Chapter 5:

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

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Abstract

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

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

5.1 Introduction

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

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

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

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

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

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

5.2 Materials and Methods

5.2.1 Milk Source

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

5.2.2 Cultures

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

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

5.2.3 Inoculation and fermentation

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

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

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

5.2.4 Acid challenge

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

3.3.2.5 Chemical analyses

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

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

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

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

5.2.6 Microbiological analyses

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

5.2.7 Statistical analyses

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

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

5.3 Results

5.3.1 Quality of raw and pasteurized Saanen goat milk

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

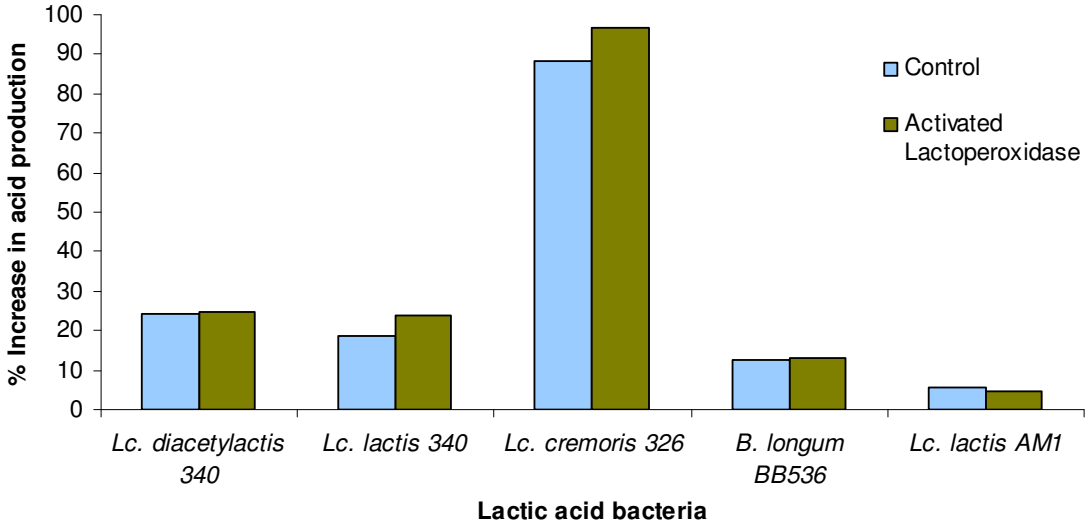

Figure 5.1: The percent increase in acid production by lactic starter cultures after 6 h fermentation of goat milk inoculated with *Escherichia coli* O157:H7 compared to 6 h fermentation of goat milk that had no *E. coli* O157:H7 present

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

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

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

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

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

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

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

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

† Standard deviation

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

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

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

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

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

5.4 Discussion

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

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

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

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

2003).

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

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

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

5.5 Conclusion

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

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Chapter 6: GENERAL DISCUSSION

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

6.1 Review of Methodology

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

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

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

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

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

that had been acidified with lactic acid to pH 3.85.

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

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

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

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

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

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

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

6.1.2 Choice of hurdles

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

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

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

6.1.3 Quantitative Real-Time PCR (qRT-PCR)

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

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

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

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

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

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

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

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

6.1.4 Microbiological analyses

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

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

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

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

6.1.5 Biochemical analysis

6.1.5.1 Fatty acid profile

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

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

6.1.5.2 Activation of the lactoperoxidase system

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

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

6.1.5.3 Lactic acid determination

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

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

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

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

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

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

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

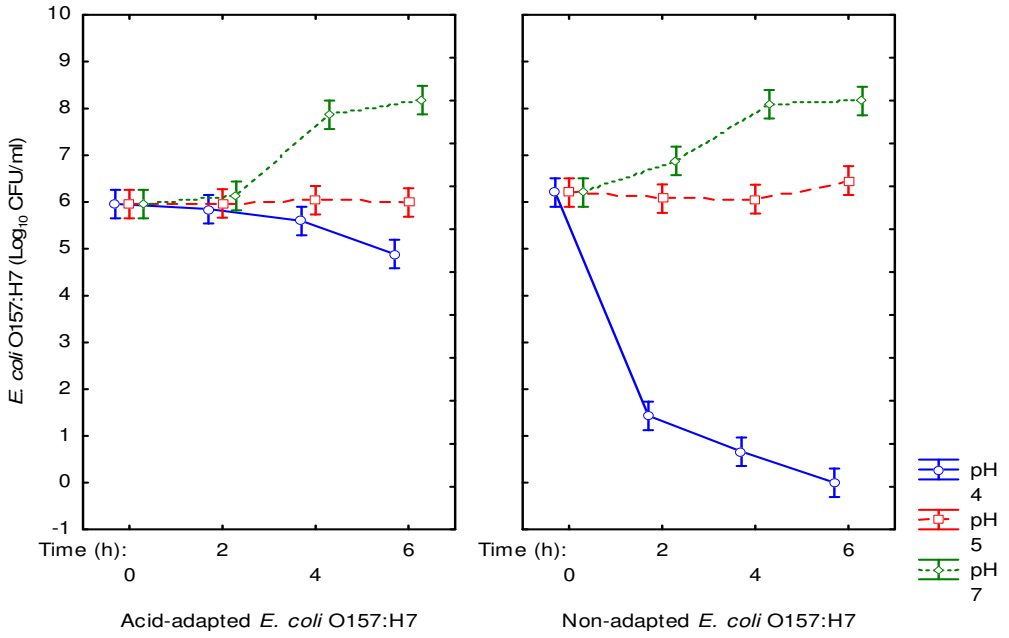

Figure 6.1: The effect of acid challenge at pH levels of 4, 5 and 7 on survival of acid-adapted and non-adapted E. coli O157:H7 in Tryptone Soy Broth incubated for 6 h at 37 °C

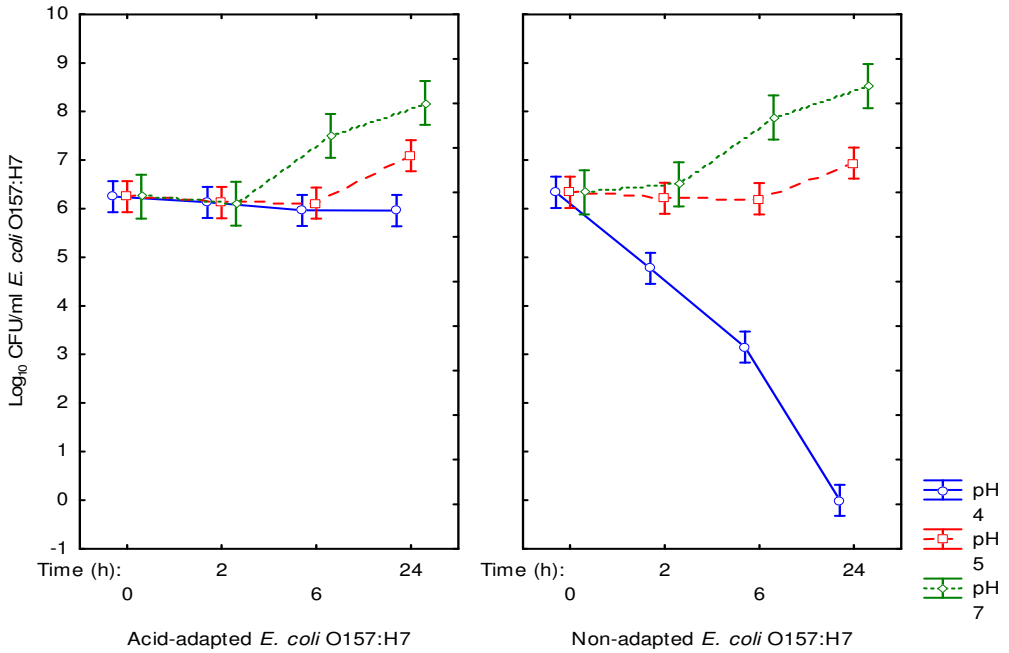

Figure 6.2: The effect of acid challenge at pH levels of 4, 5 and 7 on survival of acid-adapted and non-adapted *E. coli* O157:H7 in Tryptone Soy Broth incubated for 6 h at 25 °C

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

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

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

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

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

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

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

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

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

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

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

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

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

6.2.4 Lactoperoxidase activity in goat milk

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Chapter 7: CONCLUSIONS AND RECOMMENDATIONS

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

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

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

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

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

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