Chapter 3:

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

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Abstract

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

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

3.1. Introduction

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

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

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

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

3.2 Materials and Methods

3.2.1 Bacterial cells and culture conditions

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

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

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

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

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

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

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

3.2.3 Fatty acid analysis

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

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

3.2.4 RNA extraction and cDNA synthesis

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

3.2.5 Quantitative real-time PCR

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

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

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

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

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

3.2.6 Statistical analysis

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

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

3.3 Results

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

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

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

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

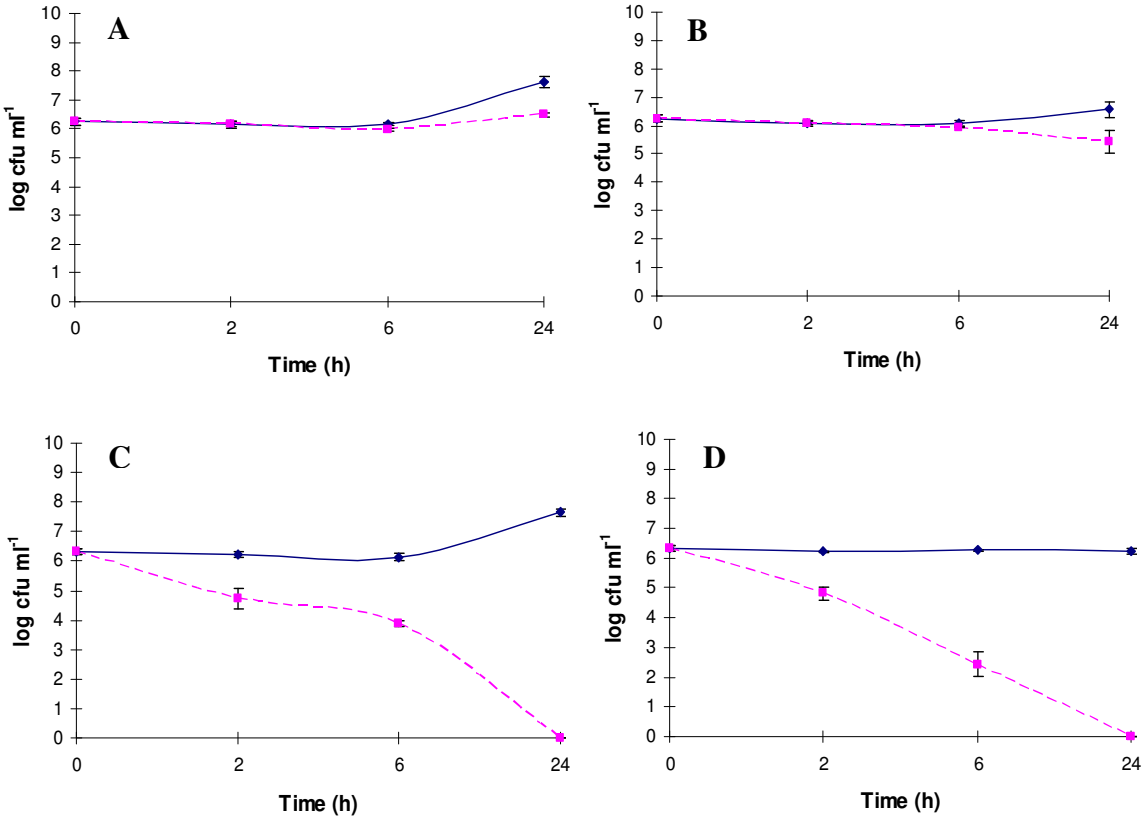


Figure 3.1: The effect of lactic acid and activated lactoperoxidase (LP) system on survival of acid-adapted (A, B) and non-adapted (C, D) *Escherichia coli* O157:H7 inocula in Tryptone Soy Broth. (A, C) Lactic acid challenge only, (B, D) Activated LP in combination with lactic acid challenge. ♦, pH 5.0;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

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

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

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

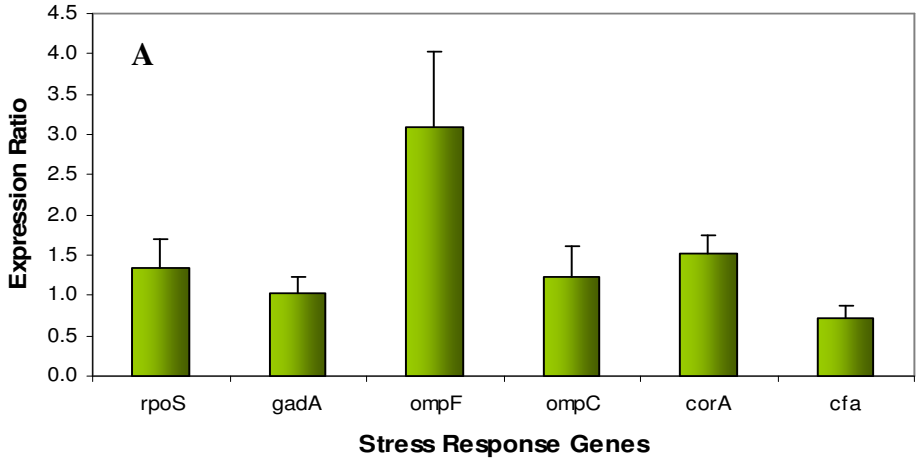
Fatty acid (%)	**A + pH7**	**N + pH7**	**A + pH5**	**N + pH5**	**A + pH4**	**N + pH4**	**A + LP**	**N + LP**
Lauric acid (C12:0)	1.98 (±1.57)	1.9 (±0.25)	ND	1.64 (±1.12)	1.11 (±0.05)	ND	1.37 (±0.43)	4.61 (±3.86)
Myristic acid (C14:0)	15.54 (±1.68)	11.74 (±2.38)	14.51 (±1.08)	9.75 (±2.11)	16.30 (±0.09)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

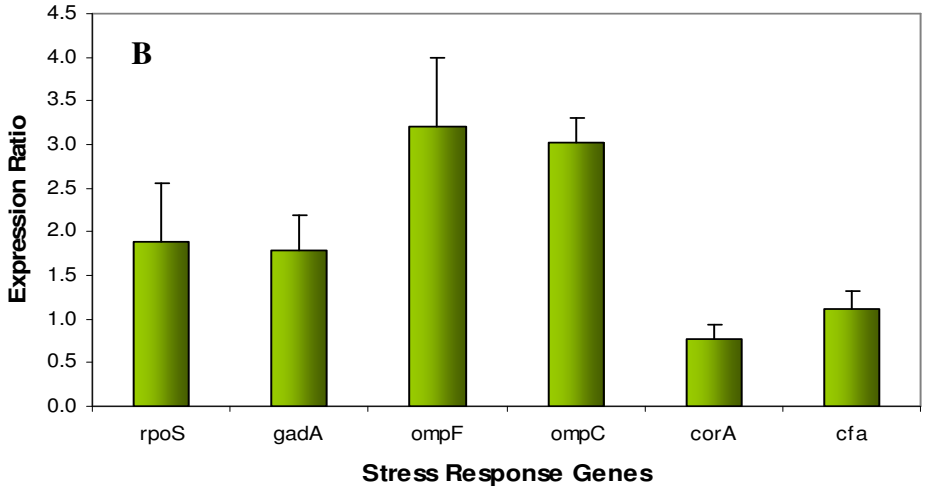
Each value presented as the mean of replicate determinations ($n = 2$) of outer membrane fatty acids ± standard deviation of replicate values; A: acid-adapted *E. coli* O157:H7; N: non-adapted *E. coli* O157:H7; LP: activated lactoperoxidase system; ND: not detected

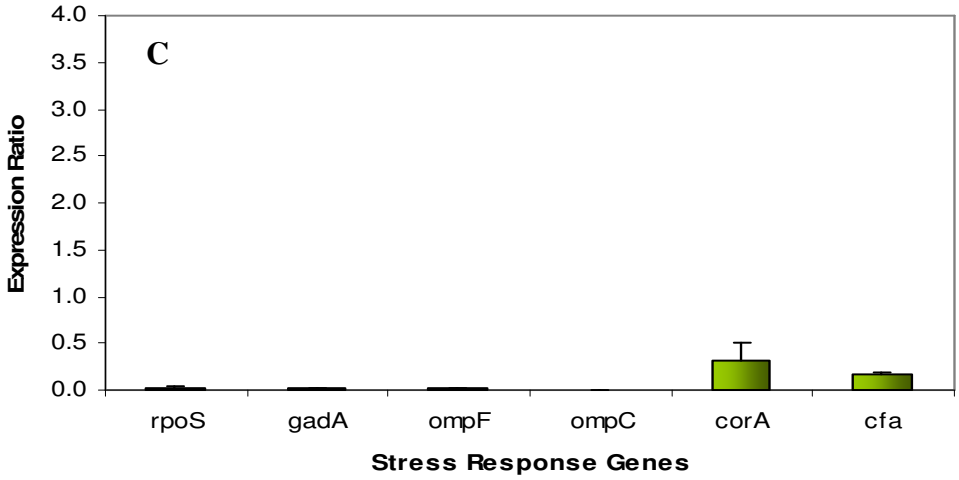
An increase in palmitic acid with a corresponding decrease in palmitoleic FAME was evident with all acid-adapted cells at pH 4.0 and pH 5.0. The acid-adapted cells had significantly higher levels of palmitic acid ($P \leq 0.05$) and significantly lower levels of palmitoleic acid ($P \leq 0.05$) compared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

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

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


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


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


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

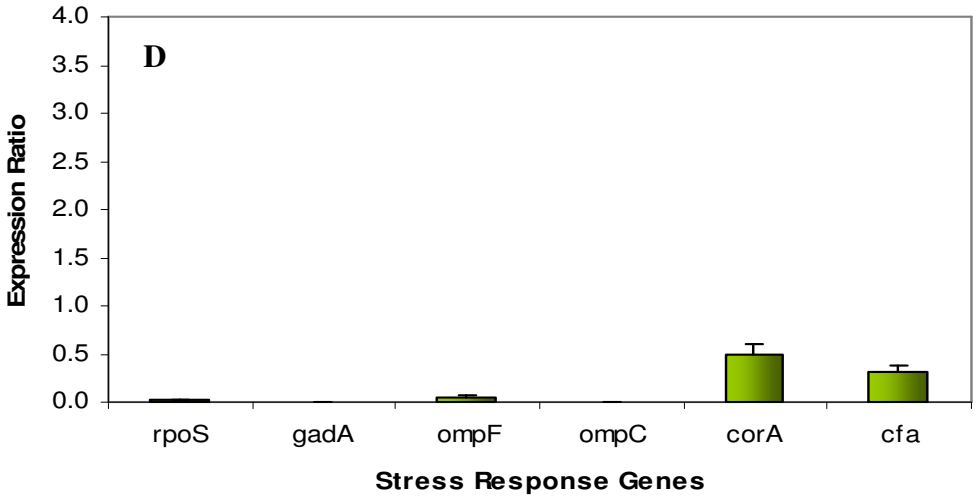


Figure 3.2: Expression of lactoperoxidase (LP) and acid inducible genes in *Escherichia coli* O157:H7 challenged against LP system and lactic acid for 6 h in Tryptone Soy Broth for 6 h at 25 °C. The bars represent the expression ratio of genes compared to untreated non-adapted *E. coli* O157:H7 cells.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

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

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

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

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

3.4 Discussion

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

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

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

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

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

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

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

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

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

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

3.5 Acknowledgements

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Chapter 4:

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

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Abstract

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

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

4.1 Introduction

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

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

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

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

4.2 Materials and Methods

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

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

4.2.2 Milk source

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

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

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

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

4.2.4. Activation of the lactoperoxidase system

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

incubated at 25 °C for 6 h.

4.2.5 Heat treatment

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

4.2.6 Microbial analyses

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

4.2.7. Statistical analysis

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

4.3 Results

4.3.1 Lactoperoxidase activity

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

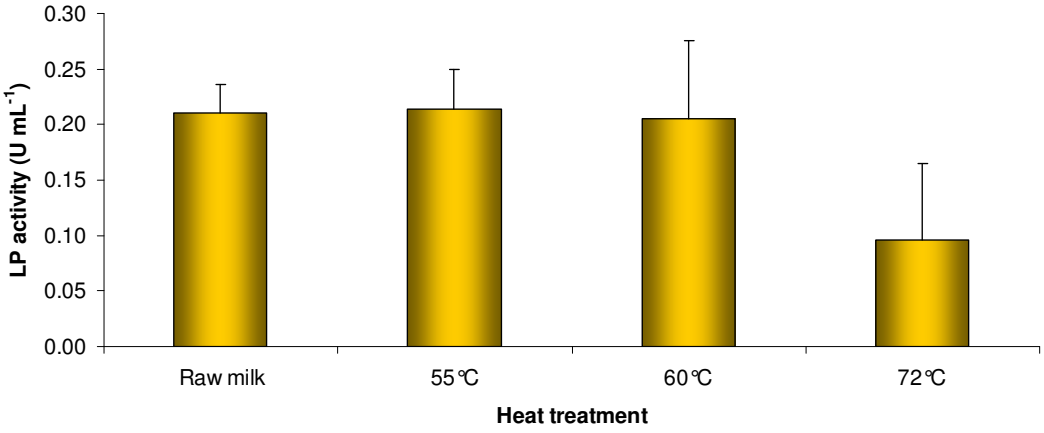

Figure 4.1: The lactoperoxidase activities of fresh goat milk (pH 6.9) before and after heat treatment at 55 °C, 60 °C and 72 °C for 15 s (excluding time taken to reach target temperature). The error bars represent standard error of the mean.

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

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

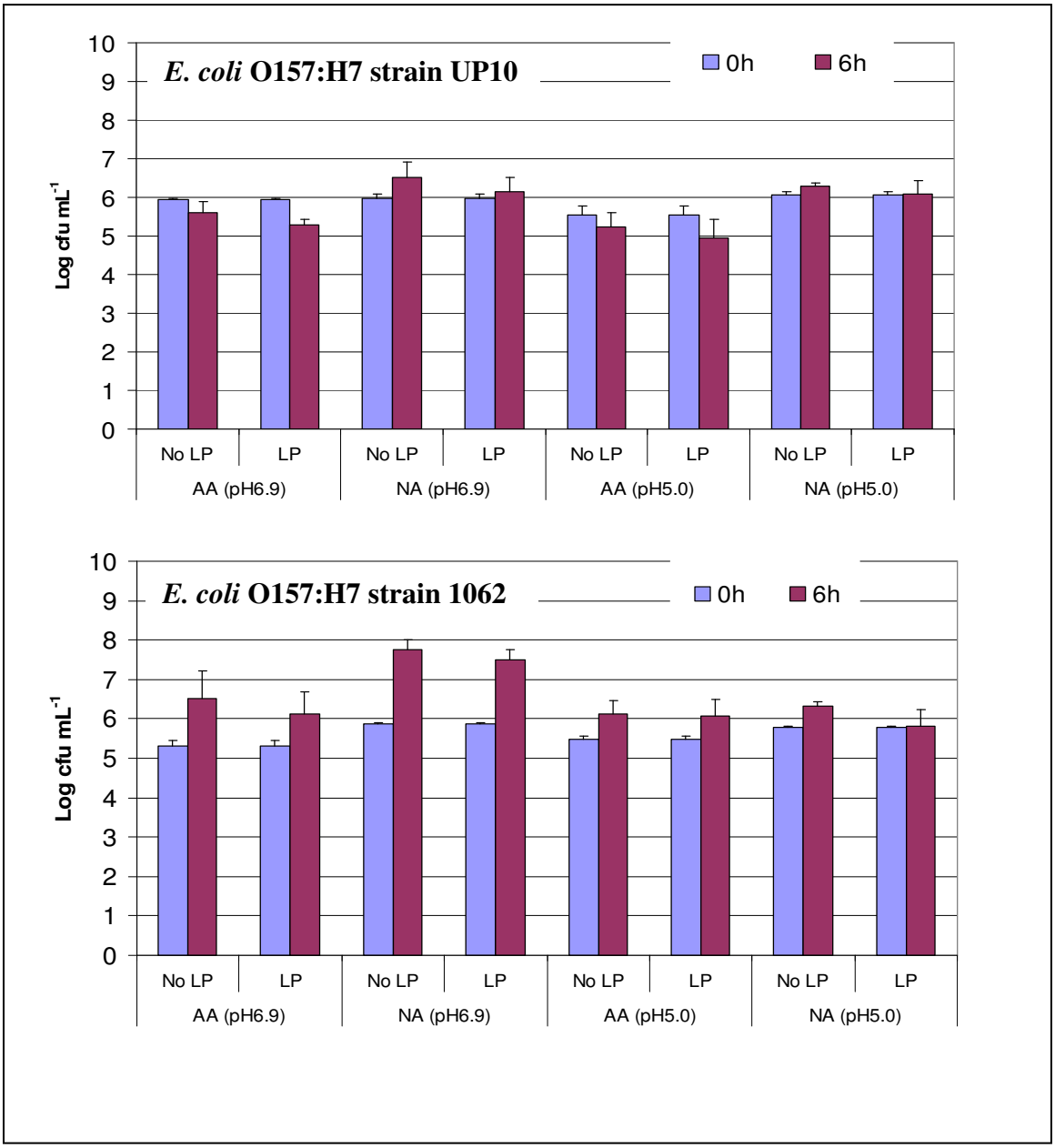

Figure 4.2: The effect of activated lactoperoxidase (LP) on acid-adapted (AA) and non-adapted (NA) *E. coli* O157:H7 strains UP10 and 1062 in fresh (pH 6.9) and acidified (pH 5.0) goat milk incubated for 6 h at 25 °C. Error bars represent standard error of the mean

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

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

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

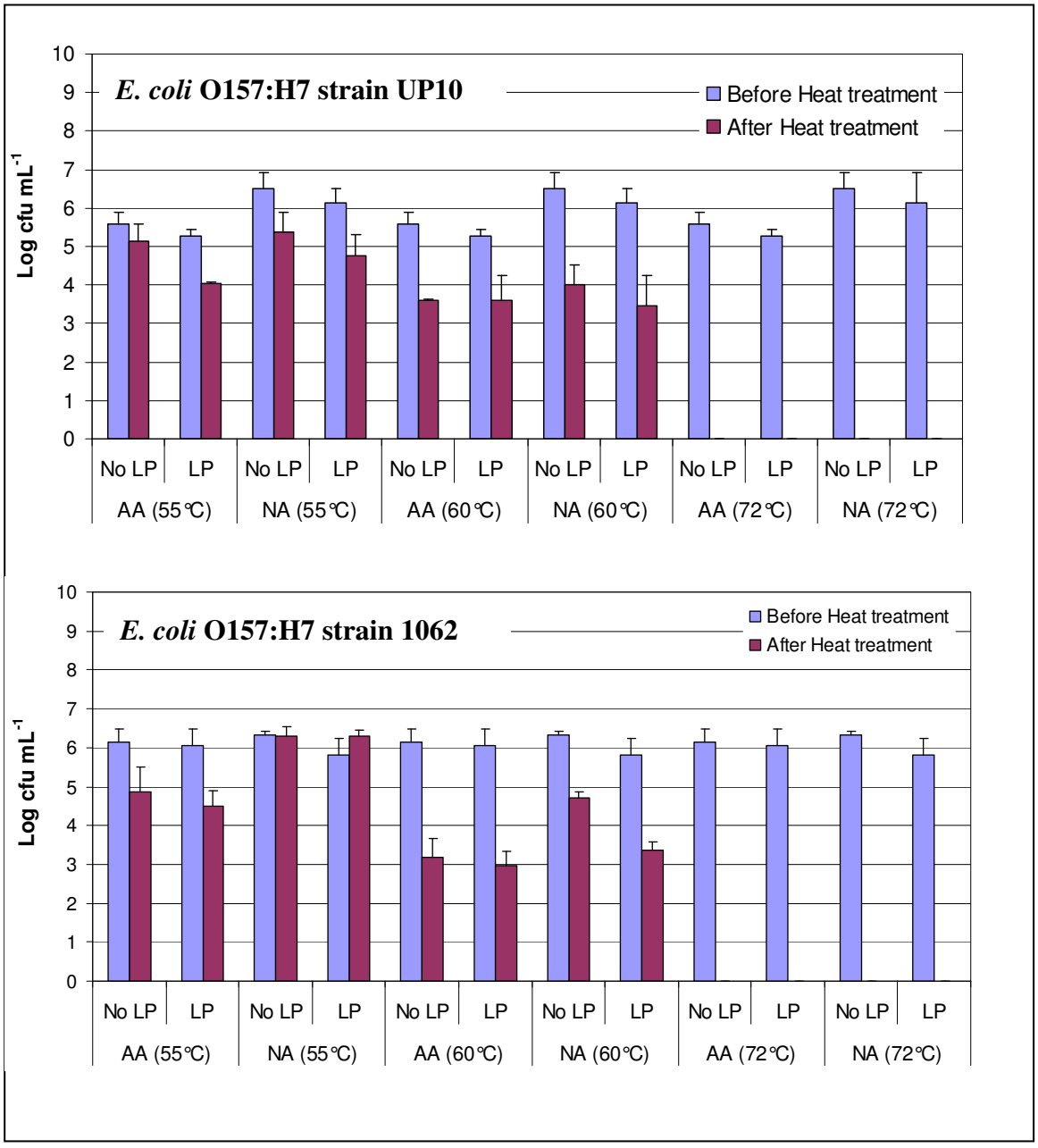

Figure 4.3: The survival of acid-adapted (AA) and non-adapted (NA) *Escherichia coli* O157:H7 strains UP10 and 1062 to the combined effect of heat (55°C or 60°C for 15 s, excluding time taken to reach target temperature) in activated lactoperoxidase (LP) goat milk at pH 6.9. Error bars represent standard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

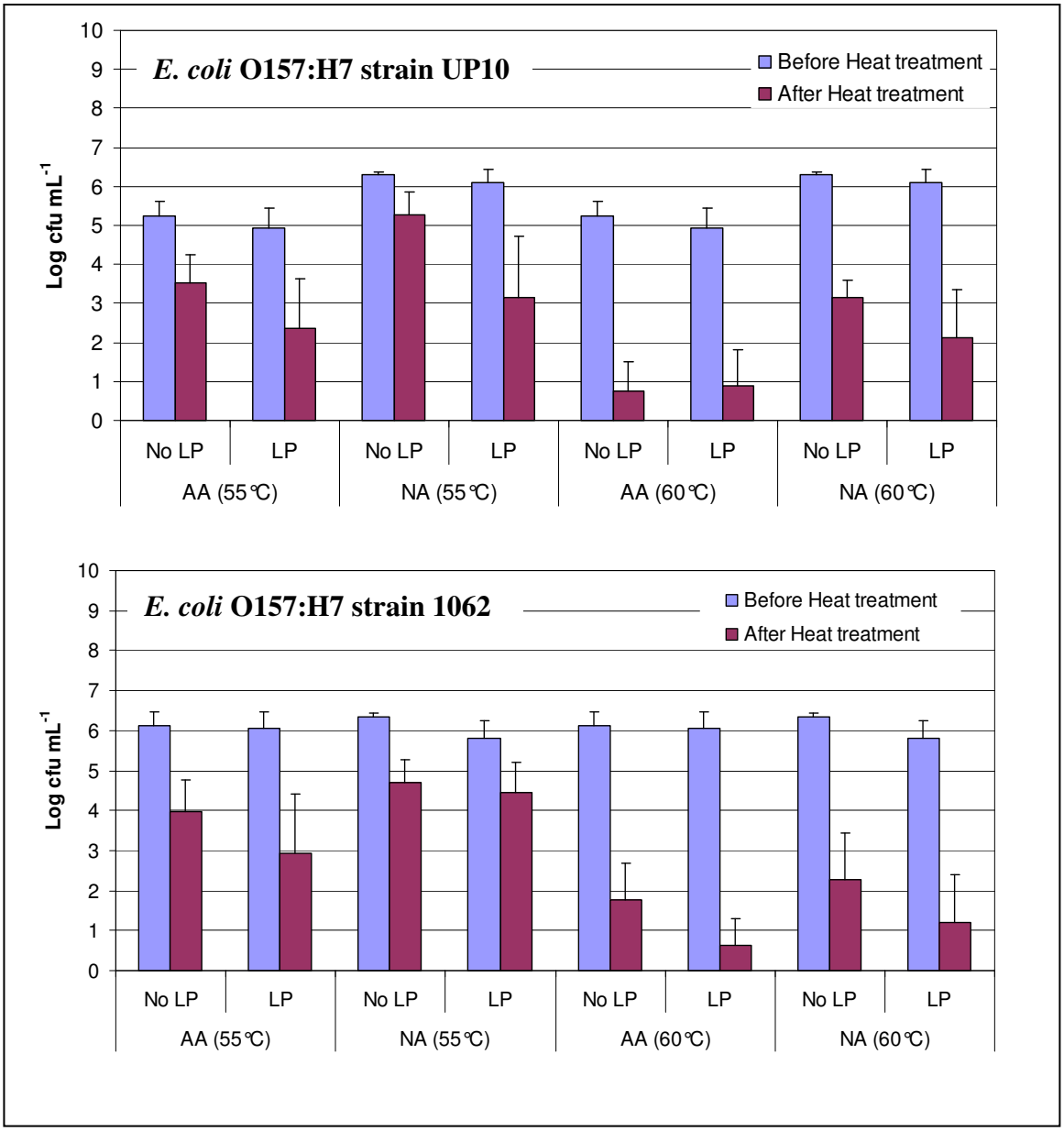

Figure 4.4: The survival of acid-adapted (AA) and non-adapted (NA) *Escherichia coli* O157:H7 strains UP10 and 1062 to the combined effect of heat (55°C or 60°C for 15 s, excluding time taken to reach target temperature), activated lactoperoxidase (LP) and low pH (pH 5.0) treatments in goat milk. Error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

4.4 Discussion

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

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

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

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

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

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

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

4.5 Conclusion

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

4.6 Acknowledgement

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