens in food systems. The other difference is the availability of other chemicals in the food that could hinder the growth of bacteria. In the current study, the survival of *E. coli* O157:H7 in the broth system was different from that observed in the product. In the broth model, *E. coli* UT 10 survived well at pH 4.5 and only decreased slightly with 0.6 log after 16 h, while in the product there was a continuous decline in *E. coli* O157:H7 counts after 24 h. The survival of the pathogen in traditional Amasi was also different from that observed in commercial Amasi and this highlights that survival of bacteria also varies with the product tested.

4.5 Acid resistance of *E. coli* O157:H7 in commercial and traditional Amasi

E. coli O157:H7 survived better in commercial Amasi than in traditional Amasi. Firstly, this is attributed to the presence of other microorganism in traditional Amasi that hinder the growth of the pathogen indirectly or directly. Indirectly, the presence of other microorganisms such as yeasts may produce toxins that could be detrimental to other microorganisms. Directly, the presence of other microorganisms may create competition for nutrients and space. Competition for nutrients may also come from other contaminating pathogens since traditional Amasi is produced under poor hygienic conditions. In the traditional inoculum used to make traditional Amasi, presumptive colonies of *Listeria* were isolated and since *Listeria* inherently possesses mechanisms to survive low pH (Dykes & Moorhead, 1999), it could have provided some competition to *E. coli* O157:H7. Backsloping is commonly practiced when making traditional Amasi and the inoculum could contain yeasts and moulds as well as other bacteria such as coliforms. The presence of high yeasts counts as well as coliforms in traditional products has been reported by Beukes *et al*. (2001). Raw milk used to make commercial Amasi is normally pasteurized and is thus devoid of other microorganisms.

4.6 Acid adaptation and low temperature storage

Acid adaptation enhances the survival of *E. coli* O157:H7 in fermented foods and this has been seen in foods such as yoghurt, buttermilk, Ergo and lactic cheeses (Tsegaye & Ashenafi,

2005; McIngrale *et al*., 2000; Massa *et al*., 1996). Storage of low pH foods at low temperatures has also been reported to further improve the survival of *E. coli* O157:H7 at low pH compared to storage at ambient temperatures (Cheng & Kaspar, 1998; Faith *et al*., 1998). Multiple systems of survival, that is, those activated by the cell at low when exposed to low pH including those activated at low temperatures, are presumably activated during storage of low pH foods at low temperatures. In the current study, acid adaptation enhanced survival of *E. coli* O157:H7 in traditional Amasi, while interestingly, in commercial Amasi, the NA strain survived better than the AA strain. The acid adaptated strain had a final pH of 4.6 when introduced into the fermenting product, which was at pH 5.6. The sudden shift to a higher pH could have resulted in a loss of adaptation. Jordan *et al*. (1999) and Schweder *et al*. (1996) reported that acid adaptation is lost upon growth at optimum conditions. Since the pH of the product was continuously declining after inoculation, the non-adapted strain could have adapted to the new environment while the adapted strain had to first lose part of its first adaptation and then adapted again to the changing environment. This could have been more energy demanding for the acid adapted strain and hence the NA strain survived better.

4.7 Conclusion and recommendations

Acid adaptation enhanced the survival of *E. coli* UT 10 and *E. coli* ATCC 25922 in broth, with the former exhibiting a better survival rate. However, when *E. coli* O157:H7 was challenged in Amasi, both the AA and NA strains showed a similar survival in traditional Amasi, while in commercial Amasi the NA strain survived significantly better than the AA strain. This confirms that acid survival tests done in broth do not truly reflect what would happen in a food system. The availability of other competing microorganisms and chemicals that are not in the broth could negatively affect the survival of target organism in the food system. In addition, survival of *E. coli* O157:H7 differs between foods as observed in commercial and traditional Amasi. Adaptation to acid was accompanied by an increase in SAFAs, indicating that lipid modification is important in enhancing survival at low pH. Furthermore, low temperature storage also enhances survival of acid adapted *E. coli* than storage at ambient temperature and this is attributed to activation of multiple acid resistant systems. As a result, *E. coli* O157:H7 can survive during the fermentation and storage of Amasi and the survival of the pathogen is

affected by fermentation time, pH and storage temperature. The food industry should therefore adapt their processing/ preservation procedures by taking the most acid tolerant pathogenic *E. coli* strains into consideration in order to ensure the safety of their products.

CHAPTER 5: REFERENCES

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