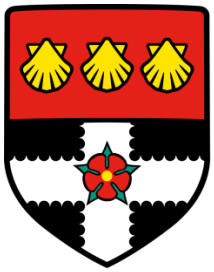

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Effects of Acid Adaptation on the Survival of *Lactobacillus plantarum* NCMIB 8826 in Fruit Juices

A thesis submitted as a partial fulfilment for the degree of Doctor of Philosophy

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Pornpoj Srisukchayakul
Reading, June 2017

Declaration of original authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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ABSTRACT

The aims of this work were to study the effect of acid adaptation on the survival of the stationary phase *Lactobacillus plantarum* NCMIB 8826 cells, a model potential probiotic strain, in several highly acidic fruit juices namely cranberry (pH 2.7), pomegranate (pH 3.5), and lemon & lime juices (pH 2.8) and to investigate the mechanisms involved in cellular response. The results indicated that exposure of the cells in both acidified solutions (buffers and de Man, Rogosa and Sharpe medium; MRS) adjusted to pH 6 to 3 by hydrochloric acid and citric acid for a short period of time significantly improved cell survival in the fruit juices, although the impact on cell viability was less than 10^7 CFU/ml for 6 weeks which was required for probiotic drinks. Furthermore, the prolonged exposure time (1 to 5 h) and low temperature (10 and 4 ^{o}C) were used to enhance the cell viability of *Lactobacillus plantarum* NCMIB 8826 but the improvements by these techniques could not make it as a promising strain for probiotic drinks. Analysis of citric and lactic acids as well as γ-aminobutyric acid (GABA) indicated that the citrate fermentation pathway and the glutamate decarboxylase system, which have been implicated in acid response in several lactobacilli, were not involved in this case while the analysis of the cellular fatty acid content showed that the cyclopropane fatty acid, cis-11,12-methylene octadecanoic acid ($C_{19cyclow7c}$), significantly increased (by ~1.7 fold) during acid adaptation, which was accompanied by a significant upregulation of the cyclopropane synthase (*cfa*) gene (by ~12 fold), as demonstrated by reverse transcription polymerase chain reaction. It was likely that these changes led to a decrease in membrane fluidity and to lower membrane permeability, which prevent the cells from proton influx during storage in fruit juices. Examination of the cell morphology by cryo-scanning electron microscopy revealed that the cell surface of acid adapted cells was rougher and thicker compared to control cells, suggesting that the composition and structure

of the peptidoglycan was possibly modified during acid exposure. A significant finding of this study was the observation that alanine, which represented the most abundant intracellular amino acid (> 45%), was significantly reduced in the case of acid adapted cells (~20%) compared to control cells, which coincided with a significant decrease in the extracellular alanine (~10%). It seems, therefore, likely that upon acid adaptation the cells utilized the available alanine to increase in the D-alanylation of wall teichoic acid, resulting in a positive cell wall with enhanced ability to reduce the influx of protons during storage in fruit juices. Moreover, proteomic analysis was also performed using 2D-gel electrophoresis, which led to the identification of eight proteins exhibiting a difference in % volume of at least 1.4 in expression levels between acid adapted and control cells. Among these, five proteins, molecular chaperone GroEL, aminopeptidase C, 30S ribosomal protein S1, D-alanine-D-ligase, and UPF0356 protein Ip_2157 were upregulated, whereas three proteins, 30S ribosomal protein S2, aspartate semialdehyde dehydrogenase, and the hypothetical protein HMPREF0531_11643 were downregulated in acid adapted cells compared to control cells.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
AgDI	Agmatine deiminase
ANOVA	Analysis of variance
ADI	Arginine deiminase
Asl	Argininosuccinate lyase
Ass	Argininosuccinate synthetase
fabH	*β*-ketoacyl-acyl carrier protein synthase III
CPS	Capsular exopolysaccharide
CO_2	Carbon dioxide
CFU	Colony forming unit
CFA	Cyclopropane fatty acid
cfa	Cyclopropane synthase
CWG	Cell wall glycopolymer
MRS	de Man, Rogosa and Sharpe medium
DAP	Diaminopimelic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EU	European Union
EPS	Exopolysaccharide
FAO	Food and agriculture organization of the united nations
GC-MS	Gas chromatography-mass spectrometry
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
GM	Genetically modified

GAD	Glutamate decarboxylase
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
IBD	Inflammatory bowel disease
IL	Interleukin
pH_i	Intracellular pH
IEF	Isoelectric focusing
pI	Isoelectric point
LAB	Lactic acid bacteria
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
MAPK	Mitogen-activated protein kinase
GlcNAc	*N*-acetylglucosamine
MurNAc	*N*-acetylmuramic acid
NK	Natural killer
OD	Optical density
PBS	Phosphate buffered saline
PMF	Proton motive force
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Scanning electron microscope
SD	Standard deviation
TNF-α	Tumour necrosis factor alpha
USA	United States of America
WTA	Wall teichoic acid
WHO	World health organization
GABA	*γ*-aminobutyric

CHAPTER 1

General Introduction

1.1. Background

The concept of using microbes to prevent and in some cases cure diseases has been established for a long time. The initial evidence of the link between consumption of beneficial microorganisms, such as lactic acid bacteria (LAB), and well-being was substantiated around 1900, when Nobel Prize-winner, Metchnikoff, revealed the association between the consumption of fermented dairy products and life expectancy of Bulgarians (Metchnikoff, 1907). In order to describe this connection, the term "Probiotic" was introduced by Lilly and Stillwell (1965). The definition of probiotics has changed considerably over the years, and the most widely accepted definition is the one by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO , 2002) "live microorganisms which confer a health benefit on the host when supplied in adequate amounts". A large number of studies have demonstrated the positive effects of probiotics on human health, which include the prevention of diarrhoea caused by pathogenic bacteria and viruses (Malaguarnera *et al*., 2012), the inhibition of pathogen colonization (Dhanani *et al*., 2011), the lowering of cholesterol level (Ooi and Liong, 2010), the stimulation of the immune system (Ashraf and Shah, 2014), the treatment of inflammatory bowel disease (IBD) (Rogers and Mousa, 2012), the prevention of intestinal and vaginal infections (Zuccotti *et al*., 2008), the reduction of food allergies (Savilahti *et al*., 2008), the prevention of cancer (Liong, 2008), the prevention of atherosclerosis (Tsai *et al*., 2009) and the amelioration of lactose intolerance (Heyman, 2000). These potential health benefits have induced the

food industry to develop appropriate methods and technologies for the incorporation of probiotics into various food products and nutraceutical formulations.

Probiotics have been incorporated mainly into dairy products, such as yoghurt, as these constitute favourable vehicles due to the acidic pH compared to other probiotic products; in yoghurt the pH is ~4 whereas in most chesses is >5.0. The development of non-dairy probiotic products such as fermented meats, cereals, vegetables, and fruit juices is a challenging task for the food industry in their effort to expand the range of probiotic containing foods (Luckow and Delahunty, 2004, Champagne *et al*., 2005). Fruit juices are a promising vehicle for the delivery of probiotics as they contain relatively high amount of sugars, essential vitamins and minerals, which could be used as energy and nutrition sources for the survival of probiotics during storage (Ding and Shah, 2008) and offer an alternative choice to consumers with lactose intolerance (Prado *et al*., 2008). Moreover, the fruit juice market is very large, as fruit juices are easily consumed by various ages. However, a key for successfully developing new probiotic products, particularly in the case of fruit juices, is to ensure the survival of the probiotic strain in the fruit juices during refrigerated storage for up to 6 weeks, which is the normal storage period for fruit juices. Considering a starting cell concentration in juices of around 10^8 to 10^9 colony forming units (CFU)/ml and the fact that the recommended minimum probiotic concentration for the efficacy is approximately 10^7 CFU/ml of product (Corcoran *et al*., 2007), this suggests that the decrease in cell concentration during storage should be less than 1 to 2 log CFU/ml. A substantial amount of work has been conducted where probiotic strains have been incorporated in a variety of fruit juices, including orange, apple, pineapple, peach, cranberry, strawberry and pomegranate; the results have demonstrated that cell survival depends on the strain and juice used but overall in many cases it was rather low due to the high acidity of the

juices and the likely presence of antimicrobial compounds, such as polyphenols and organic acids, at high concentrations (Vinderola *et al*., 2002, Sheehan *et al*., 2007a, Champagne and Gardner, 2008, Tezcan *et al*., 2009, Nualkaekul and Charalampopoulos, 2011, Mousavi *et al*., 2011).

Overall, several approaches have been used to enhance the cell viability of probiotic strains in acidic conditions, including the development and modification of the food matrices (Corcoran *et a*l., 2005), microencapsulation (Nualkaekul *et al*., 2012), and adaptation of the cells to acid stress (Saarela *et al*., 2011). Amongst these, encapsulation has been investigated the most; however, the main issue is that in many cases the size of the capsules produced by the most scalable method (extrusion) is rather large, normally between 500 μm and 3 mm, which can affect considerably the organoleptic properties of the product. In order to achieve microcapsules with size less than 200 μm, an emulsification based microencapsulation method has to be used, which is difficult to scale up and also to remove the residual oil from the capsules. A short exposure of probiotic bacteria to acid prior to their incorporation into fruit juices followed by subsequent storage at refrigerated temperature (Saarela *et al*., 2011) seems to be a viable approach for reducing cell injury and loss of viability during processing, storage, and potentially during subsequent passage through the gastrointestinal tract (GIT) (Vinderola *et al*., 2000, Shah, 2000, Gueimonde *et al*., 2004). This strategy allows the cells to activate their defence mechanisms to acid stress, such as maintenance of ΔpH homeostatic, alteration of cell envelope, and protection and restoration of proteins and DNA, in order to protect them against the adverse environment of a food matrix, i.e. the highly acidic environment of fruit juices (Van de Guchte *et al*., 2002, Spano and Massa, 2006, Lorca and de Valdez, 2009). Based on this concept, several studies have been conducted using various acids for adapting bacterial cells, including lactic acid (Lorca

and de Valdez, 2001), hydrochloric acid (HCl) (Rallu *et al*., 1996) and malic acid (Saarela *et al*., 2011). Interestingly, the study of Pudlik and Lolkema (2011b) also highlighted the positive effects of citrate in enhancing acid tolerance of *Lactococcus lactis*. This study agrees with the results obtained by Nualkaekul and Charalampopoulos (2011) and Mousavi *et al*. (2011), who suggested that the high concentration of citric acid in various fruit juices appeared to improve the survival of *Lactobacillus plantarum* during refrigerated storage.

1.2. Probiotics

1.2.1. History of definition

The relationship between the consumption of food products containing microbes and human health was first discovered by Metchnikoff (1907), a Russian scientist. As a result of this, he was awarded a Noble prize in 1908. At the same time, Tissier (1906), a French paediatrician, also suggested the administration of bifidobacteria in children in order to reduce their suffering from diarrhoea. Subsequently, the diverse positive effects of certain beneficial microbes to human health were investigated in a number of studies. For instance, the implantation of non-LAB which can prevent pathogenic bacteria in intestine was demonstrated by Nissle (1916). Additionally, the important role of the intestinal bacteria for resistance to disease was verified by Bohnhoff *et al*. (1954), Freter (1955) and Collins and Carter (1978). However, it is interesting to notice that the first use of the word “Probiotic” was introduced by Lilly and Stillwell (1965), whereas Parker (1974) was the first to use the term probiotic in the sense that it is used today. The next definition of probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" was given by Fuller (1989). In 1992, Havenaar and Huis In't Veld extended the definition of

probiotics to: “A viable mono- or mixed culture of microorganisms which when applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora” (Havenaar and Huis In’t Veld, 1992). The definition of probiotics was continually modified by various researchers, including Salminen (1996), “A live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host” and Schaafsma (1996) “Oral probiotics are living microorganisms which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition”. In 2001, Schrezenmeir and de Vrese suggested a new definition of probiotics, “A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host” (Schrezenmeir and de Vrese, 2001). Finally, probiotics are currently internationally endorsed as “Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”, a definition given by FAO/WHO (2002). This last definition is rather broad compared to the previous ones, and includes both human and animal applications of probiotics, as well as a broad spectrum of benefits to the host and not just to the gut.

1.2.2. Functional properties and health benefits of probiotics

During the last two decades, probiotics have been widely used for treatment and prevention of various diseases, such as diarrhoea and acute gastroenteritis, IBD, lactose intolerance, food allergies, hypercholesterolemia and cardiovascular diseases, and cancers. However, the causal relationships between their health claims and mechanisms of action have not been fully elucidated (Marco *et al*., 2006). In order to understand how probiotics deliver health benefits to their hosts, the current knowledge relating to

the mechanisms of action of probiotics toward human health are summarized in Table 1.1 and discussed throughout this section.

Table 1.1 Causal relationships between diseases and mechanisms of action of probiotics

Diseases	Causes	Mechanism of actions
Diarrhoea	Overgrowth of bacteria and viruses	1. Modulation of gut microbiota • Production of antimicrobial compounds • Competition for binding and receptor sites • Improvement of mucus production 2. Immunomodulation • Enhancement of production of IgA • Activation of NK cells
IBD	Chronic inflammation of all or part of GIT caused by bacterial infection	1. Anti-inflammation • Production of anti-inflammatory cytokines 2. Inhibition of intestinal epithelial apoptosis • Activation of Akt/protein kinase B • Inhibition of p38/ MAPK
Lactose intolerance	Lactose maldigestion	1. Production of lactose cleaving enzyme, *β*-galactosidase
Food allergies	Type-1 hypersensitivity	1. Anti- hypersensitivity and allergy • Inhibition of IgE
Hypercholesterolemia and cardiovascular diseases	High cholesterol level in the blood	1. Assimilation and binding of dietary cholesterol in the intestine 2. Hydrolysis of bile acids by production of bile salt hydrolase
Colon cancer	Carcinogens and mutagens produced by enteric bacteria	1. Ability to bind and degrade potential carcinogens 2. Production of anti-tumorigenic or anti-mutagenic compounds

1.2.2.1. Treatment and prevention of disorders associated with GIT

Probiotics have been reported to improve many types of diarrhoea with different degrees of success. Several probiotic strains, such as *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 have been used in an attempt to modulate the indigenous

intestinal microflora in children who develop diarrhoea (Guandalini, 2011). A number of positive effects, such as decreasing number of incidents, shortening of duration, and reducing of viral shedding have been observed after administration of these probiotics (Tuohy *et al*., 2003). Although the mechanisms of action behind these beneficial effects are still unclear, the most impressive evidence in probiotics combating pathogens and diseases is achieved by modulating the gut microbiota (e.g. the production of antimicrobial compounds, competition for binding and receptor sites and increased mucus production) as demonstrated by several *in vitro* and *in vivo* studies. For example, Ogawa *et al*. (2001) showed that the co-incubation of *Lactobacillus casei* strain Shirota or *Lactobacillus acidophilus* YIT0070 with *Escherichia coli* O157:H7 strain 89020087 in a batch fermentation system reduced the survival of *E. coli* due to the production of lactic acid. Similarly, Corr *et al*. (2007) demonstrated that the administration of *Lactobacillus salivarius* UCC118 in mice enhanced their resistance against invasion of the foodborne pathogen *Listeria monocytogenes* due to the production of the broad-spectrum class II bacteriocin *(*Abp118). Mack *et al*. (1999) reported that co-incubation of *L. plantarum* 299v and *L. rhamnosus* GG with HT-29 human intestinal epithelial cells increased the expression levels of the MUC2 and MUC3 proteins. Upregulation of these two human intestinal proteins by both bacterial strains enhanced mucus production in HT-29 human intestinal epithelial cells, which resulted in inhibiting the attachment of pathogenic *E. coli* to the cell line. However, it should be noted that the immunomodulation properties of probiotics are also involved in the treatment of diarrhoea, especially diarrhoea caused by rotavirus. For instance, *L. rhamnosus* GG and *Bifidobacterium lactis* Bb-12 were shown to enhance immunoglobulin A (IgA) production in the microfold cells of the Peyer's patches which contributed to the protection of human host against viruses. Such immunomodulatory effects might be

induced by their cell wall components (Majamaa *et al*., 1995, Fukushima *et al*., 1998). Additionally, Takeda *et al*. (2006) found that *L. casei* strain Shirota could trigger the production of interleukin (IL) -12 which elicited the response of natural killer (NK) cells against pathogen infections.

In addition to the above, the beneficial effects of probiotics in the GIT have also been reported for the management of IBD, including ulcerative colitis and Crohn's disease. In this case, the plausible mechanisms of action seem to involve the anti-inflammatory properties of probiotics, as the pathogenesis of both Crohn's disease and ulcerative colitis is associated with the chronic inflammation of all or part of the GIT. As probiotics have been proved to reduce inflammatory responses, the administration of probiotics is used to reduce the expression of proinflammatory cytokines, especially tumour necrosis factor alpha (TNF-α), IL, interferon, and nuclear factor-kappa B, which play an important role in cell signalling and regulation of inflammatory responses. For instance, Kamada *et al*. (2008) indicated that *E. coli* strain Nissle 1917 displayed a direct anti-inflammatory activity on HCT15 intestinal epithelial cells by suppressing the production of IL-8, a neutrophil chemotactic factor, leading to the reduction of TNF-α; Hart *et al*. (2004) showed that a commercial mixture of probiotics, VSL#3, consisting of four lactobacilli, three bifidobacteria, and one streptococci strain, induced the production of IL-10, an anti-inflammatory cytokine, after co-incubation with blood cells and lamina propria dendritic cells *in vitro*. However, several studies showed that the beneficial effect of probiotics for the treatment of IBD is not limited only to the reduction of inflammatory response, but also involves the inhibition of intestinal epithelial apoptosis; as this mechanism has been proved to enhance the survival of cells during development of IBD. Yan and Polk (2002) suggested that a co-culture of *L. rhamnosus* GG with either young adult mouse colon cells or HT-29 human intestinal

epithelial cells can activate anti-apoptotic factor Akt/protein kinase B and inhibit pro-apoptotic p38/mitogen-activated protein kinase (MAPK) in epithelial cells, resulting in the reduction of epithelial apoptosis via a proinflammatory cytokine dependent mechanism.

1.2.2.2. Relief of lactose intolerance

It is well known that people with lactose intolerance can tolerate lactose in yoghurt much better than in milk, even though yoghurt and milk have similar amounts of lactose (Gurr, 1987). This is due to the fact that probiotic strains in yoghurt have the ability to produce the lactose cleaving enzyme, *β*-galactosidase, leading to the reduction of lactose present in the digestive tract (De Vrese *et al*., 2001). Furthermore, many studies have also reported the effect of probiotics in the relief of lactose intolerance. For example, Almeida *et al*. (2012) showed that four-week consumption of a probiotic mixture (*L. casei* strain Shirota and *Bifidobacterium breve* strain Yakult) improved lactose intolerance symptoms (e.g. bloating, cramps, and loose stools) in lactose-intolerant patients. These results indicate that lactose intolerance can be improved by regularly consuming fermented dairy products due to the production of *β*-galactosidase enzyme by the probiotic strains present in them. This mechanism of action is perhaps the strongest evidence of a health benefit exerted by probiotics and is the only mechanism currently accepted by the European Food Safety Authority (Hill *et al*., 2014).

1.2.2.3. Reduction of food allergies

The idea of alleviating food hypersensitivity and allergy by probiotics is mainly based on their ability to suppress IgE production, as food hypersensitivity and allergy generally occur when IgE mistakenly treats harmless food compounds as a threat. This

leads to the secretion of vasoactive amines (i.e. histamines) by mast cells and basophils via type-1 hypersensitivity which then triggers an allergic reaction. Shida *et al*. (2002) reported that the administration of *L. casei* strain Shirota in mice can suppress IgE production by promoting a dominant Th1-type immune response mediated by IL-12 induction. Dev *et al*. (2008) also found that the administration of a probiotic mixture (*Bifidobacterium longum* and *Bifidobacterium infantis*) suppressed the increase of histamine receptor-1 and histidine decarboxylase, an enzyme which synthesizes histamine from L-histidine, in rat nasal mucosa. Therefore, there is less histamine production in rat fed with these probiotics. Additionally, Schiffer *et al*. (2011) showed that the injection of *L. casei* into mice inhibited IgE-induced passive systemic anaphylaxis and mast cell activation. It must be highlighted, however, that the uses of probiotics for food hypersensitivity and allergy are only able to protect against the condition rather than cure them.

1.2.2.4. Lowering of cholesterol

There is some evidence showing that probiotics could potentially lower the levels of plasma cholesterol. This benefit seems to occur through the assimilation of dietary cholesterol in the intestine by probiotics and through the deconjugation of bile acids by the bile salt hydrolase enzyme produced by probiotics (Zhuang *et al*., 2012). To this end, Liong and Shah (2005) demonstrated that *L. acidophilus* and *L. casei* could remove cholesterol when grown in culture medium under conditions similar to those found in the human intestine; however, the amount of cholesterol removed from the media was strain specific. This ability of probiotics was also demonstrated by Lye *et al*. (2010) who found that *L. acidophilus* and *L. bulgaricus* were able to remove cholesterol from the media and accumulate it onto their cell surface. These *in vitro* results

demonstrated that probiotics could lower plasma cholesterol through reduction of available cholesterol in the intestine. Moreover, Jones *et al*. (2004) found that bile salt hydrolase produced by *L. plantarum* was able to hydrolyse conjugated bile acids to deconjugated bile acids. This leads to substantial loss of bile acids which play an essential role in fat digestion, as deconjugated bile acids are less soluble and difficult to reabsorb back through the intestine than their conjugated form. Therefore, serum cholesterol was used by the liver in order to replenish the bile acids which are lost in faeces due to hydrolysis by the bile salt hydrolase produced by *L. plantarum*. However, the ability of probiotics in lowering cholesterol is still in doubt as some of these studies have reported a significant effect, while others have not.

1.2.2.5. Prevention of cancer

Although there is some evidence of cancer-preventing properties of probiotics, which have been reported in animal and human studies, the exact role of probiotics in the context of anticancer activities and the mechanisms of action have not been clearly elucidated. However, it has been suggested that a possible mechanism could involve the binding and degrading of potential carcinogens, the production of anti-tumorigenic or anti-mutagenic compounds in the colon, and enhancement the host's immune response (Hirayama and Rafter, 2000). For example, Goldin and Gorbach (1984) reported that the administration of *L. acidophilus* NCFM and N-2 reduced the levels of faecal enzymes (i.e. β-glucuronidase, azoreductase, nitro-reductase and urease) which can convert procarcinogens to carcinogens in human subjects. Pool Zobel *et al*. (1996) also showed that a probiotic mixture (*L. acidophilus*, *Lactobacillus gasseri* P79, *Lactobacillus confusus* DSM 20196, *Streptococcus thermophilus* NCIM 50083, *Bifidobacterium breve* and *Bifidobacterium longum*) prevented the induction of DNA

damage by *N*-methy-*N'*-nitro-*N*-nitrosoguanidine in colon cells of rats. Overall, however, the existing amount of evidence is not sufficient to establish a correlation between probiotic administration and cancer treatment, in human subjects. More studies are therefore required before such a claim can be made.

1.2.3. Selection of probiotic strains

Probiotics have been recognized as functional food ingredients due to the advancement of scientific evidence indicating their potential health-promoting effects, as discussed above. Several microbial strains, mainly *Bifidobacterium* species and *Lactobacillus* species (Table 1.2), have been incorporated into various food products and claimed as probiotics. The vast amount of strains used seems to lead to skepticism amongst the general population (i.e. potential customers) regarding the efficacy of probiotic strains. These issues have been diminished by the guidelines for the evaluation of probiotics in food, produced by FAO/WHO (2002). These guidelines state that probiotics need to meet certain requirements before they can be claimed as probiotics. More specifically, i) genus, species, and strain need to be identified and declared, ii) positive health benefits need to be tested *in vitro* and in animal studies, iii) safety information needs to be presented, iv) clinical trials need to be conducted, and v) content, dosage, shelf-life, and health claim need to be labelled. These criteria should ensure that probiotics are regulated and made in a way which is safe for human consumption and effective. However, recently the concept of what can be defined as probiotics was revised in 2014 by an expert group of international scientists working on behalf of FAO/WHO (Hill *et al*., 2014). The principal concepts were the same as previous guidelines with the exception of one modification; a core group of well-studied probiotic species such as *Bifidobacterium adolescentis*, *Bifidobacterium animalis*,

Bifidobacterium bifidum, Bifidobacterium breve, L. acidophilus, L. casei, L. fermentum, L. gasseri, L. johnsonii, L. paracasei, L. plantarum, L. rhamnosus, and *L. salivarius,* can ascribe the general beneficial effects on gut. Clinical trials are not essential to be conducted on specific strains belonging to these species in order to claim their general health effects. However, these guidelines have not explicitly stated the essential properties that microorganisms should have in order to be considered as probiotics. For example, Collins *et al*. (1998) suggested that a good probiotic strain should have some of the following properties: i) it should originate from humans so that it can adhere to and colonise the human GIT; ii) it should have a history of being non-pathogenic even in immunocompromised hosts so that it is safe to use in humans; iii) it should tolerate the low pH of the gastric juice and high concentrations of both conjugated and unconjugated bile acids so that it can survive during passage through the upper GIT; iv) it should exhibit antibacterial properties (e.g. produce lactic acid) so that it can inhibit the growth of potentially pathogenic bacteria; v) it should stimulate or inhibit immune response via epithelial cells, dendritic cells, monocytes/macrophage and lymphocytes in the digestive tract so that it can modulate the host's immune responses; vi) it should survive during product manufacturing and storage until the time of consumption so that it can deliver the desired health benefits to the host. Using these criteria for microbial selection should increase the possibility for obtaining potential probiotic strains that can be used in food products. However, it needs to be emphasised that certain of the above properties are strain specific, thus effective probiotic screening programs are important for selecting appropriate strains. Additionally, the knowledge derived from human microbiome research using a range of emerging molecular methods, such as metagenomics, metatranscriptomics and metabolomics will further enhance our understanding about the correlations between microbiota and their impact on human

health or diseases and also assist in the identification of novel probiotic strains from indigenous microbial species (Hemarajata and Versalovic, 2012). Finally, it is essential to note that there were a number of reasons for selecting *L. plantarum* NCIMB 8826 as the model potential probiotic strain in this thesis. The strain is of human origin and has a good safety history in clinical trials (De Vries, 2006). The strain has demonstrated good survival *in vitro* and *in vivo*, e.g. it can survive well in model gastric solutions at pH<3 (Charalampopoulos *et al*., 2003, Parente *et al*., 2010), it can persist in the mouse intestine for 10 days after oral administration (Pavan *et al*., 2003), and it can resist the conditions of the human digestive tract (up to ileum) with 7% survival (Vesa *et al*., 2000). A number of potential health benefits have been shown (e.g. modulation of gut microbiota, anti-inflammation, anti-allergic, and immunomodulation) *in vitro* and in animal models. For example, the modulation of gut microbiota through competition for binding and receptor sites was shown by Sánchez *et al*. (2009) and Hevia *et al*. (2013). The serine/threonine-rich proteins which were secreted by *L. plantarum* NCIMB 8826 displayed an ability to bind to mucin and fibronectin located on human epithelial cells. From these results, they suggested that the production of these proteins helped in improving the adhesion and colonization of this bacterium to the human intestinal mucosa. The anti-inflammatory properties of this strain were reported by Foligné *et al*. (2006). They showed that daily gavage administration of *L. plantarum* NCIMB 8826 with a cell concentration of 10^8 to 10^9 CFU helped to reduce colitis induced by trinitrobenzene sulfonic acid in mice. They demonstrated that this process occurred due to the production of IL-10, an anti-inflammatory cytokine, by this strain after co-incubation with human peripheral blood mononuclear cells *in vitro* (Foligne *et al*., 2007). The immunomodulation activity of *L. plantarum* NCIMB 8826 was demonstrated by Dong *et al*. (2012). They showed that this strain was able to induce the

production of IL-12 *in vitro* which helps to promote the development of T-cells and stimulate the secretion of IFN-γ. These lead to the activation of T-cells and NK cells which respond to viral infection and tumour formation. The anti-allergic activity of this strain was also reported by Pochard *et al*. (2005). They found that this strain could reduce the effect of mite allergen (Der p 1) on dendritic cells via the reduction of IL-4 which was involved in the development of B-cells, as well as decreased IL-5 which stimulates B cell growth, and increase immunoglobulin secretion. From these results, it seems likely that the administration of *L. plantarum* NCIMB 8826 may have led to change in IL-4 and IL-5 production/level by host cells which resulting in the lower production of antibodies in allergic patients after challenge with mite allergen compared with healthy people.

Certain health benefits have been also demonstrated in human trials. Karczewski *et al*. (2010) reported that oral administration of *L. plantarum* NCIMB 8826 was able to decrease the epithelial barrier dysfunction in humans by increasing the localization of zonula occludens-1 and transmembrane proteins, which are the essential proteins for strengthening the tight junctions, via the toll-like receptor 2 signalling pathway. The complete genome sequence of this bacterium is available. This information should allow the molecular investigation of certain genes, (e.g. those responsible for the synthesis of antimicrobial compounds and exopolysaccharide (EPS) production, and sugar metabolism) which can affect the adaptability and survival of the cells in foods as well as in the GIT. *L. plantarum* NCIMB 8826 can thus be considered a potential probiotic strain as it meets the requirements underlined in the guidelines for the evaluation of probiotics by FAO/WHO (2002) and it is also one of most well-defined probiotic strains which can ascribe the general beneficial effects on gut physiology and human health of *L. plantarum* at species level (Hill *et al*., 2014).

Table 1.2 List of microorganisms considered as probiotics adapted from Holzapfel *et al*. (2001)

***Lactobacillus* species**	***Bifidobacterium* species**
Lactobacillus acidophilus *Lactobacillus amylovorus* *Lactobacillus brevis* *Lactobacillus casei* *Lactobacillus casei* subsp. *rhamnosus* *Lactobacillus crispatus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus fermentum* *Lactobacillus helveticus* *Lactobacillus gallinarum* *Lactobacillus gasseri* *Lactobacillus johnsonnii* *Lactobacillus paracasei* *Lactobacillus plantarum* *Lactobacillus reuteri* *Lactobacillus rhamnosus*	*Bifidobacterium adolescentis* *Bifidobacterium animalis* *Bifidobacterium bifidum* *Bifidobacterium breve* *Bifidobacterium infantis* *Bifidobacterium lactis* *Bifidobacterium longum*
Other LAB	**Non-LAB and yeast**
Enterococcus faecalis *Enterococcus faecium* *Lactococcus lactis* subsp. *cremoriss* *Lactococcus lactis* subsp. *lactis* *Leuconostoc mesenteroides* *Pediococcus acidilactici* *Sporolactobacillus inulinus* *Streptococcus thermophilus*	*Bacillus cereus* var. *toyoi* *Escherichia coli* strain Nissle *Propionibacterium freudenreichii* *Saccharomyces cerevisiae* *Saccharomyces boulardii*

1.2.4. Products with probiotics on the market

At present, there are various dairy products supplemented with probiotic bacteria on the market, e.g. fermented dairy products, cottage cheese, ice cream, frozen dairy desserts (Shah, 2007), but the top selling product has always been yoghurt. It must be noted though that the commercial success of yoghurt ultimately depends on its taste, appearance, price, and in addition to those, to potential health benefits to consumers (Heller, 2001). From a technical point of view, yoghurt is considered as a very good probiotic carrier because of the high buffering capacity of milk (Salminen and Playne,

2001). Moreover, the pH of yoghurt normally ranges from pH 4.0 to 4.4 (Hamann and Marth, 1984) which is suitable for long term refrigerated storage of *Lactobacillus* and *Bifidobacterium* species (Rivera-Espinoza and Gallardo-Navarro, 2010).

The development of non-dairy probiotic products, such as fermented meats (e.g. dry sausages), cereals (e.g. sourdough), vegetables (e.g. sauerkraut) and fruit juices, is a challenging task for the food industry in its efforts to expand the range of probiotic containing foods (Luckow and Delahunty, 2004, Champagne *et al*., 2005). Table 1.3 presents a list of potential non-dairy probiotic products which have been investigated. According to Luckow and Delahunty (2004), Champagne *et al*. (2005), and Rivera-Espinoza and Gallardo-Navarro (2010), a number of factors need to be considered for the successful development of probiotic products including: i) strain selection, ii) physiological state of the probiotic strain, iii) physical conditions during processing and storage, iv) physicochemical parameters of the product, e.g. pH, water activity, carbon, nitrogen, mineral and oxygen content, and v) organoleptic properties and consumer acceptability. Considering the above criteria, it can be suggested that fruit juices are promising vehicles for the delivery of probiotics as they contain relatively high amounts of sugars, minerals, and vitamins, which could be used as energy and nutrition sources for the survival of probiotics during storage (Ding and Shah, 2008) and offer an alternative choice to consumers with lactose intolerance (Prado *et al*., 2008). Moreover, fruit juices have sensory profiles which are acceptable to all age groups and are also perceived by consumers as being healthy and refreshing foods (Rivera-Espinoza and Gallardo-Navarro, 2010).

Table 1.3 List of non-dairy products containing probiotics adapted from Granato *et al*. (2010)

Category	Product
Fruit and vegetable based products	- Vegetable-based drinks - Fermented banana pulp - Fermented banana - Beets-based drink - Tomato-based drink - Many dried fruits - Green coconut water - Peanut milk - Cranberry, pineapple, and orange juices - Ginger juice - Grape and passion fruit juices - Cabbage juice - Carrot juice - Noni juice - Onion - Banana puree - Non-fermented fruit juice beverages - Blackcurrant juice
Soy based products	- Non-fermented soy-based frozen desserts - Fermented soymilk drink - Soy-based stirred yoghurt -like drinks
Cereal based products	- Cereal-based puddings - Rice-based yoghurt - Oat-based drink - Oat-based products - Yosa (oat-bran pudding) - Mahewu (fermented maize beverage) - Maize-based beverage - Wheat, rye, millet, maize, and other cereals fermented beverages - Malt-based drink - Boza (fermented cereals) - Maize, sorghum, and millet malt fermented beverages - Millet or sorghum flour fermented beverage
Other non-dairy products	- Starch-saccharified drink - Cassava-flour product - Meat products - Dosa (rice and chickpea)

1.2.5. Probiotic-containing fruit juices

A considerable amount of research has been conducted to address the cell survival relevant to the incorporation of probiotics in various fruit juices including orange, apple, kiwi, pineapple, peach, cranberry, cashew apple, strawberry, and pomegranate juices (Table 1.4). Strain selection is of high importance considering that not all species or strains can tolerate a highly acidic environment, such as that of fruit juices (pH 2.5 to 3.5). Among the LAB, lactobacilli are considered more robust than other genera, such as lactococci and streptococci. Besides the intrinsic properties of the strains, the type of juice used affects cell survival significantly. For example, *L. casei*, *L. rhamnosus* and *L. paracasei* survived very well in orange and pineapple juice for more than 12 weeks of refrigerated storage, whereas in cranberry juice they died very quickly (<9 days) (Sheehan *et al*., 2007a). Very low survival was also observed for *L. delbruekii* and *L. plantarum* in pomegranate juice after 2 weeks of storage (Mousavi *et al*., 2011). Research carried out by our research group demonstrated that the cell viability of *L. plantarum* in cranberry juice decreased by more than 8 log CFU/ml within a week of storage (Nualkaekul and Charalampopoulos, 2011). These results were similar to the work of Sheehan *et al*. (2007a), who demonstrated that the cell viability of *L. salivarius*, *L. casei* and *Bifidobacterium lactis* decreased by more than 4 log CFU/ml while that of *L. rhamnosus* and *L. paracasei* decreased by approximately 3 log CFU/ml after 2 days of storage in cranberry juice (Sheehan *et al*., 2007a). The main physicochemical factor influencing cell survival is the pH of the juice; for example cranberry and pomegranate juices have a pH between 2.5 and 2.8, whereas the pH of orange, apple and pineapple is higher, ranging from 3.3 to 4.0. Besides pH, most juices have large amounts of organic acids, in particular citric and malic acid (Flores *et al*., 2012), which are likely to have adverse effects on cell viability in their undissociated form (this happens at a pH higher

than their pKa value), as they can easily enter into the cell leading to a decrease in intracellular pH (pH_i) and eventually to cell death (Beales, 2004). Moreover, fruit juices and in particular juices from berries contain considerable amount of polyphenols, some of which have antimicrobial activities against LAB, including lactobacilli (Puupponen-Pimiä *et al*., 2001).

Table 1.4 Survival of probiotic bacteria in various types of fruit juices

Microorganism	Fruit juices	Starting cells (CFU/ml)	Duration of storage when cell viability was higher than 10^6 (CFU/ml)	References
- *L. salivarius* subsp. *salivarius* UCC118 - *L. salivarius* subsp. *salivarius* UCC500 - *B. animalis* subsp. *lactis* Bb-12 - *L. paracasei* subsp. *paracasei* NFBC4338 - *L. rhamnosus* GG - *L. casei* DN- 114 001	Orange	10^8	1 week 1 week 6 weeks >12 weeks >12 weeks >12 weeks	(Sheehan *et al*., 2007a)
- *L. salivarius* subsp. *salivarius* UCC118 - *L. salivarius* subsp. *salivarius* UCC500 - *B. animalis* subsp. *lactis* Bb-12 - *L. paracasei* subsp. *paracasei* NFBC4338 - *L. rhamnosus* GG - *L. casei* DN- 114 001	Pineapple	10^8	1 week 1 week 4 weeks >12 weeks >12 weeks >12 weeks	
- *L. salivarius* subsp. *salivarius* UCC118 - *L. salivarius* subsp. *salivarius* UCC500 - *B. animalis* subsp. *lactis* Bb-12 - *L. paracasei* subsp. *paracasei* NFBC4338 - *L. rhamnosus* GG - *L. casei* DN- 114 001	Cranberry	10^8	<1 week	
- *L. acidophilus* - *L. brevis* - *L. rhamnosus* - *L. fermentum* - *L. plantarum* - *L. reuteri*	Blend of pineapple, apple, orange, pear and/or grape, passion fruit, lemon and purees (peach, strawberry, mango and kiwi)	10^7	4 weeks >11 weeks >11 weeks >11 weeks >11 weeks >11 weeks	(Champagne and Gardner, 2008)
- *L. acidophilus* DSMZ 20079 - *L. paracasei* DSMZ 15996 - *L. plantarum* DSMZ 20174 - *L. delbrueckii* DSMZ 20006	Pomegranate	10^7	<1 week <1 week 1 week 1 week	(Mousavi *et al*., 2011)
- *L. acidophilus* LA39 - *L. casei* A4 - *L. delbrueckii* D7 - *L. plantarum* C3	Beetroot	10^6	2 weeks 4 weeks 4 weeks 4 weeks	(Yoon *et al*., 2005)
- *L. casei* NRRL B-442	Cashew apple	10^7	6 weeks	(Pereira *et al*., 2011)
- *L. plantarum* NCIMB 8826	Orange Grapefruit Blackcurrant Pineapple Lemon Pomegranate Cranberry	10^8	6 weeks 6 weeks 6 weeks 6 weeks 6 weeks 2 weeks <1 week	(Nualkaekul and Charalampopoulos, 2011)

1.2.6. Genetically modified (GM) probiotics in foods

Although a variety of techniques has been developed to generate GM microorganisms, such as cloning systems, chromosome modification systems and expression systems, few GM products find their way into the food market because the food products produced with the aid of GM microorganisms are banned in many countries (Sybesma *et al*., 2006). For example, the regulation for GM foods in the European Union (EU) is very stringent. Any genetic modifications that do not occur naturally are considered as GM microorganisms which are unacceptable in food production (Kondo and Johansen, 2002). However, the regulatory standpoint on GM foods is different from country to country. In the United States of America (USA), the commercial opportunities for GM foods are more promising for the future than in EU because the FDA regulation for GM foods, which is known as generally recognized as safe (GRAS), focuses on the nature of the products, rather than the process in which they are created (Kondo and Johansen, 2002). This means that the method of deriving new GM foods is not the primary concern in the evaluation of safety but instead the safety of the final product is given the highest consideration. As a result many products produced from GM microorganisms are launched into the food market in USA, such as cellulase enzyme derived from a GM-*Myceliophthora*, eicosapentaenoic acid-rich triglyceride oil derived from a GM-*Yarrowia lipolytjca*. However, it needs to be noted that no products with live GM LAB has been released on the market (Sybesma *et al*., 2006) or even accepted as GRAS yet. This might be due to the fact that GM LAB do not have a history of safe use as food ingredient which is the most important aspect of the GRAS criteria. Moreover, it is not possible to guarantee they are safe for human consumption based on limited scientific evidence currently available. To achieve this, the safety of GM microorganisms needs to be demonstrated including genetic stability,

potential gene transfer, and the interactions with indigenous microflora (Feord, 2002). Research in this area is very important as GM probiotics might be able to provide considerable, technological, functional and health benefits in the future.

1.2.7. Survival of GM-probiotics in the gut

It is well established that the beneficial effects of probiotics can be expected only when the cells are viable and thus are able to survive in the human gut. However, many potential probiotics die during passage through the GIT because they are challenged by many stressful conditions, such as acid stress, bile stress, oxygen stress, etc. Improving stress responses of probiotic strains by genetic modification might be an answer for enhancing the survival of probiotics during passage through the human gut. However, this strategy might not always ensure good survival in the GIT because of unintended effects occurring during genetic modification (e.g., inhibition of specific metabolites from the overproduction of certain proteins, toxicity associated with overexpression of target proteins), which might impact cell survival.

To our knowledge, there is a limited number of studies on the survival of engineered stress resistant probiotics in the gut. The introduction of *betL* gene, encoding for a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes*, into *Bifidobacterium breve* UCC2003 was performed to improve the survival of *Bifidobacterium breve* UCC2003 in the gut, as this gene has been found to increase the pressure stress (Smiddy *et al*., 2004) and viability in certain foods (Sleator *et al*., 2003). It was found that the strain after insertion of the *betL* gene exhibited significantly increased tolerance to gastric juice and to conditions of elevated osmolarity mimicking the gut environment when compared to the control strain lacking *betL* gene (Sheehan *et al*., 2007b). Moreover, the strain having the *betL* gene was able to survive

and colonize better in murine intestine than the control strain (Sheehan *et al*., 2007b). Similarly, the transformation of *bilE* genes involved in bile stress response in *Listeria monocytogenes* were cloned into *Lactococcus lactis* NZ9000 and *Bifidobacterium breve* UCC2003. The results showed that both strains harbouring *bilE* genes exhibited 2.5 log CFU/ml higher survival rate than the wild type strains when grown in porcine bile at concentrations similar to that found in the intestine. Furthermore, they persisted longer in the murine GIT and were recovered in higher amounts from murine faeces compared to the control strains (Watson *et al*., 2008). Based on these promising results, it seems likely that a LAB strain engineered to be resistant to a variety of stresses could possibly be able to survive in the human gut better than wild type strains. This is a line of research worth exploring in an effort to improve the technological and functional properties of probiotics.

1.3. LAB

1.3.1. The taxonomy of LAB

As mentioned above, LAB are the bacteria that are most commonly used as probiotics. Species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are typical LAB associated with foods (Vandamme *et al*., 1996). The term LAB was introduced because these bacteria have the ability to ferment and coagulate milk and form lactic acid as a major end product. In 1919, Orla Jensen classified LAB based on their morphological and physiological characteristics. For several decades, the taxonomy of LAB was based on classical approaches. On account of the advances in molecular techniques, polyphasic approaches including chemotaxonomic and phylogenetic studies have become key criteria for the

classification of LAB. With the application of new bacterial systematics, LAB are recognized as a part of Gram-positive bacteria with low G+C content in DNA (≤55 mol%) and belong to the phylum Firmicutes, class Bacilli, and order Lactobacillales (Garrity and Holt, 2001). They are represented as a group of bacteria distributed in six families: *Aerococcaceae* (7 genera), *Carnobacteriaceae* (16 genera), *Enterococcaceae* (17 genera), *Lactobacillaceae* (3 genera), *Leuconostoccaeae* (4 genera) and *Streptococcaceae* (3 genera) (Tsakalidou and Papadimitriou, 2011). In total, more than 40 genera of LAB have been established; the largest genus is *Lactobacillus* which was first proposed by Beijerinck in 1901 (Versalovic and Wilson, 2008). Lactobacilli are Gram-positive bacteria, usually non-motile, non-spore forming rods and lack cytochromes as well as porphyrins. They are catalase negative, aerotolerant anaerobes, fastidious, acid-tolerant, and strictly fermentative. Their optimum growth temperature and pH range between 30 and 40 °C and from 5.5 to 6.2, respectively (Axelsson, 2004). At the time of writing, this genus comprised 214 recognized species. However, some species of the *Lactobacillus* genus have not been confirmed, as the phylogenetic relationship alone is not enough to elucidate the taxonomy. In order to resolve this issue, Claesson *et al*. (2008) used an array of whole-genome and single-marker phylogenetic approaches to establish four sub-generic groups, namely, Group A (*L. acidophilus*, *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus*, *L. johnsonii*, and *L. gasseri*), Group B (*L. salivarius*, *L. plantarum*, *L. reuteri*, *L. brevis*, and *Pediococcus pentosaceus*), Group C (*L. sakei* and *L. casei*), and Group D (*Leuconostoc mesenteroides* and *Oenococcus oeni*).

1.3.2. Overview of stress response in LAB

The LAB is a highly heterogeneous group, as these bacteria can be isolated from various environments, which reflects the diverse properties of this group. For example, *Lactobacillus suebicus* is able to grow at pH 2.8, *Enterococcus faecium* is able to survive to pH as high as 9.6, *Carnobacterium viridans* tolerates up to approximately 26 % NaCl, *Leuconostoc gelidum* can survive at low temperature (0 to 2 °C), and *L. delbrueckii* subsp. *delbrueckii* can endure high temperatures (55 °C) (Tsakalidou and Papadimitriou, 2011). The reason that LAB can survive in adverse environments is that they have developed stress-sensing systems and defence mechanisms (Hartke *et al*., 1996, Van de Guchte *et al*., 2002, Spano and Massa, 2006, Lorca and de Valdez, 2009). Like most other living organisms, LAB have genes/regulators responsible for each stress; the activation of defence mechanisms against a specific stress is commonly induced by the expression of several gene clusters resulting in a series of changes in their global regulatory network (VanBogelen *et al*., 1999, Van de Guchte *et al*., 2002, De Angelis and Gobbetti, 2004). For example: i) Heat shock induces the synthesis of a specific set of proteins known as heat shock proteins which are controlled by several regulators (Class I: HrcA, Class II: sigma factor, Class III: CtsR, and Class IV: unknown regulators). These proteins protect the cells by reducing the degradation and aggregation of proteins caused by high temperature. ii) Cold stress induces the synthesis of another set of proteins called cold shock proteins for which the control regulator is not clear. These proteins are synthesized in order to maintain membrane fluidity by increasing the degree of unsaturated fatty acids and increase the rate of transcription as well as translation by declining the coiling of a DNA strand. iii) Acid stress induces the synthesis of several proteins that are related to various stress response systems, such as F-ATPase, arginine/agmatine deiminase (ADI/AgDI), decarboxylases, cell membrane

and cell envelope biosynthesis. These proteins are produced to control ΔpH homeostasis, protect proton influx and repair damage of other proteins in response to acid stress. Moreover, the time required to initiate a stress response depends on the type of stress. For example, the response to acid, heat and osmotic shock requires less time (min), with acid stress requiring less than 20 min for the majority of changes to take place in the cell membrane, whereas the response to cold shock requires more time (h) (Fozo and Quivey Jr, 2004, Tsakalidou and Papadimitriou, 2011).

1.3.3. Responses of LAB to acid stress

Acid stress response has been previously reported in several LAB, e.g. *Lactococcus lactis* (Budin-Verneuil *et al*., 2005), *L. bulgaricus* (Fernandez *et al*., 2008), *L. reuteri* (Rollan *et al*., 2003), *L. delbrueckii* subsp. *bulgaricus* (Zhai *et al*., 2014), *L. casei* (Wu *et al*., 2012b), *L. rhamnosus* (Koponen *et al*., 2012), *L. acidophilus* (Lorca and de Valdez, 2001), *L. helveticus* (Guerzoni *et al*., 2001), *L. plantarum* (Pieterse *et al*., 2005) and *L. sanfranciscensis* (De Angelis *et al*., 2001). According to these studies, multiple systems have been proposed as the systems used by LAB to maintain their viability in low pH environments; the main ones include ΔpH homeostasis and alkalization of the external environment, and cell envelope alteration.

The main biochemical pathways involved in ΔpH homeostasis and the alkalization of the external environment include: increased H^+-ATPase or F-ATPase activities (Kullen and Klaenhammer, 1999), the ADI and AgDI pathway (De Angelis *et al*., 2002, Lucas *et al*., 2007), glutamate decarboxylase (GAD) (Sanders *et al*., 1998, Su *et al*., 2011) and the citrate fermentation pathway (Martín *et al*., 2004). H^+-ATPase or F-ATPase enzymes pump protons outside the cell via adenosine triphosphate (ATP) hydrolysis. This activity increases as the pH decreases and it has been demonstrated that

it is essential for cell viability at low pH (Kobayashi *et al*., 1984, Kobayashi *et al*., 1986, Nannen and Hutkins, 1991, Yokota *et al*., 1995, Miwa *et al*., 1997). The ADI pathway converts arginine to ammonia, ornithine/putrescine and carbon dioxide (CO_2). Ammonia production contributes to survival at low pH through the neutralization of the medium (Poolman *et al*., 1987). GAD converts glutamate to γ-aminobutyric (GABA) and CO_2, a proton consuming reaction, which also contributes towards the reduction of pH_i (Sanders *et al*., 1998, Azcarate-Peril *et al*., 2004, Feehily and Karatzas, 2013). The citrate fermentation pathway involves the release of lactate from the cytoplasm out of the cells through the exchange with divalent citrate present in the media. This system generates a proton motive force (PMF) which can be used for ATP synthesis in order to pump protons out of the cells (Pudlik and Lolkema, 2011a).

The second system of acid stress in LAB involves structural changes of the cell envelope. The aim of these changes is to decrease the influx of protons into the cells. More specifically, in order to decrease proton permeability into the cell, LAB can change the fatty acid composition of the cell membrane, in particular the ratio of unsaturated and saturated fatty acids as well as the concentration of cyclopropane fatty acid (CFA) (Guerzoni *et al*., 2001, Streit *et al*., 2008, Montanari *et al*., 2010, Wu *et al*., 2012b). Additionally, there are suggestions of an association between peptidoglycan synthesis and teichoic acid in the cell wall, which is responsible for binding of cations (Swoboda *et al*., 2010), and sensing environmental stress (Jordan *et al*., 2008).

1.3.3.1. The role of F-ATPase in acid stress response

A number of studies have established that the membrane bound F-ATPase is involved in enhancing the survival of bacteria such as *Salmonella typhimurium* (Kobayashi *et al*., 1984, Kobayashi *et al*., 1986), *Listeria monocytogenes* (Cotter *et al*.,

2000), *Lactococcus lactis* and *L. casei* (Nannen and Hutkins, 1991, Yokota *et al*., 1995) when challenged by acidic conditions. The main function of F-ATPase is to remove protons from the cytoplasm by pumping them through the membrane-bound subunit, at the expense of the ATP which is produced during glycolysis. The result is a cytoplasm that is more alkaline than the external environment. Amplification of the proton-translocating ATPase was shown to be involved in the regulation of cytoplasmic pH in *Enterococcus hirae* (Kobayashi *et al*., 1984). Subsequent studies showed that the differences in proton movement were directly attributed to the amount of F-ATPase being produced (Bender *et al*., 1986, Belli and Marquis, 1991). Correspondingly, Nannen and Hutkins (1991) demonstrated that the specific activity of F-ATPase from LAB increases as the extracellular pH moves from neutral to acidic. The F-ATPase structure was first established in *E. coli* by Walker *et al*. (1984) and consists of eight subunits. Five subunits (α, β, γ, δ, and ε) constitute a globular structure F_1, which is an extrinsic membrane domain, while the remainders (a, b, and c) form an intrinsic membrane-bound subunit, F_0. The F-ATPase in *E. coli* is encoded by the *apt* operon containing nine corresponding genes which are designated as A, B, C, D, E, F, G, H, and I (*atpIEBFHAGDC*), whereas F-ATPase in *L. acidophilus* is organized somewhat differently. It contains only eight corresponding genes, *atpBEFHAGDC*, in which the *atp*I, an unknown function gene, is absent (Kullen and Klaenhammer, 1999). The *atpBEF* gene encodes the membrane-bound element of the enzyme while the *atpHAGDC* establishes the catalytic site for ATP hydrolysis. Overall, the role of F-ATPase in the survival of LAB during acid stress is not clearly elucidated. However, in a previous study, Yokota *et al*. (1995) compared the survival of a *Lactococcus lactis* strain with that of its mutants, which were lacking F-ATPase, under growth conditions at pH 6 and 4. The parent strain showed significantly better survival in acidic conditions

than the mutant strains, indicating that membrane-bound ATPase is essential for this microorganism to survive at low pH, probably through its function of proton pumping for maintaining cytoplasmic pH levels. This confirmed previous work, which showed an upregulation of genes related to F-ATPase in LAB at low pH. For example, Kullen and Klaenhammer (1999) showed a significant upregulation of the *atpBEF* genes in *L. acidophilus* when incubated in de Man, Rogosa and Sharpe medium (MRS) at pH 3.5 compared to pH 5.6, whereas Ventura *et al*. (2004) demonstrated a rapid 15 fold increase in the expression of the *atpD* gene in *Bifidobacterium lactis* after exposure to MRS at pH 3.5 compared to pH 6.0. Duary *et al*. (2010) also demonstrated that increased transcription of the *atpD* gene following incubation of *L. plantarum* in MRS at different pH (pH 2.5, 3.5, 4.5 and 6.5) was correlated with increased acidity of the medium.

1.3.3.2. The role of ADI/AgDI pathway in acid stress response

The production of ammonia, which helps to raise the pH_i, appears to be one of the key mechanisms of acid resistance in LAB (Rollan *et al*., 2003, Wu *et al*., 2012a, Zhang *et al*., 2012). Ammonia can be produced through the fermentation of arginine and the less abundant amino acid agmatine. Some genera of LAB, including lactococci, streptococci, and lactobacilli can utilize the ADI pathway to survive in acidic environments. The ADI system converts arginine to ornithine, ammonia and CO_2, while concomitantly generating one mole of ATP, as shown in figure 1.1. The generation of ammonia increases the pH_i through the alkalization of the cytoplasm. This system is repressed by carbohydrates and is induced by arginine, but it is not clear whether induction is influenced by environmental pH (Poolman *et al*., 1987, Curran *et al*., 1995). The genetic organization of this system (*arcABCTD*) has been examined and consists of

arcA (encoding arginine deiminase), *arcB* (encoding ornithine transcarbamylase), *arcC* (encoding carbamate kinase), *arcT* (encoding putative transaminase), and *arcD* (encoding putative arginine/ornithine antiporter) (Zuniga *et al*., 1998). In addition to the ADI system, some LAB such as *Streptococcus mutans* (Griswold *et al*., 2004) and *Lactobacillus brevis* (Lucas *et al*., 2007) utilize agmatine as a substrate through the AgDI pathway to produce ammonia resulting in acid protection. The biochemical pathway is similar to that of the ADI system; in this case agmatine is converted to putrescine (Figure. 1.1). The genetic organization of the AgDI pathway operon in *Lactobacillus brevis* is *aguRBDAC* (Lucas *et al*., 2007). The role of ADI pathway in response to acid stress was confirmed by Wu *et al*. (2012a) and Zhang *et al*. (2012) who reported that the cell survival of *L. casei* in acidified MRS using lactic acid at pH 3.3 was improved (by ~1.4 to ~3.4 times) after adding 50 mM of aspartate or arginine into the media. More specifically, added aspartate can be absorbed and converted to arginine by argininosuccinate synthetase (Ass) and argininosuccinate lyase (Asl), while added arginine can be directly utilized leading to the production of ammonia via the ADI pathway in order to reduce the pH_i.

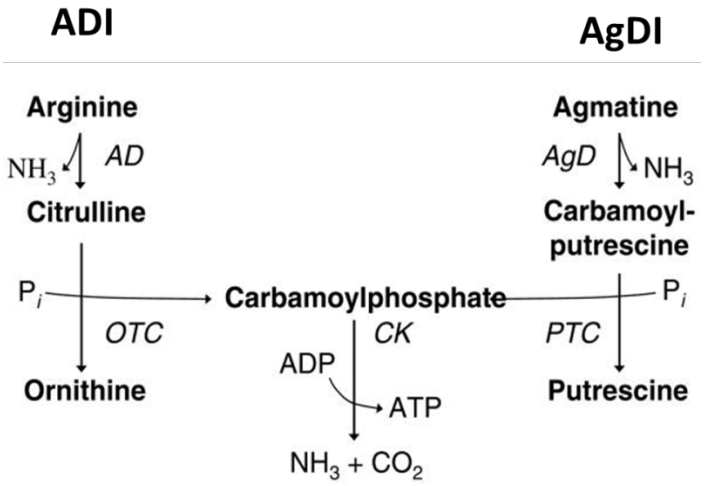


Figure 1.1 Simplified schemes describing the ADI and AgDI pathways in LAB (Griswold *et al*., 2009).

1.3.3.3. The role of decarboxylation reaction in acid stress response

Although several systems are utilized by LAB to withstand low pH, the GAD system is deemed to be one of the most important systems for acid resistance. GAD is present in many bacteria, but it varies across species. Lactobacilli seem to possess only one decarboxylase and one antiporter, which are *gadB* and *gadC*, respectively (Feehily and Karatzas, 2013). The GAD, *gadB*, converts internalized glutamate to GABA through the consumption of a proton with the concomitant production of a molecule of CO_2, while the putative membrane protein, *gadC*, is suggested to be involved in the antiporter of glutamate and GABA. The net result of the combined action of *gadCB* is the removal of a proton from the cytosol, which leads to an increase in the internal pH. However, this system requires the presence of glutamate and Cl^- ions (Sanders *et al*., 1998). Chloride-dependent activation of *gadCB* is controlled by *gadR*, a regulatory gene located just upstream of *gadCB*. Overall, a putative GAD system has been shown to confer acid resistance to LAB in media supplemented with glutamate (Komatsuzaki *et al*., 2005, Su *et al*., 2011). Although there are no published work confirming whether the GAD system can enhance the survival of LAB, it is interesting to note that *L. reuteri* wild type cells incubated in phosphate buffer with 10 mM glutamate (pH 2.5 using HCl, 37 °C, 24 h) were able to produce and secrete GABA out of the cells but this was not the case of the mutant strain which lacked *gadB* (Su *et al*., 2011).

1.3.3.4. The role of the citrate fermentation pathway in acid stress response

The citrate fermentation pathway has been implicated in the acid adaptation of LAB and has been studied in detail, primarily in *Lactococcus lactis* (García-Quintáns *et al*., 1998). This pathway involves the uptake of citrate into the cell by exchange of lactate produced from the glycolysis pathway. Once citrate is inside the cell, it is

converted to pyruvate; for this to be done three enzymes are required, i.e. citrate permease, citrate lyase and oxaloacetate decarboxylase (Hugenholtz, 1993). The first step is the conversion of citrate to acetate and oxaloacetate by citrate lyase. Then, oxaloacetate is converted to pyruvate and CO_2 by consuming a proton which is catalyzed by oxaloacetate decarboxylase as shown in figure 1.2. It has been suggested that the presence of citrate in the growth medium induced the activity of citrate lyase (Bekal-Si Ali *et al*., 1999, Bott, 1997, Martín *et al*., 2000). The genetic organization for citrate fermentation in *Lactococcus lactis* (*citM-citCDEFXG*) has been examined and consists of *citM* (encoding malic enzyme), *citCXG* (encoding accessory genes required for the synthesis of an active citrate lyase complex), and *citFED* (encoding the α, β, and γ subunits of citrate lyase (Martín *et al*., 2004). According to Martín *et al*. (1999) and Pudlik and Lolkema (2011a) the citrate fermentation pathway also generates a PMF through the electrogenic membrane exchange of divalent citrate and monovalent lactate, which is catalyzed by citrate permease (*citP*). The expression of this gene is induced at a transcriptional level by the acidification of the medium (García-Quintáns *et al*., 1998, Martín *et al*., 1999, Martín *et al*., 2004). The citrate fermentation pathway is utilized by LAB, including members of the genera *Lactoccocus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* (Poolman *et al*., 1991). In contrast, the malolactic fermentation pathway uses the PMF generated from the exchange of malate instead of citrate to secrete lactic acid out of the cytoplasm in order to help the cells survive in acidified conditions (Sheng and Marquis, 2007). Although there is no information currently demonstrating an improvement of cell viability after acid stress and the potential role of the citrate fermentation pathway, it must be noted that citric acid has been shown to support *L. plantarum* DSM 20174 growth under acidic conditions (pH 4 to 5) when used in

combination with glucose, although to a lesser extent than when used as the sole carbon source (Kennes *et al*., 1991).

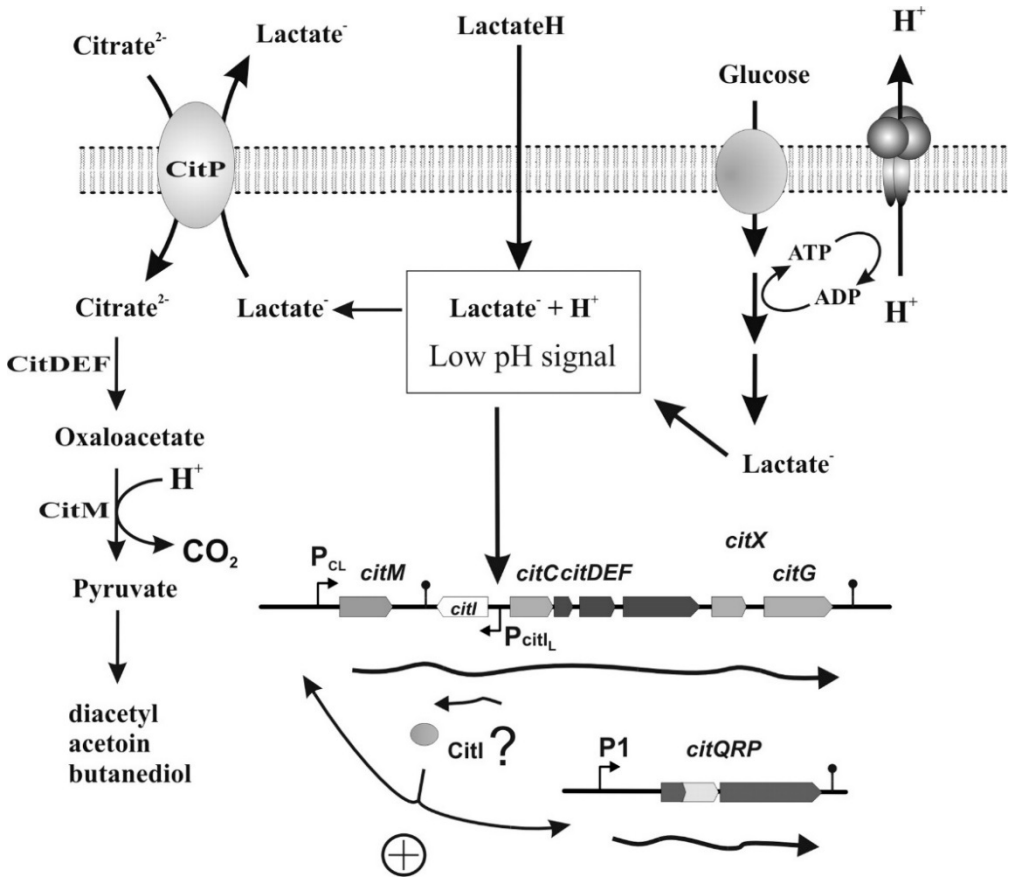


Figure 1.2 Response of the *cit* operons to acidic stress and the contribution to pH homeostasis in *Lactococcus lactis* CRL264 (Martín *et al*., 2004)

1.3.3.5. The role of cell membrane fatty acids in acid stress response

It is well documented that the proton permeability through the cell membrane is regulated by the membrane fluidity, which depends on the membrane fatty acid composition (Lemos *et al*., 2005). Bacteria are able to change their membrane composition in response to external stimulants, in order to maintain a degree of membrane fluidity which improves their survival (Beney and Gervais, 2001). Even though there are several external influences that affect the integrity and properties of the cell membrane in harsh environments, acid stress is one of the factors that has an impact on the cell membrane fluidity. Many previous reports have suggested that a relationship

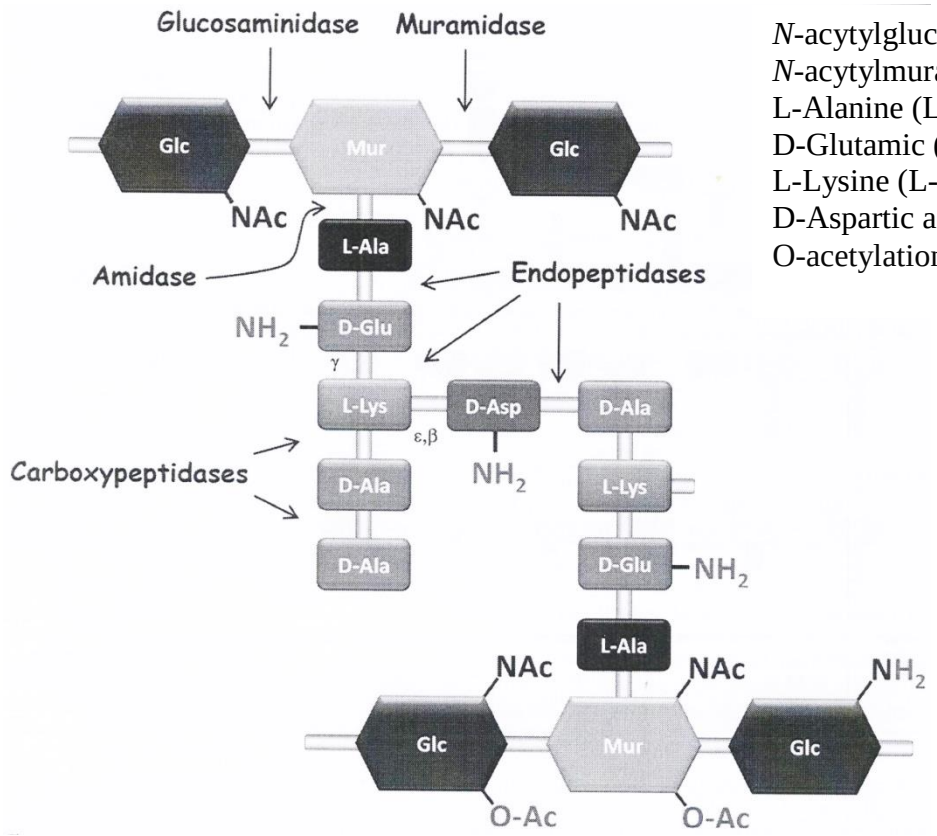
exists between acid stress and change in membrane fluidity (Lemos *et al*., 2005, Zhang and Rock, 2008). At low pH, the cellular adaptive mechanisms induce a decrease in the unsaturated membrane fatty acid content, which leads to an increase in the fluid-to-solid phase transition and to a decrease in membrane fluidity; this phenomenon which is known as homeoviscous adaptation has been reported for a number of LAB. Broadbent *et al*. (2010) reported that acid adaptation of *L. casei* cells at low pH, using MRS acidified with HCl at different pH (pH 3 to 5, 37 °C, 10 to 20 min), led to a decrease in the proportion of the unsaturated to saturated fatty acid and to an increase in the amount of CFA when the cell were exposed to MRS acidified with HCl (pH 2, 37 °C, 140 min). These changes led to a decline in membrane fluidity. A similar change in cyclopropane composition during acid stress, as the means for maintaining their membrane fluidity, was also reported for *L. helveticus* (Montanari *et al*., 2010). Moreover, Wu *et al*. (2012b) demonstrated that a *L. casei* mutant strain, which was obtained by serial sub-culturing the cells in MRS medium (pH 4.3) for 70 days, showed better survival in acidified solutions (pH 2.5 and 3.0) than the parent strain. They attributed this partly to the higher amount of cyclopropane (octadecanoic acid, $C_{19cyclo}$) in the mutant strains (3 % higher cyclopropane than the parent strain initially (i.e. 0) and 9 % higher cyclopropane after 60 min in acidified solutions). The overexpression of the cyclopropane synthase (*cfa*) gene in *L. plantarum* during acid stress was reported (Seme *et al*., 2015), although fatty acid analysis was not done in that study. Overall, the above indicate that during acid stress, changes in the membrane fatty acids composition, particularly increases in the degree of saturated fatty acids and the levels of cyclopropane, most likely affect to membrane fluidity and thus the influx of protons, and consequently could possibly play an important role in the survival of *L. plantarum* at low pH.

1.3.3.6. The role of cell wall peptidoglycan in acid stress response

Peptidoglycan is a major component of the Gram positive cell wall and its role is to maintain the cell shape and structure, which are important features influencing cell viability (Tiyanont *et al*., 2006). Peptidoglycan consists of sugars and amino acids; the sugar components are made of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) linked by β-1,4 bonds, whereas amino acids, or peptide chains containing 3 to 5 amino acids, are covalently linked through the MurNAc. These peptide chains have diverse amino acid composition, depending on the different species, and can be cross-linked directly or indirectly by an interpeptide cross-bridge, i.e. a short chain of one or more amino acids (Figure 1.3). Typically, the stem peptide sequence in LAB is L-Alanine-γ-D-Glutamate-X-D-Alanine, where the third amino acid (X) is L-lysine in *Lactococcus lactis* and most lactobacilli; however it can also be diaminopimelic acid (DAP) in *L. plantarum* or L-ornithine in *L. fermentum* (Chapot-Chartier and Kulakauskas, 2014).

An important group of molecules found within the cell wall of Gram positive bacteria, including lactobacilli such as *L. plantarum*, is wall teichoic acids (WTA) (Bron *et al*., 2012, Tomita *et al*., 2012). WTA are covalently attached to peptidoglycan by a disaccharide as shown in the figure 1.4. Recent studies have reported the binding of D-alanine to WTA, which can reduce the negative charge of the cell envelope (Giaouris *et al*., 2008, Swoboda *et al*., 2010, Brown *et al*., 2013), which might have potential implications in acid stress response of bacteria with potential effects on how WTA bind to D-alanine via a process known as D-alanylation or teichoic acid alanylation. The phosphodiester linkage between the D-alanyl ester residue and the *sn* glycerol 1-phosphate of teichoic acids, which is controlled by *dltABCD* operon (Weidenmaier and Peschel, 2008, Brown *et al*., 2013), can reduce the negative charge of the cell

envelope; this can be attributed to the protonated amino group of the D-alanyl ester residue, which acts as a counter ion to the negatively charged phosphate group of the glycerol 1-phosphate unit of teichoic acid (Neuhaus and Baddiley, 2003). It seems likely that the higher the D-alanine content, the more positively charged the cell envelope. Moreover, proteomic analysis of acid treated *L. casei* indicated that the MurA, MurG and Ddl enzymes, which are involved in peptidoglycan biosynthesis, were overexpressed during acid stress at pH 3.5 compared to pH 6.5 (Wu *et al*., 2012a) indicating a potential link between peptidoglycan biosynthesis and acid stress response. To our knowledge, the potential involvement of teichoic acid in response to acid stress has not been investigated for LAB. The hypothesis investigated in this thesis is that the changes occurring in the configuration of teichoic acid due to D-alanylation, which should reduce the overall negative charge, are responsible for controlling the influx of protons inside the cytoplasm and hence contribute to acid tolerance of cells.



N-acytylglucosamine (Glc)
N-acytylmuramic acid (Mur)
L-Alanine (L-Ala)
D-Glutamic (D-Glu)
L-Lysine (L-Lys)
D-Aspartic acid (D-Asp)
O-acetylation (O-Ac)

Figure 1.3 Schematic representation of the main aspects of the structure of peptidoglycan (Chapot-Chartier and Kulakauskas, 2014).

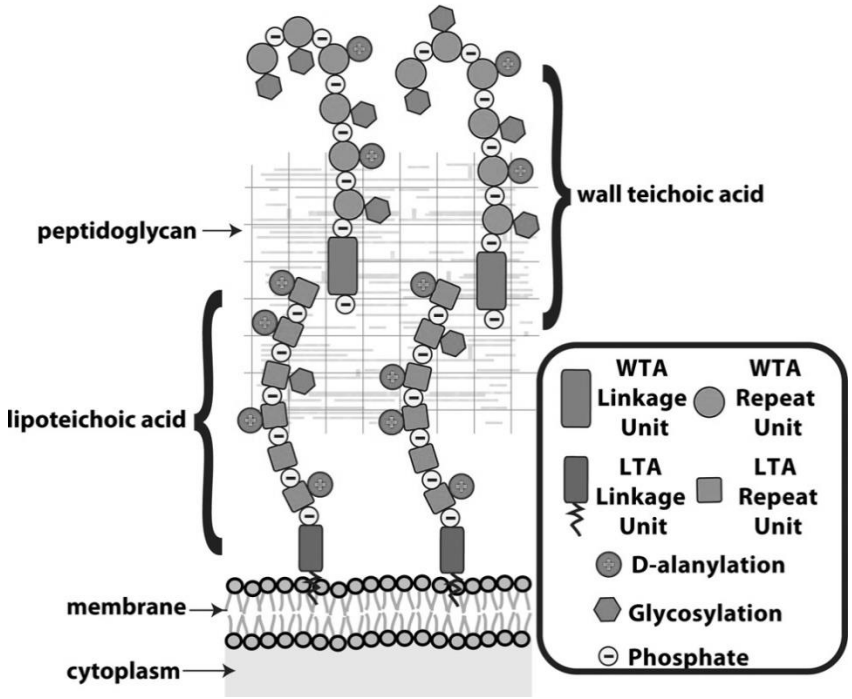


Figure 1.4 Teichoic acid polymers located within the Gram-positive cell wall (Brown *et al*., 2013).

1.4. Aims and objectives

The aims of this study were to evaluate the potential of using an acid adaptation method to enhance the survival of a model potential probiotic strain (*L. plantarum* NCIMB 8826) during storage in various fruit juices, and to investigate the possible mechanisms involved. The main objectives were:

1. To study the effects of acid adaptation on subsequent cell survival during storage in fruit juices and optimize the conditions for acid adaptation (e.g. acid type, acid solution, time).
2. To investigate the mechanisms leading to potential improvement in the survival of acid adapted cells in fruit juices using complementary approaches, including cell morphology analysis, extracellular and intracellular compounds analysis (e.g. organic acids, GABA, amino acids), and proteomic analysis.

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CHAPTER 2

The effect of citric acid adaptation on the subsequent survival of *L. plantarum* NCIMB 8826 in fruit juices during refrigerated storage

2.1. Introduction

The development of new products is an essential part of a successful business strategy to ensure that business is productive and competitive within the global market. In this respect, the development of non-dairy products, such as fermented meats, cereal-based beverages and fruit juices containing probiotics is a challenging task with high potential for commercialization by the food industry (Luckow and Delahunty, 2004, Champagne *et al*., 2005). Fruit juices are suitable vehicles for the delivery of probiotics as they contain high amounts of sugar, minerals and vitamins which could be used as energy and nutrition sources for the survival of probiotics during storage (Ding and Shah, 2008). Moreover, they not only are consumed frequently and loyally by consumers but also are an alternative choice for lactose intolerant individuals who are looking for non-dairy functional products to include in their daily diet (Prado *et al*., 2008).

The survival of probiotics within any carrier food matrix is a major issue for new product development, as a large number of cells die during processing and storage and also during passage through the GIT following ingestion (Vinderola *et al*., 2000, Shah, 2000, Gueimonde *et al*., 2004). In the case of fruit juices, their low pH (ranging between 2.5 and 3.8) has an adverse effect on bacterial survival (Champagne *et al*., 2005, Sheehan *et al*., 2007). In order to convey their health benefits, the minimal dose required for efficacy is suggested to be over 10^7 CFU/g or ml of product (Corcoran *et*

al., 2007). However, the cell concentration might vary between different probiotic products (e.g. 10^9 CFU/serving is recommended by FAO/WHO; Hill *et al*., 2014) as the beneficial effects of probiotics are influenced by various factors, including the intrinsic properties of the strain used, the type of product and storage conditions, as well as the target site in the GIT. Therefore, the minimal dose required for a probiotic product to exert a beneficial health effect needs to be established ideally by clinical trials before making a potential claim. To meet the minimal dose requirements for probiotic applications, many approaches have been used to ensure probiotic survival during food production, storage, and in the GIT, including selecting suitable food systems (Corcoran *et al*., 2005), microencapsulation (Nualkaekul *et al*., 2012), and stress adaptation (Saarela *et al*., 2011). Among these, the ability of bacterial cells to adapt to unfavourable environments through the induction of various stress responses prior to their incorporation into the food matrix seems to be a promising approach, as it would require less process alterations, capital investment and product re-design compared to other approaches, such as encapsulation.

For many LAB species a short acid adaptation period, before transferring them to harsh acidic environments often results in cells which are able to survive longer compared to those with non-adapted cells. This strategy allows the cells to activate their defence mechanisms for protecting themselves before passing through adverse conditions. Such mechanisms include the upregulation of stress response proteins, particularly chaperone proteins which play an important role in protein homeostasis (Hartke *et al*., 1996, Lim *et al*., 2000, Wu *et al*., 2014) and the reduction of membrane fluidity through the alteration of cell membrane fatty acids which can protect against the influx (Montanari *et al*., 2010, Wu *et al*., 2012). As a result, acid adaptation has been used to improve cell survival of LAB in acidic conditions in many studies. For instance,

exposure of *L. acidophilus* cells for 15 to 60 min in MRS acidified to different pH values (pH 3.8 to 6.0) resulted in an increase in their acid tolerance during subsequent incubation in MRS at pH 3 (Lorca *et al*., 1998). Similarly, 16 h exposure of *Bifidobacterium longum* in MRS supplemented with 0.05% L-cysteine, acidified at pH 4 and 5 resulted in more resistant cells which survived well in artificial gastric solution (pH 2) (Sanchez *et al*., 2007). Exposure of *L. casei* to MRS at pH 4.5 for 1 h was able to enhance its survival in acidified MRS at pH 3.5 compared to 1 h exposure in MRS at pH 5 and 6 (Wu *et al*., 2014). Moreover, 30 min exposure of *L. plantarum* in acidified MRS (pH 4.5 and 5.0 using HCl), extensively increased its tolerance in acidified MRS (pH 2) for up to 90 min compared to the control, (30 min in MRS at pH 7) (Seme *et al*., 2015). Based on this concept, exposure of probiotic cells to acid prior to their incorporation into fruit juices seems to be a promising approach for reducing cell injury and loss of viability during storage in juices, caused by the high acidity of the juices. Although this has been shown previously by Saarela *et al*. (2011) with *Bifidobacterium animalis* subsp. *lactis*, the improvement in cell viability obtained in their study was not enough for the method to be translated into a commercial application. In order to develop a suitable acid adaptation method, a number of factors should be investigated including the physiological state of the cells at the time of exposure to the acid solutions, the types of acid used, the time of exposure, and the intrinsic properties of the probiotic strains.

It is well known that stationary phase cells are more robust to various types of stresses than exponential phase cells (Lee *et al*., 1994, Storz and Hengge, 2011), because bacterial cells enter the stationary phase of growth when the environment is not appropriate for them to grow; due to the depletion of nutrients and/or the accumulation of waste products. In order to survive in such conditions, the cells need to change their

physiology and morphology (e.g. upregulation of genes) enabling them to rapidly adapt to those stresses (Bačun-Družina *et al*., 2011). Consequently, only cells which can adapt remain viable during a prolonged stationary phase. This means that the viable cells obtained from this stage of cell growth should be more robust toward stresses than those derived from exponential phase which have not been exposed to considerable stresses. For these reasons, studies on the long-term survival of cells in acidic food formulations (e.g. acidified milks, juices) often employ stationary phase cells. For example, treatment of stationary phase *Lactobacillus* cells with acid has been shown to increase the tolerance of the cells to low pH during subsequent storage in acidic solutions and media (Bâati *et al*., 2000, Lorca and de Valdez, 2001, Saarela *et al*., 2004, Seme *et al*., 2015). Regarding the acid type, many studies have been conducted using different acids for bacterial adaptation, including lactic acid (Lorca and de Valdez, 2001), HCl (Rallu *et al*., 1996) and malic acid (Saarela *et al*., 2011), but none have compared the degree of acid tolerance caused by the different types of acid. Using different types of acid for cell adaptation could lead to different levels of acid adaptation and therefore cell survival. Moreover, Beales (2004) suggested the occurrence of synergistic effects between pH and the undissociated form of a weak acid on cell survival, leading to increased acid tolerance than when using a strong acid. Interestingly, the study of García-Quintáns *et al*. (1998) highlighted the positive effect of citrate adaptation in improving the acid tolerance of *Lactococcus lactis* grown in M17 medium with no pH control. This finding relates to the results by Nualkaekul and Charalampopoulos (2011) and Mousavi *et al*. (2011) who suggested that the high concentration of citric acid in fruit juices improved the survival of *L. plantarum* during refrigerated storage. However, the exact role of citric acid adaptation in the survival of LAB during storage is not understood.

Following on from their work, in this study citric acid was one of the acids used to adapt stationary phase *L. plantarum* cells in order to elucidate its mode of action.

Besides acid exposure, cold adaptation has also been suggested as a suitable strategy for enhancing the shelf life of probiotics during refrigerated storage (Panoff *et al*., 1994, Kim and Dunn, 1997). Some reports have demonstrated that an acid adaptation can also improve the cryotolerance of *L. reuteri* (Palmfeldt and Hahn-Hägerdal, 2000), *L. acidophilus* (Wang *et al*., 2005) and *L. delbrueckii* (Streit *et al*., 2008). From these studies, it seems likely that there is a link between acid and cold adaptation. To this end, combinations of acid and cold adaptation were investigated in this study as the means for enhancing cell survival during subsequent storage in fruit juices at 4 °C.

Various studies have been conducted to identify the potential use of fruit juices as carrier food vehicles for probiotics, including orange, apple, kiwi, pineapple, peach, cranberry, pomegranate and strawberry juices (Vinderola *et al*., 2002, Sheehan *et al*., 2007, Mousavi *et al*., 2011). Some fruit juices displayed very low cell viability during refrigerated storage, particularly cranberry juice (no viable cells after 1 week of refrigerated storage) most likely due to its low pH (~2.5) and high phenolic compound content (Sheehan *et al*., 2007, Nualkaekul and Charalampopoulos, 2011).

L. plantarum NCIMB 8826 has been selected for this thesis as it meets several criteria proposed in the FAO/WHO guidelines (2002) for the evaluation of probiotics in foods and it is one of the core groups of well-studied probiotic species that can claim the general beneficial effects on gut physiology and health at species level without conducting clinical trials (Hill *et al*., 2014). More specifically, this strain has been isolated from human saliva (Hols *et al*., 1997), has a good safety record (De Vries, 2006), has been shown to survive well in model gastric solutions at pH < 3

(Charalampopoulos *et al*., 2003, Parente *et al*., 2010) and in conditions of the upper human digestive tract (mouth to ileum) (Vesa *et al*., 2000), and has been shown to exert immune (Pochard *et al*., 2005) as well as anti-inflammatory activities *in vitro* (Foligne *et al*., 2007) and in animal studies (Foligné *et al*., 2006). Moreover, it has been shown that it can decrease the epithelial barrier dysfunction in human subjects (Karczewski *et al*., 2010).

2.2. Materials and methods

2.2.1. Bacterial strain

L. plantarum NCIMB 8826 (National Collection of Industrial and Marine Bacteria, UK) was used throughout this study. The stock culture was stored at -80 °C in MRS (Oxoid, UK) containing 10% (v/v) glycerol (Sigma-Aldrich, UK).

2.2.2. Fruit juices

Three commercial fruit juices, namely cranberry (Oceanspray™), pomegranate (Pureplus™) and lemon & lime (This water®), available on the UK market, were used in this study. The cranberry juice consisted of water (83%), cranberry juice from concentrate (27%), sugar (11 g/100 ml), and vitamin C (32 mg/100 ml). The pomegranate juice consisted of water (68%), pomegranate juice from concentrate (32%), sugar (11.7 g/100 ml), protein (0.2 g/100 ml) and fat (0.2 g/100 ml). The lemon & lime juice consisted of fresh lemon and lime juice (100%). All fruit juices used in this experiment had no preservatives or additives.

2.2.3. Growth of *L. plantarum* NCIMB 8826

Initially, the growth of *L. plantarum* NCIMB 8826 was monitored every 3 h in MRS broth incubated at 37 °C for 30 h in order to identify the early stationary phase of

the cell growth and thus the optimum harvest time for the cell adaptation experiments. To prepare the inoculum, a two-step propagation procedure was used; the cells were thawed from a cryovial and transferred onto MRS agar by streak plating. After incubation (3 days, 37 °C), a single colony was inoculated into 25 ml of MRS broth and grew overnight for 18 h at 37 °C. The growth of the bacterial culture was measured using a spectrophotometer (Biomate 3, Thermo Scientific, UK) at 600 nm. It needs to be noted that the cell culture was serially diluted with fresh MRS before measuring the optical density (OD). The final OD_{600} value was calculated back to the original concentration by multiplying with dilution factor. Then, an appropriate volume of the overnight culture (18 h) was calculated and inoculated into fresh 250 ml of MRS broth to obtain a starting OD_{600} of ~0.2. The new culture was incubated at 37 °C in an orbital shaker (KS 501 digital, IKA, Germany) set at 200 rpm for 30 h. A 15 ml aliquot of this culture was collected every 3 h and the pH was immediately measured using a pH meter (S20 SevenEasy™, METTLER TOLEDO, UK), while the cell viability was determined by the spread plate method on MRS agar, incubated at 37 °C for 3 days. The colonies were counted using a colony counter (Colony counter SC5, Stuat™, UK). The results were expressed as CFU/ml. Biological triplicate comprising technical triplicate were conducted for this experiment. The data shown are expressed throughout as mean ± standard deviation (SD).

2.2.4. Effects of acid adaptation on subsequent cell survival in cranberry juice

2.2.4.1. Adaptation of cells in buffered solutions at various pH

In the first set of adaptation experiments, the role of pH and types of acid (weak acid versus strong acid) on the survival of early stationary phase cells of *L. plantarum* NCIMB 8826 were evaluated. To this end, the cells were incubated at 37 °C for 1 h in

two different buffers, phosphate and citrate buffer, with the pH adjusted to pH 2, 3, 4, 5 and 6 using HCl (Sigma-Aldrich, UK) in the case of the phosphate buffer, and citric acid (Sigma-Aldrich, UK) as well as sodium citrate (Sigma-Aldrich, UK) in the case of the citrate buffer. Cells incubated in 0.1 M phosphate buffered saline (PBS, Oxoid, UK) with a pH of 7.3 were used as the control.

2.2.4.1.1. Preparation of stationary phase cells

As discussed previously, late stationary phase cells are more robust to various stresses compared to other cell stages. The reason for selecting early stationary phase cells instead of late stationary phase cells was that the latter is generally more stressed due to the lack of nutrients and the accumulation of toxic waste products. Based on the growth pattern of *L. plantarum* NCIMB 8826 observed in the section 2.3.1, the cells needed to be cultured for at least 15 h before reaching early stationary phase. To this end, a single colony prepared as described in section 2.2.3 was inoculated into 10 ml of MRS broth and grown overnight for 18 h at 37 °C. The OD_{600} of the cell culture was measured by a spectrophotometer and then an appropriate volume of the cell culture was calculated and added into fresh 100 ml of MRS broth in order to obtain a starting OD_{600} of ~0.2. The cells were then incubated at 37 °C on an orbital shaker set at 200 rpm for 15 h; at which point, the concentration of the early stationary phase cells was ~10^{10} CFU/ml.

2.2.4.1.2. Preparation of citric acid and HCl exposed cells

Early stationary phase cells, prepared as mentioned above, were harvested from 50 ml of culture by centrifugation 3,500 g for 15 min; (Heraeus™ Multifuge™ X3 Centrifuge, Thermo Scientific, UK). The pellets were washed twice with 0.1 M PBS (pH 7.3) and resuspended in 5 ml of 0.1 M PBS (pH 7.3), resulting in a 10-fold

concentration of bacterial cells; this way a cell concentration of ~10^{11} CFU/ml was obtained. The cell suspension (250 μl) were then added into two types of filter sterilised (0.2 μm, Minisart, Sartorius AG, Germany).acidified buffers (pH 2, 3, 4, 5, 6), namely 0.1 M PBS (25 ml) and 0.15 M citrate buffer (25 ml), as well as a control solution (pH 7.3), namely 0.1 M PBS (25 ml).The cell concentration in the acidified solution was ~10^{9} CFU/ml in all cases; the cells were incubated at 37 °C for 1 h.

2.2.4.1.3. Addition of 1 h acid adapted cells into cranberry juice

After incubation, the cells were collected from each acidified solution by centrifugation at 3,500 g for 15 min. The pellets were washed twice with 0.1 M PBS (pH 7.3), harvested by centrifugation at 3,500 g for 15 min and resuspended in 2.5 ml of cranberry juice. An aliquot (250 μl) of this cell suspension was used to inoculate 25 ml of cranberry juice contained in 50 ml sterile plastic container. Consequently, the initial cell concentration in the juices was approximately ~10^{8} CFU/ml. The juice was stored at 4 °C. Based on previous work by Nualkaekul and Charalampopoulos (2011), it was known that *L. plantarum* NCIMB 8826 was able to survive in cranberry juice for less than a week; therefore, the cell survival in cranberry juice in this particular experiment was monitored immediately upon inoculation of the juices and then every day until cell death. The cell viability was measured by the same method described in section 2.2.3. Biological triplicate comprising technical triplicate were conducted for each pH. The effects of acid adaptation on the survival of *L. plantarum* in cranberry juice were analysed using one-way analysis of variance (ANOVA) and Tukey's post-hoc test at 95% confidence level.

2.2.4.2. Adaptation of cells in MRS at various pH

A second set of adaptation experiments using acidified MRS instead of acidified buffers was performed in order to evaluate whether MRS, which contains glucose, peptone and yeast extract, can serve as a nutrient and an energy source for the cells during acid adaptation. This might be important for enhancing the survival of the cells and avoid starvation which might be taking place when the cells were adapted in the acidified buffers. Moreover, from an industrial point of view, it would be more efficient and economically viable to adapt the cells in the fermentation medium at the end of the fermentation process rather than harvesting, washing and re-suspending the cells in a buffer. To this end, the control and citric adapted cells were prepared and cultured as mentioned in section 2.2.4.1.2 but instead of using acidified buffers the cells were incubated for 1 h at 25 °C in 25 ml of MRS acidified to pH 3, 4, 5 and 6 with citric acid, while unmodified MRS with a pH of 6.4 was used as the control. It must be noted that acid adaptation at pH 2 was excluded from this experimental set as MRS became cloudy when it was acidified to pH 2. Moreover, the incubation temperature during acid adaptation in the case of MRS (25 °C) was lower than that used for the buffers (37 °C) in order to prevent cell growth. After incubation, the cells collected from each solution were added into 25 ml of cranberry juice following the method in section 2.2.4.1.3. Cell viability was measured immediately upon inoculation of the juices and then after 1, 2, and 3 days of storage using the spread plate method, as described in section 2.2.3. Biological triplicate comprising technical triplicate were conducted for each pH. The effects of acid adaptation on the survival of *L. plantarum* NCIMB 8826 in cranberry juice were analysed using one-way ANOVA and Tukey's post-hoc test at 95% confidence level.

2.2.5. Influence of exposure time in citrate buffer at pH 3

In the third set of adaptation experiments, the aim was to evaluate the effect of exposure time on acid adaptation. It should be noted that citrate buffer acidified to pH 3 was used in this experiment, because these cells showed the greatest survival during storage in cranberry juice (section 2.3.2.1). Although the cells exposed with MRS acidified to pH 3 showed the same results (section 2.3.2.2), the acidified citrate buffer was more appropriate as it avoided cell growth at 37 °C during acid adaptation process. To this end, the control and citrate exposed cells were prepared and cultured as mentioned in section 2.2.4.1.2; however, the cell cultures were incubated only in citrate buffer acidified to pH 3 at 37 °C for various periods of time (1, 2, 3, 4, and 5 h). After incubation, the collected cells from each solution were added into 25 ml of cranberry juice using the methods stated in section 2.2.4.1.3. Cell viability was measured immediately after inoculation of the juice and then after 1, 2, and 3 days of storage, by the spread plate method as described in section 2.2.3. Biological triplicate comprising technical triplicate were conducted. The effects of exposure time on the survival of *L. plantarum* in cranberry juice were analysed using one-way ANOVA and Tukey's post-hoc test at 95% confidence level.

2.2.6. Effect of cold adaptation on subsequent cell survival in cranberry juice

In the fourth set of adaptation experiments, the effect of temperature on the survival of cells in cranberry juice was investigated. More specifically, *L. plantarum* NCIMB 8826 cells were incubated at various temperatures, including 4, 10, 25, and 37 ^{o}C for 2.5 and 5 h (pH 7.3) to evaluate whether cold adaptation can improve subsequent cell survival in acidic condition. It must be noted that 0.1 M PBS (pH 7.3) was selected as the incubation solution rather than unmodified MRS in order to

minimise the influence of pH and nutrients during cell adaptation, which would skew the results. Moreover, the reason for selecting 10 ^{o}C and 2.5 and 5 h incubation times was based on Kim and Dunn's findings that cell viability of *Lactococcus lactis* subsp. *lactis* was significantly improved, by 25 % to 37 %, during 24 h storage at -20 °C following cold shock at 10 °C for 2.5 h (Kim and Dunn, 1997). Cell viability further increased when the incubation time was extended to 5 h. Refrigerated temperature of 4 °C was also examined due to the fact that it is the temperature generally used to preserve fruit juices.

2.2.6.1. Preparation of control and cold adapted cells

Early stationary phase cells were prepared as previously (2.2.2.4.1.2). Aliquot (250 μl) of cell suspension were added into 25 ml of 0.1 M PBS (pH 7.3) in 50 ml conical centrifuge tubes (VWR international, UK) and then incubated at 4, 10, 25, and 37 ^{o}C, respectively. One set of cell suspensions at each temperature was incubated for 2.5 h and a second set was incubated for 5 h. After incubation, the collected cells were added into 25 ml of cranberry juice following the methods stated in section 2.2.4.1.3. The cell viability was measured immediately after inoculation of the juices and then after 1, 2, and 3 days of storage by the spread plate method as described in section 2.2.3. Biological triplicate comprising technical triplicate were conducted for each treatment. The effects of temperatures and exposure time on the survival of *L. plantarum* NCIMB 8826 in the cranberry juice were analysed using two-way ANOVA and Tukey's post-hoc test at 95% confidence level.

2.2.7. Effect of combined acid and cold adaptation on subsequent survival of *L. plantarum* NCIMB 8826 cells in cranberry juice

In the fifth set of adaptation experiments, *L. plantarum* NCIMB 8826 cells were incubated in citrate buffer acidified to pH 3 at 4 °C for 3 h (versus 37 °C as the control) to investigate the potential synergistic effect of pH and temperature during cell adaptation. The pH, type of acid, and exposure time used in this experiment were selected based on the results from the experiments described in sections 2.3.2 (acid adaptation) and 2.3.3 (exposure time) whereas the temperature was obtained from section 2.3.4 (cold adaptation). More specifically, the adapted cells in acidified citrate buffer (pH 3) for 3 h showed the greatest cell survival in cranberry juice (72 h) while the adapted cells in 4 °C for both 2.5 and 5 h displayed the greatest cell survival in cranberry juice (24 and 48 h). For this reason the conditions selected for the combined acid and cold adaptation were pH 3 at 4 °C for 3 h; 37 °C was the set temperature for the control. The control and citrate exposed cells were prepared and cultured as described in section 2.2.4.1.2. After adaptation for 3 h, the collected cells were incorporated into 25 ml of cranberry juice following the methods stated in section 2.2.4.1.3. The cell viability was measured immediately after inoculation and then after 1, 2, and 3 days of storage by the spread plate method, as described in section 2.2.3. Three sets of biological replicate experiments were conducted for each treatment. The synergistic effects of acid and cold adaptation on the survival of *L. plantarum* NCIMB 8826 in cranberry juice were analysed using paired t-test at 95% confidence level.

2.2.8. Survival of acid adapted cells in pomegranate, and lemon & lime juices

In the sixth set of adaptation experiments, the aim was to evaluate whether the improvement in cell survival due to acid adaptation, which was seen in some cases in

cranberry juice, would be sustained during longer storage periods or in other juices. MRS was used in this case to adapt the cells based on the rationale that it might provide nutrients and energy sources for the cells which would help them to adapt during acid adaptation, although the effect of media was not seen in this study (section 2.3.2.2). Pomegranate and lemon & lime juices were selected as previous work had shown they supported relatively good cell survival during refrigerated storage. The cells were exposed to MRS acidified to pH 3 at 25 °C for 1 h and then transferred to pomegranate and lemon & lime juices following the methods described in section 2.2.4.2. Cell viability in this experiment was measured every week for up to 6 weeks in pomegranate, and lemon & lime juices based on the findings from a previous study (Nualkaekul and Charalampopoulos, 2011).

2.3. Results and discussion

2.3.1. Growth of *L. plantarum* NCIMB 8826 in MRS

The growth of *L. plantarum* NCIMB 8826 measured by OD_{600} and viable cell counts followed the typical sigmoidal curve obtained for most bacteria (Figure 2.1A). No lag phase was observed during the growth of cells. The cell concentration observed by viable cell counts increased from ~10^7 to ~10^{10} CFU/ml within approximately 15 h, after which the slope became stable. The curve of OD_{600} started from ~0.2 and rose to ~8 after approximately 18 h, after which it remained stable. Although 3 h deviation between two methods was observed, the 15 h time point was selected as the point of harvest, as the cells at that point were entering the early stationary phase based on the viable cell count. It must be noted that the spectrophotometric method does not distinguish between live and dead cells, while in addition to this, any morphological

changes commonly taking place during the end of growth (e.g. cells becoming longer and bigger) would overestimate the result.

In terms of pH, the graph exhibited a downward sigmoidal curve (Figure 2.1B) as expected, since the cells produce lactic acid during growth and the pH of the MRS broth was not controlled in this study. The pH decreased from pH 7.0 to a pH of 4.6 after 15 h, the time when the cells reached the early stationary phase, and decreased further down to 4.3 after 24 h. It must be noted though that the early stationary phase cells obtained at 15 h, were already acid exposed at pH between 5.0 and 4.6 for at least 6 h before conducting the acid adaptation experiments. Nevertheless, the effect of further acid exposure (in buffers or MRS) can still be evaluated when compared with the control cells (no subsequent acid exposure).

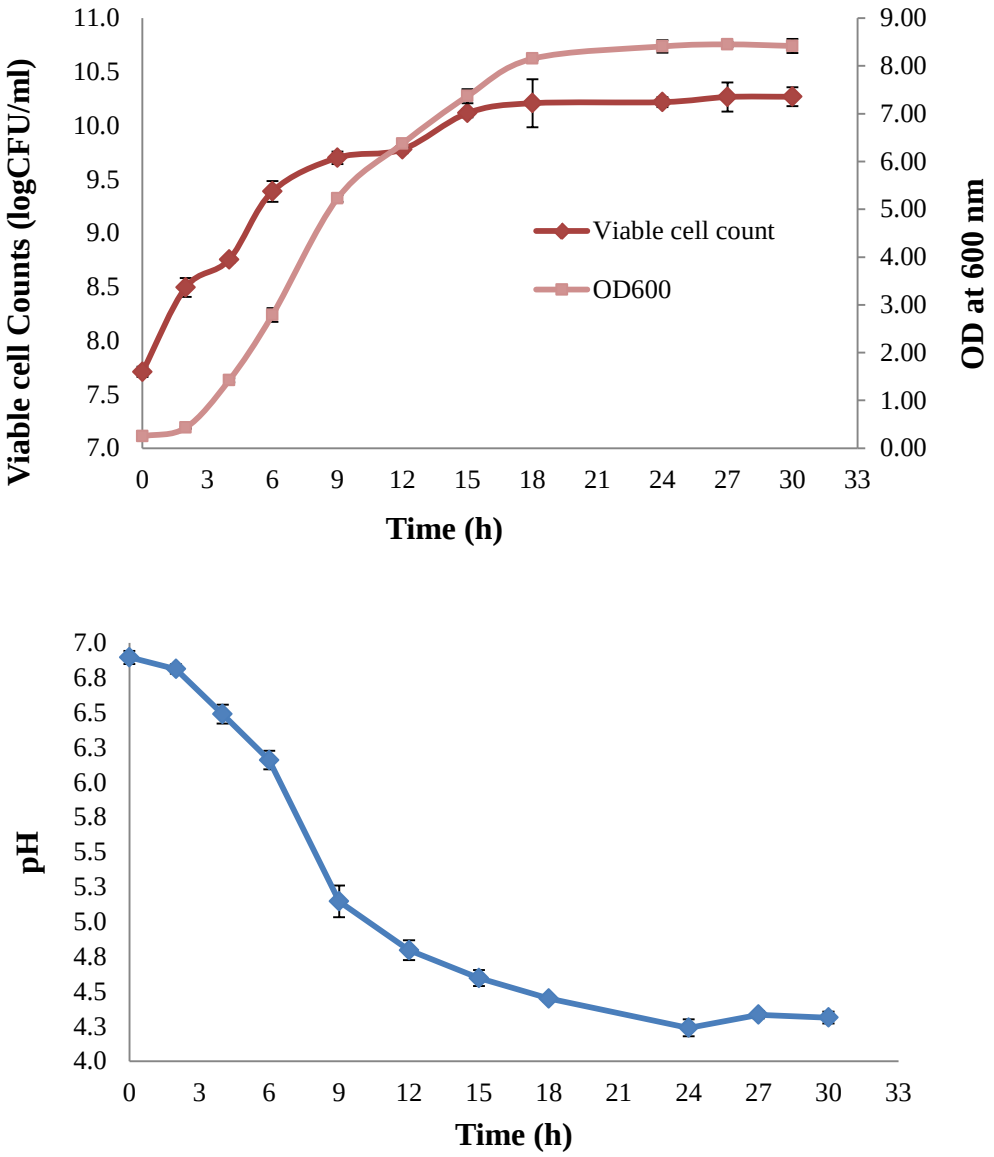

Figure 2.1 Growth of *L. plantarum* NCIMB 8826 in MRS incubated at 37 °C for 30 h and shaken at 200 rpm. (A) The cell concentration was measured by OD_{600} and viable cell counts; and (B) pH of medium during growth of *L. plantarum* NCIMB 8826. Data are expressed in mean ± SD (n=3)

2.3.2. Effects of acid adaptation on subsequent cell survival in cranberry juice

2.3.2.1. Adaptation of cells in buffered solutions at various pH

Exposure of the cells for 1 h in both acidic solutions (HCl and citric acid), even at a pH as low as 2, did not significantly impact on cell viability (0 h) compared to the PBS control (pH 7.3) (Figure 2.2). This might be due to the short period of time used for acid adaptation (1 h) in this experiment. Overall, the results indicated that acid adaptation of cells collected at 15 h of culture (MRS, pH 4.6) in both acidic solutions at pH values higher than 2 significantly improved subsequent cell survival in cranberry juice (24 to 48 h) compared to the control cells (PBS, pH 7.3); the greatest results were obtained after adapting the cells in pH 3, followed by pH 4, 5 and 6. Acid adaptation at pH 2 was detrimental towards the survival of the cells in cranberry juice as the viable cells were below the detection limit (100 CFU/ml) after only one day of storage. Although the results were promising, acid adaptation was not able to improve sufficiently the survival of this particular strain during storage in cranberry juice, as the viable cell counts were below those required for probiotic application ($>10^7$ CFU/ml).

This effect of pH has been observed in the previous work (Seme *et al*.,2015), although a different strain, *L. plantarum* KR6, and different experimental set up were used. Non-acid stressed cells of *L. plantarum* KR6 exposed to acidified MRS (pH 4.5 using HCl) for 30 min, increased survival in acidified MRS (at pH 2) by about 100 times compared to the control (cells exposed to MRS at pH 7 for 30 min). The researchers suggested that this behaviour might relate to the upregulation of genes involved in membrane fatty acid biosynthesis, and in particular *cfa*, as determined by qPCR analysis. To this end, the higher survival rates of *L. plantarum* NCIMB 8826 in cranberry juice (24 to 48 h) after 1 h acid adaptation (pH 6, 5, 4 and 3) might be

associated with changes in the composition of the membrane fatty acids which is used as a mechanism by the cells in order to regulate the proton permeability through their cell membrane (Lemos *et al.*, 2005, Zhang and Rock, 2008). This hypothesis will be investigated later in chapter 3. It is interesting to note that compared to the control cells (PBS, pH 7.3) some improvements in cell survival were observed in the case of cells adapted at pH 5 and 6; i.e. values higher than the pH at which the cells were collected at the 15 h time point (pH ~ 4.6). This indicates that any changes in pH, even from an acid to a more alkaline environmental could affect the response of the cells during subsequent exposure to the acidic environment of fruit juices.

Similar viability profiles of *L. plantarum* NCIMB 8826 were seen during the first 2 days storage in cranberry juice for adapted cells irrespective of type of acid used to acidify the buffers (HCl versus citric acid) (Figure 2.2). However, the adapted cells in acidified citrate buffer (pH 3 and 4) were able to survive in cranberry juice beyond 2 days of storage, albeit at low concentrations (~10^3 and ~10^2 CFU/ml, respectively), indicating that changes caused by citric acid have more positive effect on cell survival in cranberry juice than that by HCl.

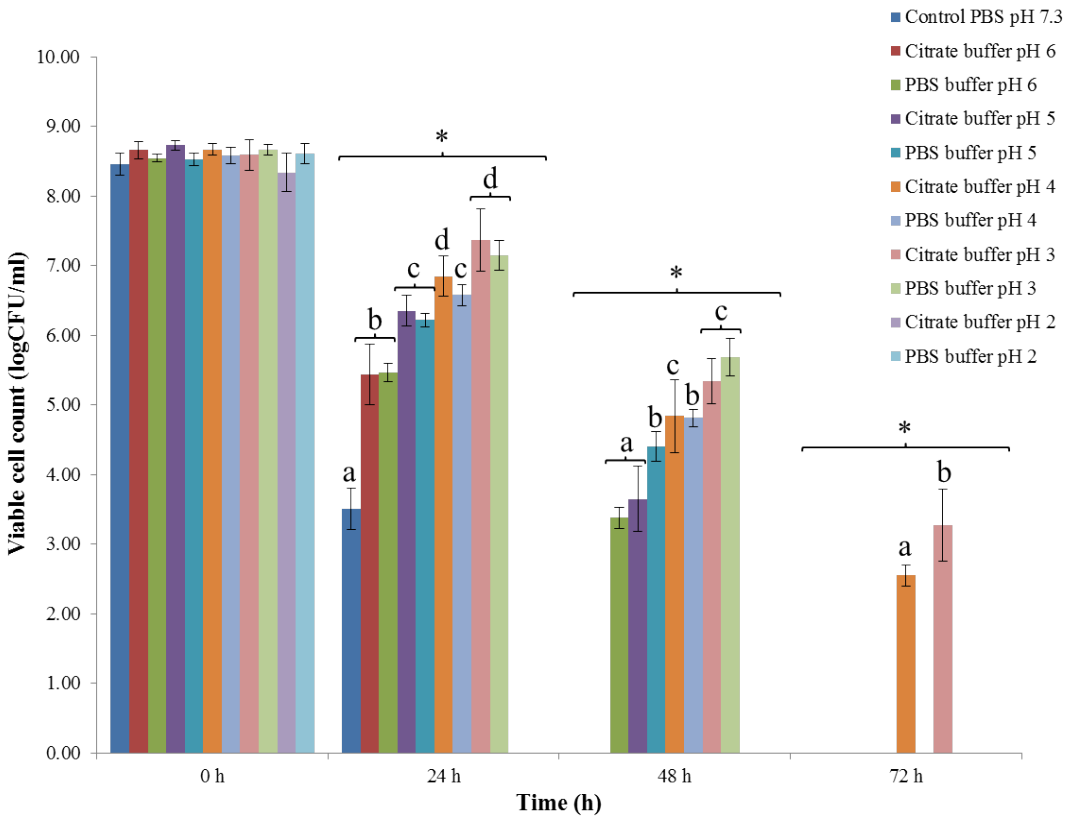

Figure 2.2 Cell concentration (log CFU/ml) of *L. plantarum* NCIMB 8826 during refrigerated storage in cranberry juice after pre-treatment in 0.15 M citrate buffer acidified to different pH using citric acid, and in 0.1 M PBS acidified to different pH using HCl. Results are expressed in mean ± SD (n=3). Significant differences between different pH within each group at a specific time point were determined by one-way Anova and Tukey's post-hoc tests. *indicates a significant difference determined by one-way Anova (at least one difference in each group means significant difference) while ^{a}, ^{b}, ^{c}, different lettering indicates significant differences between each pH determined by Tukey's post-hoc tests.

This phenomenon could be explained on the basis of the study of Pieterse *et al*. (2005), who investigated the effect of the dissociated and undissociated forms of lactic acid and that of hydrogen ions alone on *L. plantarum* WCFS1. The levels of gene/operon expression were measured during continuous steady state experiments, in which the pH was controlled by automatic titration with 10 M sodium hydroxide. The degree of undissociation/dissociation was varied by adding 300 mM of sodium lactate at the two different pH values, at pH 6.0 (lactate effect; dissociated>undissociated form)

and pH 4.8 (undissociated lactic acid effect; undissociated>dissociated form). Gene expression analysis indicated that during acid stress a small number of genes/operons were differentially expressed in response to the hydrogen ions and the dissociated form of lactic acid. On the other hand, stressing the cells with the undissociated form of lactic acid resulted in the overexpression of several genes/operons in *L. plantarum* WCFS1. More specifically, 18 genes or operons which are involved in multiple cell surface proteins were overexpressed. Such overexpression of these key proteins might have taken place in the case of the experiment in the present study, in which another weak organic acid was used, i.e. citric acid instead of lactic acid.

Another potential mechanism responsible for the improved survival of citric acid treated cells compared to the HCl treated cells, could have involved the transportation of citrate inside the cells, as part of the citrate fermentation pathway, which has been reported to be more active in *Lactococcus lactis* MG 1363 at pH 4.5 compared to that at pH 6.5 (García-Quintáns *et al*., 1998). The transport of citrate in exchange of lactate which is catalyzed by citrate permease (*citP*) results in a considerable increase in the membrane potential, a difference in the electric charge across the membrane, a transmembrane pH gradient, and a difference in the concentration of ions across the membrane (García-Quintáns *et al*., 1998, Pudlik and Lolkema, 2011). Both the membrane potential and the transmembrane pH gradient are known as PMF. More specifically, the uptake of citrate into the cell not only generates energy due to the electric potential but also drives a proton into the cell due to the pH gradient. Once inside the cell, the energy from the PMF can be used for ATP production by ATP synthase in order to pump protons outside the cell, while the proton can be used for citrate metabolism to produce pyruvate and CO_2 by oxaloacetate decarboxylase (Pudlik and Lolkema, 2011). The above indicates that the citrate fermentation pathway is a

likely mechanism used to protect the cells during acid stress. Although the increase in the transportation of citric acid at low pH was reported in *Lactococcus lactis* CRL 264 (Magni *et al*., 1999) and in *Leuconostoc paramesenteroides* J1 (Martín *et al*., 1999), this is a mechanism that can possibly occur in other lactic acid bacteria, as the existence of the citrate fermentation pathway was reported in growth experiments with *Leuconostoc mesenteroides* 19D (Marty-Teysset *et al*., 1995), *Lactococcus lactis* CRL 264 (Magni *et al*., 1999), *Lactobacillus rhamnosus* ATCC 7469 (de Figueroa *et al*., 1996) and *L. plantarum* DSMZ 20174 (Kennes *et al*., 1991).

2.3.2.2. Adaptation of cells in MRS at various pH

The adapted cells in acidified MRS with citric acid (Figure 2.3) displayed a similar survival profile as that observed in acidified citrate buffer (Figure 2.2), although some differences were observed e.g. improvement in cell survival after 72 h of storage (by ~1 log CFU/ml) for pH 3 and 4, and extension of protection time (from 24 to 48 h) for pH 6. Overall however, the use of acidified MRS, which contains nutrients and energy sources for cells, was not able to improve considerably the survival of *L. plantarum* NCIMB 8826 in cranberry juice, compared to the use of acidified buffers. It seems likely that the response of the cells as a result of the acid adaptation process was primarily influenced by the pH of acidified solutions (pH 3 to 6) and type of acids (weak versus strong acids) rather than by the presence of nutrients and energy sources in the medium.

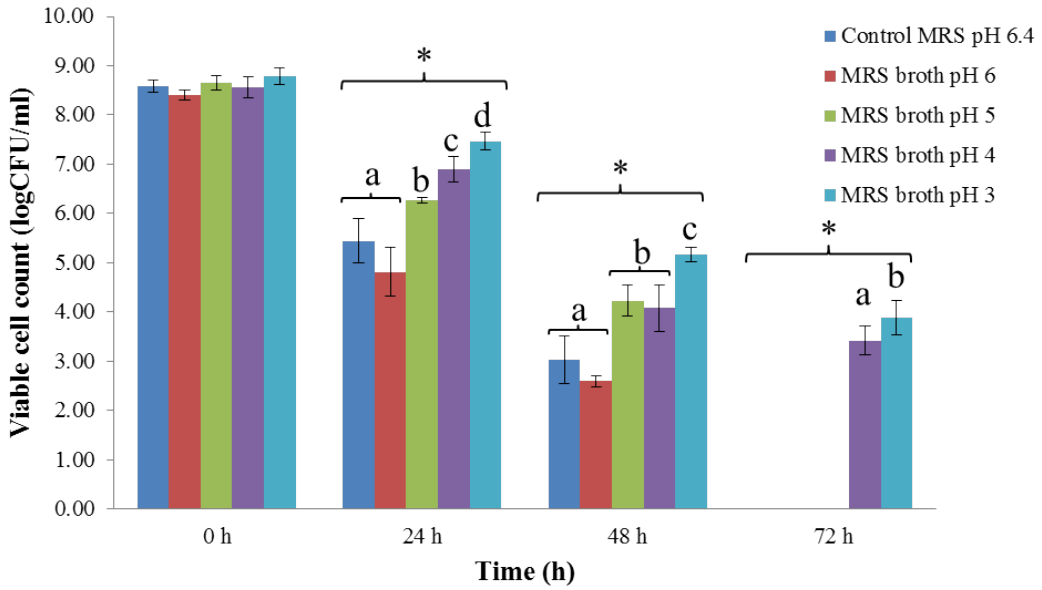

Figure 2.3 Survival of *L. plantarum* NCIMB 8826 during refrigerated storage in cranberry juice after pre-treatment in MRS acidified to different pH using citric acid. Results are expressed in mean±SD (n=3). Significant differences between different pH within its group at a specific time point were determined by one-way Anova and Tukey's post-hoc tests. *indicates significant difference determined by one-way Anova while ^{a}, ^{b}, ^{c}, different lettering indicates significant differences between each pH determined by Tukey's post-hoc tests.

2.3.3. Influence of exposure time in citrate buffer at pH 3

Increasing the exposure time of the cells in acidified citrate buffer (pH 3) from 1 h up to 5 h has an effect on cell survival in cranberry juice (Figure 2.4). Exposure of the cells in acidified citrate buffer (pH 3) for 2 and 3 h resulted in the highest survival in cranberry juice (48 h), followed by 1, 4, 5 h exposure. It seems likely that an incubation time of less than 1 h would not be enough to impact protection to the cells, whereas incubation for more than 3 h imparted negative effects on cell survival. This negative effect might be associated with cell injury or damage during prolonged acid adaptation caused by the antimicrobial effects of the undissociated form of citric acid (Beales, 2004). However, this hypothesis cannot be confirmed without the examination of the surface cell morphology under scanning electron microscope (SEM) at different

exposure times. Overall, the results suggest that the cells should be incubated for at least 2 to 3 h in order to gain maximum protection from further acid exposure.

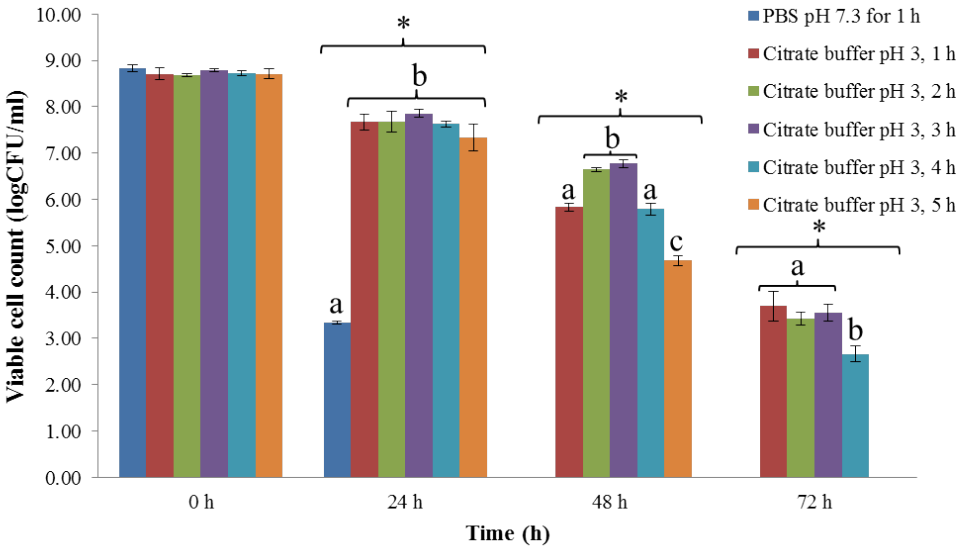

Figure 2.4 Survival of *L. plantarum* NCIMB 8826 during refrigerated storage in cranberry juice after pre-treatment in acidified citrate buffer (pH 3, 37 °C) for different times. Results are expressed in mean±SD (n=3). Significant differences between different pH within its group at a specific time point were determined by one-way Anova and Tukey's post-hoc tests. *indicates significant difference determined by one-way Anova while [a], [b], different lettering indicates significant differences between each exposure time determined by Tukey's post-hoc tests.

2.3.4. Effect of cold adaptation on subsequent cell survival in cranberry juice

There were no significant differences between the 2.5 and 5 h incubation time for all temperatures (Figure 2.5), while exposure of the cells to 4 and 10 °C significantly improved the cell viability in cranberry juice (24 and 48 h) compared to exposure to 25 and 37 °C (~1 to ~3 log CFU/ml). This suggests that exposure time did not affect cell survival in cranberry juice like temperatures (4 and 10 °C). However, no viable cells were observed at 72 h of refrigerated storage in cranberry juice (Figure 2.5), in contrast to the results with the cells in acidified citrate buffer (pH 3 and 4) (Figure 2.2). This

indicates that cold adaptation was less effective than acid adaptation in protecting the cells in cranberry juice. Taking into account the results from two studies including, i) the increase in the $C_{16:0}$ and $C_{18:2}$ membrane fatty acids in *L. acidophilus* CRL 640 when grown at 25 °C compared to 37 °C (Murga *et al*., 2000), and ii) the increase in $C_{18:1}$ fatty acid content in *L. plantarum* when the cells were grown at 10 °C compared to 30 and 40 °C (Russell *et al*., 1995), the improvement in cell survival in cranberry juice (24 to 48 h) by cold adaptation (4 and 10 °C) might be associated with changes in the cell membrane fatty acid composition which is the same process as in acid adaptation. Additionally, it has been suggested that cellular adaptation to cold stress not only results in the alteration of the cell membrane fatty acid composition, but may also be related to the expression of certain heat shock proteins which are involved in acid tolerance, such as, DnaK and GroEL (Salotra *et al*., 1995). To understand better the possible influence of cold stress exposure towards acid tolerance, the cellular fatty acid composition was determined, and the results are reported and discussed in chapter 3.

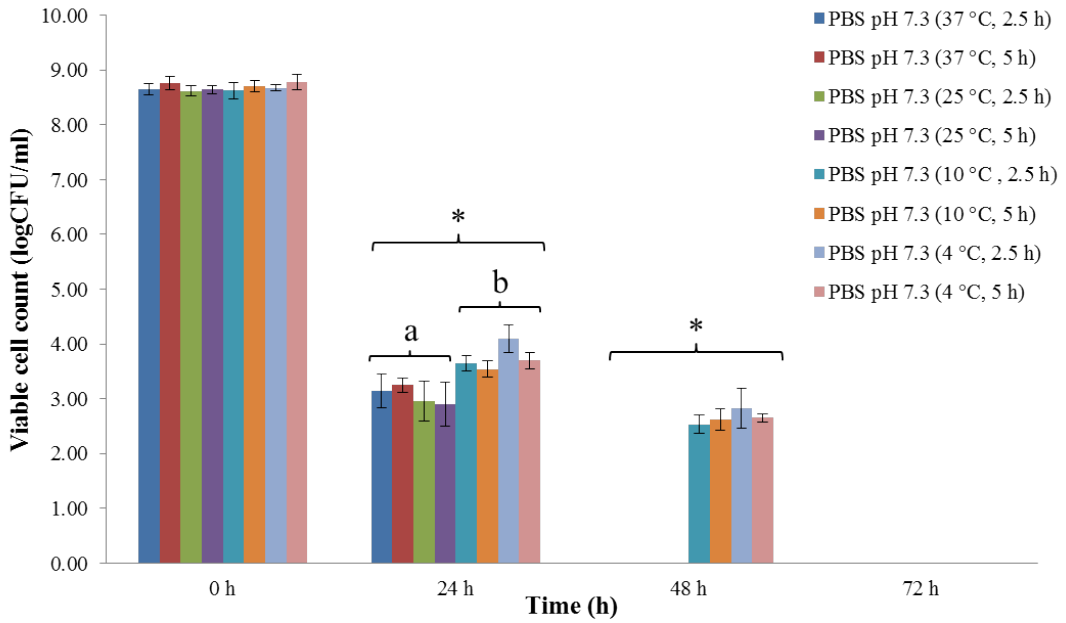

Figure 2.5 Survival of *L. plantarum* NCIMB 8826 during refrigerated storage in cranberry juice after pre-treatment in PBS (pH 7.3 at 37, 25, 10, and 4 °C) for both 2.5 and 5 h. Results are expressed in mean±SD (n=3). Significant differences for temperature and exposure time within each group at a specific time point were determined by two-way Anova and Tukey's post-hoc tests. *indicates significant difference determined by two-way Anova (at least one difference in its group means significant difference) while [a], different lettering indicates significant differences between each treatment determined by Tukey's post-hoc tests

2.3.5. Effect of combined acid and cold adaptation on subsequent survival of *L. plantarum* cells in cranberry juice

There were no significant differences in terms of cell viability between the cells adapted in acidified citrate buffer (pH 3) at 37 and 4°C during storage in cranberry juice from 24 to 72 h (Figure 2.6). This indicates that a synergistic effect of pH and temperature did not occur during cell adaptation, although both pH (pH 3) and temperature (4°C) had separate positive effects on the survival of *L. plantarum* NCIMB 8826 during storage in cranberry juice as previously discussed. There are two possible explanations for this; either the cellular mechanism in the case of temperature (4°C) might not be functional when the cells are exposed to acidic environment (pH 3) or a

similar mechanism was used in the case of cold and acid adaptation. This hypothesis will be investigated and discussed in chapter 3.

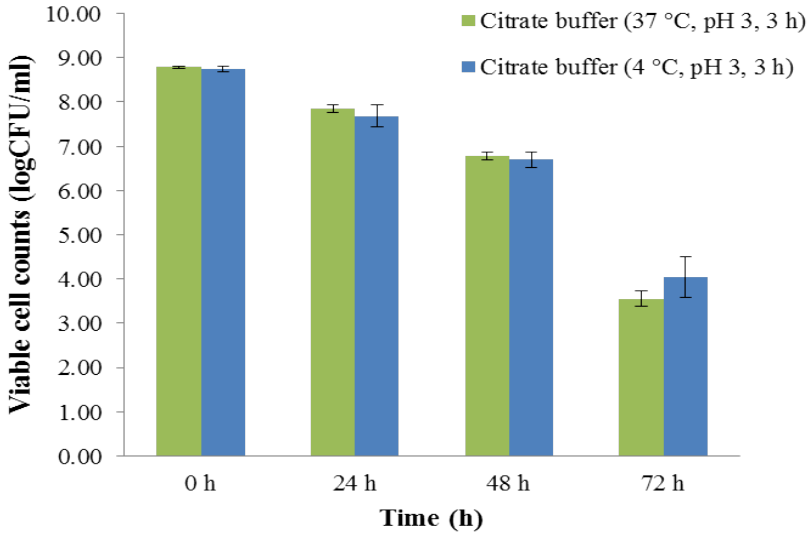

Figure 2.6 Comparison of cell viability between acid adaptation and combination of acid and cold adaptation stored in cranberry juice at 4 °C. Results are expressed in mean±SD (n=3). Significant differences at specific time point were determined by paired t-test. *indicates significant difference.

2.3.6. Survival of acid adapted cells in pomegranate and lemon & lime juices

Acid adaptation (MRS, pH 3) for 1 h improved cell survival in pomegranate and lemon & lime juices compared to the control (MRS, pH 6.4) as previously seen in cranberry juice, although different degrees of cell protection were observed (Figure 2.7). More specifically, the adapted cells exhibited better survival, by more than 1 log CFU/ml for the first and second week compared to the control cells; however, no significant differences were observed for the third week and beyond in both juices. These results indicate that acid adaptation had a positive effect on cell survival in pomegranate and lemon & lime juices but only for the initial stages of storage, suggesting that the alteration process by acid adaptation was not permanent.

The significant loss in the cell viability of *L. plantarum* NCIMB 8826 observed in all fruit juices used in this study during storage, is most likely due to the low pH of the juices as the pH, which for most juices ranges between 2.5 and 3.8, has an adverse effect on bacterial survival (Champagne *et al*., 2005, Sheehan *et al*., 2007). More specifically, the pH of cranberry, pomegranate and lemon & lime juices were 2.7, 3.5, and 2.8, respectively. Interestingly, although the pH values of cranberry and lemon & lime juice were very similar, the survival rates were considerably different; the cells died within the first week of storage in cranberry juice whereas in lemon & lime juice they were viable until week 5. This most likely indicates that other compounds present in cranberry juice might have negative effects on cell survival. Considering the high levels of total phenolic compounds (1.5 g/l) that have been reported for the same cranberry juice product (Nualkaekul and Charalampopoulos, 2011), these could be certain phenolic compounds which have strong antimicrobial effects such as benzoic acid (Sheehan *et al*., 2007) and coumaric acid (Landete *et al*., 2007). Moreover, the presence of phenolic compounds in the fruit juices used in this study might also explain why the cells died quicker in pomegranate juice compared to lemon & lime juice (4 weeks versus 5 weeks) despite the fact that the pH in the former was higher. However, this cannot be confirmed as the phenolic compounds were not determined for all juices.

Considering the average shelf life of refrigerated fruit juices (~6 weeks) (Esteve and Frígola, 2007) and the concentration of viable cells required for probiotics application (~10^7 CFU/ml) (Corcoran *et al*., 2007), the cell concentrations of *L. plantarum* NCIMB 8826 in each fruit juice should be higher than ~10^7 CFU/ml after 6 weeks in order to exert its beneficial effects upon consumption. However, the viable cells higher than ~10^7 CFU/ml were only observed at week 2 and 3 in pomegranate and lemon & lime juices, respectively. These results suggest that the improvement in cell

survival after acid adaptation was not significant enough to make these particular fruit juices suitable commercial products for the delivery of *L. plantarum* NCIMB 8826. However, the pomegranate and lemon & lime juices still have the potential for being used as probiotic carriers as they provide a relatively mild environment for the cells, compared to the harsh environment of cranberry juice. To this end, new strategies that could prolong probiotic survival in these fruit juices, such as use of microencapsulation techniques to encapsulate the cells within polymeric matrices that can protect the cells from acid or use of other bacteria strains that are more robust to acidic environments than *L. plantarum* NCIMB 8826 or using other mild fruit juices should be considered before conducting a new experiment in the future.

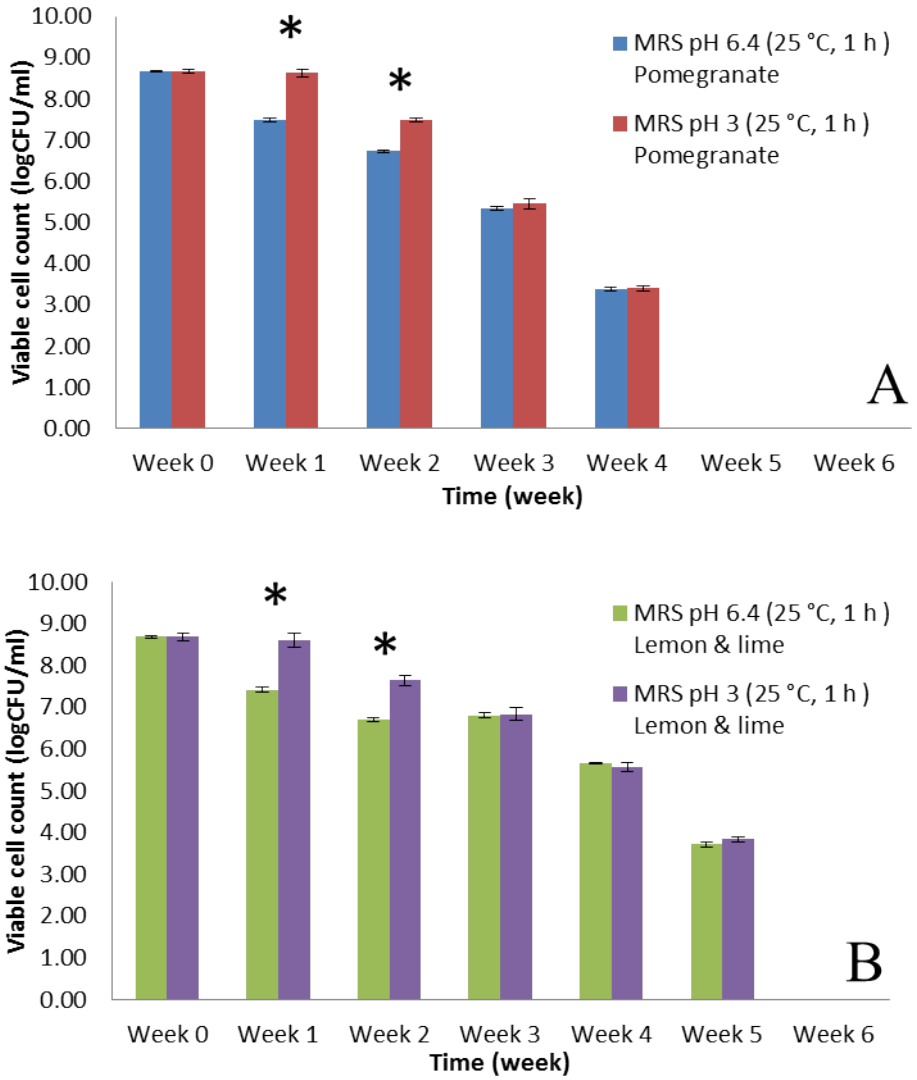

Figure 2.7 Survival of *L. plantarum* NCIMB 8826 during refrigerated storage in pomegranate (A), and lemon & lime (B) after pre-treatment in acidified MRS (pH 3, 25 °C, 1 h). Results are expressed in mean±SD (n=3). Significant differences at specific time point were determined by paired t-test. * indicates significant difference.

2.4. Conclusions

The results from this study demonstrated that acid adaptation for 1 h in different acidic solutions (HCl versus citric acid) with pH higher than 2 improved cell survival during subsequent storage in cranberry juice, while adaptation at pH 2 the cells died within just one day storage in cranberry juice. The presence of nutrients in the MRS medium did not improve cell survival in contrast to expectations. Exposure time between 1 and 3 h in acidified citrate buffer (pH 3) was the most suitable time for adapting the cells, while incubation for more than 3 h had negative effects on cell viability. Moreover, the use of weak acid (citric acid) resulted in better cell survival in cranberry juice (pH 3 and 4, 72 h) compared to strong acid (HCl). This is most likely due to the combined effect of the undissociated form and the pH in the case of weak acid, while strong acid exerts only a pH effect. Besides pH, temperatures of 4 and 10 °C were able to enhance cell survival in cranberry juice compared to higher temperatures (25 and 37 °C), although the improvement was less than that observed after acid adaptation (pH 3). The cell survival data obtained indicate that acid adaptation improved the survival of *L. plantarum* NCIMB 8826 during storage in the three fruit juices; however, the protection was not significant enough to meet the requirements for probiotic applications (~10^7 CFU/ml for 6 weeks). To this end, new approaches, such as improving acid tolerance cells by other techniques (e.g. serial subculturing of cells after storage in these fruit juices, varying the types of acid used for acid adaptation, changing the type of fruit juice) could be used in future experiments to prolong the survival of *L. plantarum* NCIMB 8826.

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CHAPTER 3

Role of cell membrane during citric acid exposure of *L. plantarum* NCIMB 8826 in enhancing subsequent storage in fruit juices

3.1. Introduction

From the previous chapter, it was found that adaptation of *L. plantarum* NCIMB 8826 cells in acidic solutions (HCl & citric acid) at different pH, particularly pH 3 and 4, resulted in better survival during refrigerated storage in fruit juices compared to the control cells. This improved survival is most likely associated with the fact that LAB, including lactobacilli, have developed a variety of mechanisms to withstand harsh conditions, such as low pH. These include the maintenance of ΔpH homeostasis possibly through the citrate fermentation pathway, the alteration of membrane integrity and functionality, and the upregulation of proteins relevant to acid adaptation (Wu *et al*., 2014). This chapter aims to examine the potential mechanisms of acid exposure on subsequent cell survival in low pH environments, namely the citrate fermentation pathway and the compositional and structural changes of the cell membrane.

The citrate fermentation pathway involves the transportation of monovalent lactate out of the cells by exchanging it with divalent citrate during acid exposure. This system protects the cell by generating a PMF which can be used for ATP production to pump protons out of the cell (Pudlik and Lolkema, 2011). Moreover, there is evidence indicating an increase in citric acid consumption, most likely due to induction of *cit* genes including citrate lyase and citrate transporter genes, which has been shown to take place during growth of *Lactococcus lactis* under acidic conditions (M17 medium, pH 4.5) (García-Quintáns *et al*., 1998, Magni *et al*., 1999, Martín *et al*., 1999, Martín *et al*.,

2004). Although the citrate fermentation pathway has never been reported in *L. plantarum* NCIMB 8826, citric acid has been shown to support *L. plantarum* DSM 20174 growth under acidic conditions (pH 4 to 5) when used in combination with glucose; though to a lesser extent when used as the sole carbon source (Kennes *et al*., 1991). However, the likelihood of the citrate fermentation pathway taking place by stationary phase cells during highly acidic conditions and its potential link to acid resistance have not been investigated.

The second potential mechanism involves the overexpression during acid stress of several genes/operons encoding multiple cell surface proteins; this for example has been shown to occur during growth of *L. plantarum* WCFS1 in MRS at pH 4.8 (Pieterse *et al*., 2005). Moreover, the striking morphological differences at the surface of acid stressed and non-stressed *L. plantarum* WCFS1 cells were observed under SEM by Ingham *et al*. (2008). However, the relationship between acid tolerance and surface proteins has not been investigated in depth.

A third potential mechanism is the alteration of the cell membrane integrity and functionality, as the basic function of the cell membrane is to protect the cell from the environment. It is well documented that proton permeability through the cell membrane is regulated by the membrane fluidity, which depends on the composition of membrane fatty acids (Lemos *et al*., 2005, Zhang and Rock, 2008). As the cell membrane of *Lactobacillus* species typically consists of saturated, unsaturated and cyclopropane fatty acids (Johnsson *et al*., 1995), changes in the ratios of these fatty acids caused by external stimulants, especially acid stress, could potentially improve cell survival in adverse conditions including highly acidic environments (Beney and Gervais, 2001). More specifically, it is well known that upon acid stress LAB decrease their membrane fluidity by increasing the degree of saturation, the level of cyclopropane formation, and

the carbon chain length, and by changing the branching position as well as cis/trans isomerization of the membrane fatty acids (Montanari *et al*., 2010). Although these changes have been observed in many LAB and can be regarded as a universal response to acid stress, the regulation of the genes involved in membrane homeostasis of fatty acid biosynthesis, which is the main controlling system in response to acid stress, is not well characterized. To this end, the upregulation of only a small number of specific genes has been investigated in LAB. Two studies have shown the overexpression during acid stress of the *cfa* gene in *L. plantarum* KR6 (Seme *et al*., 2015) and of the *β*-ketoacyl-acyl carrier protein synthase III (*fabH*) gene in *L. bulgaricus* (Fernandez *et al*., 2008). The *cfa* gene stimulates the conversion of monounsaturated fatty acids to their cyclopropane derivatives and the *fabH* gene is a key enzyme in controlling the synthesis of saturated fatty acids. Accordingly, these target genes were examined in this study, with the aim of understanding their role toward cell membrane fatty acid biosynthesis in *L. plantarum* NCIMB 8826.

3.2. Materials and methods

3.2.1. Determination of citric and lactic acid concentration by high performance liquid chromatography (HPLC)

In order to investigate whether the citrate fermentation pathway could have been activated during acid adaptation or storage in fruit juices, HPLC method was used to analyse the citric and lactic acid concentrations in three groups of samples including: i) in acidified solutions (citrate buffer and MRS medium) before and after adding the cells, ii) in three fruit juices (cranberry, pomegranate, and lemon & lime) upon inoculation of the cells and at the end of the storage period, and iii) inside acid adapted and control cells (both from acidified solutions & fruit juices).

3.2.1.1. Citric and lactic acid determination in acidified solutions and in juices

The citric and lactic acid concentrations were determined in the acidified solutions used for cell adaptation and also in the supernatants from the fruit juices (prepared as described in section 2.2.4.1.2 and 2.2.4.1.3, respectively) before and after adding the cells. More specifically, the citric and lactic acid concentrations were determined in acidified citrate buffer (pH 3, 37 °C, 1 h) and acidified MRS with citric acid (pH 3, 25 °C, 1 h) before and after adding the cells; 0.1 M PBS (pH 7.3, 37 °C, 1 h) and unmodified MRS (pH 6.4, 25 °C, 1 h) were used as the controls. Additionally, both citric and lactic acid were determined in the fruit juices before and after adding the cells, and then after 72 h of storage for cranberry juice, 5 weeks for pomegranate juice and 6 weeks for lemon & lime juice. The concentrations of citric and lactic acid were analysed using an Agilent 1100 HPLC system with a ultraviolet detector (Agilent technologies, UK) according to a previous method (Nualkaekul and Charalampopoulos, 2011). The separation was carried out using a 250 mm x 4.6 mm x 5 μm organic acid column (Prevail^{TM}, Alltech, UK). A solution of 25 mM potassium dihydrogen phosphate (KH_2PO_4) at pH 2.5 was used as the mobile phase and the flow rate was set at 1 ml/min. The organic acids were detected at 210 nm. Samples were analysed in triplicate. A calibration curve was constructed for each organic acid using standards with different concentrations (5 to 300 mM for each organic acid).

3.2.1.2. Determination of intracellular citric and lactic acid concentrations

The intracellular citric and lactic acid concentrations of the acid adapted and control cells and of the cells in the fruit juices were determined. The cells were collected by centrifugation at 3,500 g for 15 min, washed twice with 0.1 M PBS (pH 7.3), and then rapidly quenched with 25 ml of pre-chilled 0.5 mM tricine in 60% (v/v)

methanol (Sigma Aldrich, UK) in dry ice. Next, the pellets were collected by centrifugation at 3,500 g for 15 min and were resuspended in 2.5 ml of sterile distilled water. Acid-washed 106-mm-diameter glass beads (Sigma Aldrich, UK) were added to fill about 1/3 of a 2 ml microtube before 1 ml of the cell suspension was added into the microtube. The samples were disrupted thrice using a Mini-Beadbeater (Biospec, UK) for 1 min and cooled for 1 min on ice during the breaking process. After cell disruption, the samples were centrifuged for 10 min at 15,000 g at 4 °C. The supernatants were transferred to a new sterile 1.5 ml microtube and analysed by HPLC based on the method described in section 3.2.1.1

3.2.2. Morphological investigation of the surface of acid adapted and control cells

The surface of *L. plantarum* NCIMB 8826 cells was examined by cryo-SEM in order to visualize possible changes on the cell surface caused by acid adaptation. To this end, 25 ml of early stationary phase cells with a concentration of ~10^{10} CFU/ml as described in section 2.2.4.1.1 was incubated in acidified MRS (pH 3, 25 °C, 1 h); the cells incubated in unmodified MRS (pH 6.4, 25 °C, 1 h) were used as a control. After 1 h incubation, the pellets were harvested by centrifugation at 3,500 g for 15 min, washed twice with 0.1 M PBS (pH 7.3), and transferred into a microtube. The pellets were mounted onto a copper holder with colloidal graphite and plunged into liquid nitrogen. The frozen specimens were then loaded into a transfer chamber where they were fractured with a cold scalpel blade. The specimens were etched at -90 °C for 10 min and coated by sputtered argon. They were then transferred under vacuum onto the cold stage and imaged using a high vacuum field emission SEM (QUANTA 600F, FEI, USA). The images were captured and analysed by the xT microscope control software.

3.2.3. Determination of cellular fatty acid composition

In order to evaluate the potential effect of the acid adaptation and the combinatory effect of acid and cold adaptation on the membrane composition, the concentrations of intracellular fatty acids in the adapted and control cells at different pH (pH 3.0 and 6.4) and temperatures (4 and 37 °C) were determined by gas chromatography using a mixture of fatty acids from 9 to 20 carbons in length and five hydroxyl acids as external standards (Sherlock MIS System, MIDI Inc, USA). To this end, 50 ml of early stationary phase cells with a concentration of ~10^{10} CFU/ml, obtained as described in section 2.2.4.1.1, were incubated in MRS (pH 3.0 and pH 6.4, 1 h at 25 °C) and acidified citrate buffer (pH 3, 3 h at 4 and 37 °C); in all cases citric acid was used to acidify the solutions. After incubation, the pellets were harvested by centrifugation at 3,500 g for 15 min, washed twice with 0.1 M PBS (pH 7.3), and frozen at -80 ^{o}C overnight before freeze drying (VirTis BenchTop™ K Series, SP Scientific, UK). Finally, the freeze-dried samples were sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, Germany fatty acid analysis. At DSMZ, the fatty acids were extracted from 20 mg of each freeze-dried sample according to a standard protocol for fatty acid analysis (Miller, 1982, Kuykendall *et al*., 1988). The mixtures of fatty acid methyl esters produced were analysed using the Hewlett-Packard 5898A microbial identification system (Microbial ID Inc, USA) equipped with a gas chromatography system (Hewlett-Packard model 5980), a flame ionization detector (Hewlett-Packard models 3392), and an autosampler (Hewlett-Packard models 7673). The detection was performed using a 25 m x 0.2 mm 5% phenylmethyl silicone capillary column. All peaks were automatically integrated and the percentages of each fatty acid were calculated.

3.2.4. Gene expression analysis by reverse transcription-polymerase chain reaction (RT-PCR)

In an effort to support the results obtained from the intracellular fatty acid analysis, the level of gene expression between adapted (pH 3) and control cells (pH 6.4) during citric acid adaptation was evaluated by RT-PCR. *cfa* and *fabH* were selected as the target genes while the 16S rRNA gene was used as the reference gene in this study because it is one of the housekeeping genes (5 gene copies) which exhibit constant RNA transcription in *L. plantarum* NCIMB 8826 cells (Lee *et al*., 2008). It needs to be noted that *cfa* was selected to evaluate the level of cyclopropanation, as it catalyzes the conversion of the cis-double bond of unsaturated fatty acids to the cyclic ring of their cyclopropane derivatives; for example oleic acid ($C_{18:1w9c}$) and cis-vaccenic acid ($C_{18:1w7c}$) are converted to dihydrosterculic acid ($C_{19cyclow9c}$) and lactobacillic acid ($C_{19cyclow9c}$), respectively (Johnsson *et al*., 1995). *fabH* was selected to evaluate the synthesis of straight chain fatty acids as this enzyme plays an important role in the first step of the elongation process of fatty acids. Consequently, the upregulation of this gene reflects the amount of saturated fatty acids being produced, as depicted in figure 3.1.

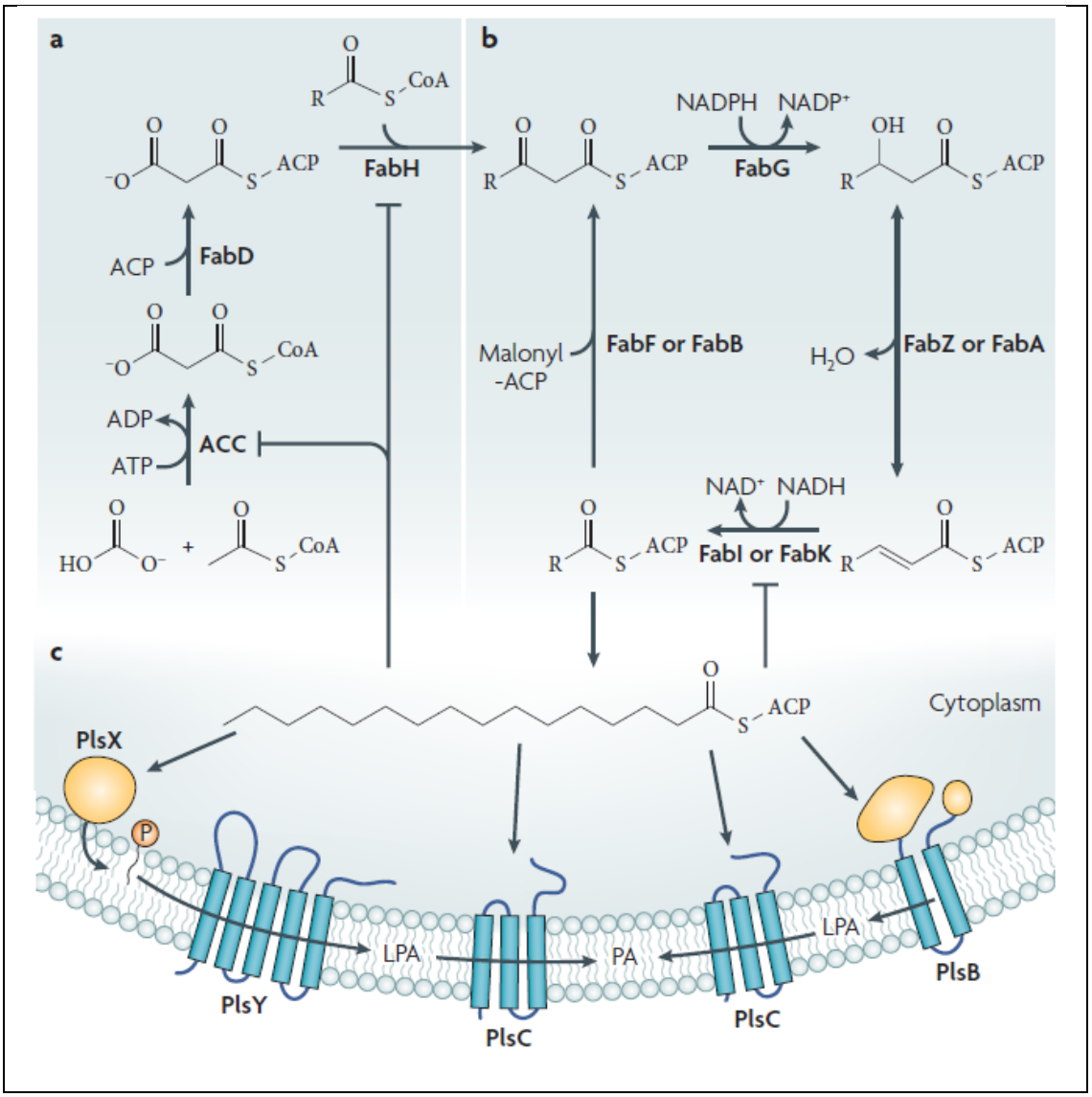

Figure 3.1 Conserved pathway for the formation of fatty acid in bacteria. a) The first step in fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). Next, the malonyl group is transferred to acyl carrier protein (ACP) by malonyl transacylase (FabD). Then, condensation is initiated by FabH to form the first β‑ketoacyl-ACP intermediate. b) The second step, chain elongation, is continued and repeated by serial enzymatic reactions using; i) β-ketoacyl-ACP reductase (FabG), ii) β‑hydroxyacyl-ACP dehydrase (FabA or FabZ), iii) enoyl-ACP reductase (FabI) until the long chain ACP end product is obtained. c) The long chain ACP end product is finally inserted into the membrane phospholipid by acyl transferase via the PlsX–PlsY pathway (Zhang and Rock, 2008).

3.2.4.1. Design of primers for RT-PCR

Primer-BLAST, a free primer designing tool (http://www.ncbi.nlm.nih.gov/ tools/primer-blast/), was used to design the specific primers for the targeted genes. After obtaining the results, each pair of designed primers was checked for their specificity toward the genome of *L. plantarum* WCFS1 using NCBI Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, the primer dimer and hairpin structure were calculated by OligoEvaluator (http://www.oligoevaluator.com/Login.jsp) before the PCR experiments were performed.

3.2.4.2. Preparation of samples for RNA extraction

The pellets of adapted (pH 3, 25 °C, 1 h) and control cells (pH 6.4, 25 °C, 1 h) prepared as described in section 3.2.3 were used for RNA extraction. Importantly, prior to RNA extraction, these cell pellets needed to be treated with 25 ml of RNA stabilization reagent (RNA*later*, Qiagen, UK) in order to prevent RNA degradation, as gene expression studies require high quantity of RNA. The stabilized cells were collected by centrifugation at 3,500 g for 15 min. They were then resuspended in 2.5 ml nuclease free water (Qiagen, UK) and then 1 ml of this cell suspension was transferred into a 2 ml microtube containing acid-washed 106-mm-diameter glass beads. The samples were disrupted thrice by a Mini-Beadbeater following the method described in section 3.2.2. After cell breaking, the aqueous phase was collected by centrifugation for 10 min at 15,000 g (4 °C) and transferred into a new sterile 1.5 ml microtube. The RNA of each sample was isolated using RNeasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. All RNA preparations were treated with RNase-free DNase for 20 min at 37 °C during mRNA extraction. Finally, the RNA pellet was dissolved in 20 µl of nuclease-free water and stored at -20 °C until use. The concentration and purity

of each RNA sample were determined using NanoDrop ND-1000 (Thermo Scientific, UK).

3.2.4.3. RT-PCR quantification

By real time RT-PCR, the genes of interest including the reference gene (Table 3.1) were amplified using QuantiFast SYBR Green RT-PCR Kit (Qiagen, UK). Three concentrations of mRNA (1:10, 1:100, and 1:1000 dilutions) were used as the template for each set of primers. The amplification was run in triplicate for each sample in white 96 well plates (Roche Applied Science) using the LightCycler® 480 RT- PCR system. Before the RT-PCR products were analysed, preliminary RT-PCR experiments were conducted by making a 10 fold serial dilution of the extracted mRNA to set up the optimum reaction for amplifying the target genes. Reactions were carried out in a total volume of 25 µl, containing 2 µl of mRNA, 1 µl of each primer (0.01 nM), 12.5 µl of 2X QuantiFast SYBR Green RT-PCR Master Mix, 0.25 µl of QuantiFast RT Mix and 8.25 µl of RNase free water. A non-template control was included in each run to identify possible DNA contamination. The RT-PCR cycler conditions were set as follows: Reverse transcription at 50 °C for 10 min, PCR initial activation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 10 sec, annealing at 60 °C for 30 sec, and elongation with measurement of fluorescence at 72 °C for 30 sec. The cycles were followed by a melting curve analysis at 95 °C for 1 min, 55 °C for 30 sec, and a slow increase to 95 °C with continuous fluorescence acquisition. The relative expression ratio of each candidate gene was determined by comparison with the expression levels of the reference gene. The RT-PCR efficiency (E) for each pair of primers was calculated by using serial 1:10 dilutions of mRNA of the LightCycler®480 RT- PCR System.

Table 3.1 Target genes and primers used in RT-PCR analysis

Locus tag[a]	gene	Function	Primer(5'-3')	*E* value
lp_rRNA01	*16S rRNA*	16S ribosomal RNA	**Forward primer:** TCTGTAACTGACGCCTGAGGC **Reserve primer:** CTGTATCCATGTCCCCGAAG	1.98
Ip_1696	*cfa*	Cyclopropane fatty acyl phospholipid synthase	**Forward primer:** AGCAGCGTCATTTGGAGGAA **Reserve primer:** GACCTGTTGTTTCGACCTGCT	2.23
Ip_1671	*fabH2*	3-oxoacyl ACP synthase	**Forward primer:** GTGCGGGCTTTGTTTATGGG **Reserve primer:** CAGTGCCCAGTGGTCGTATT	1.97

[a]Designated gene loci for annotating the location on *L. plantarum* WCFS1 chromosome.

3.3. Results and Discussion

3.3.1. Concentrations of citric and lactic acids after acid adaptation and during storage in fruit juices

During cell adaptation in acidified citrate buffer (pH 3, 37 ^{o}C, 1 h), no consumption of citric acid was observed and no lactic acid was produced during acid adaptation (data not shown). This suggests that the citrate metabolism most likely did not take place during incubation of early stationary phase *L. plantarum* NCIMB 8826 cells. It might be due to the lack of glucose which is necessary for cell growth as citrate metabolism was shown to take place during growth of *L. plantarum* DSM 20174 in rich media at acidic pH (pH 3.6 to 5.0) when citric acid was used as a carbon source; acetate and CO_2 were the major end products, whereas small amounts of lactic acid were also detected (Kennes *et al*., 1991). Interestingly, when the experiment was carried out with a minimal base medium, citric acid did not support cell growth as the sole carbon source, but in the presence of citric acid and glucose the growth rate increased compared to just glucose (Kennes *et al*., 1991). Although no detailed metabolite analysis was done, it suggests that in the presence of citric acid the cells were

potentially able through the citrate fermentation pathway to expel the lactic acid (produced through glycolysis) faster, thus increasing growth rate; this was shown to take place following a similar experiment with *Lactococcus lactis* CRL 264 (Magni *et al*., 1999). Taking the above into account and the citrate and lactate results from the acid adaptation experiment, in which the buffer used did not contain additional carbon rich nutrients, it can be deduced that acid adaptation alone is insufficient to activate citrate fermentation pathway, but growth under acidic condition in presence of citric acid maybe required. That is, early stationary phase cells did not use the citrate fermentation pathway as no lactic acid was being produced, and the citrate fermentation pathway is a PMF-generating pathway associated specifically with lactic acid toxicity (Magni *et al*., 1999).

To evaluate whether using a rich medium to carry out the acid adaptation would induce the citrate fermentation pathway, the cells were exposed to acidified MRS (pH 3, 25 ^{o}C, 1 h), containing substantial levels of carbon sources (20 g/l glucose) and nitrogen (10 g/l peptone; 8 g/l Lab-Lemco' powder, 4 g/l yeast extract). The results show that there were no significant differences in citric and lactic acid concentration between the different MRS samples (MRS with no cells at pH 6.4 versus MRS with cells at pH 6.4 versus MRS with cells at pH 3.0) (data not shown); this indicated that citrate fermentation was not activated even in the case of MRS, which contained glucose. Overall, the two sets of experiments presented above demonstrated that the citrate fermentation did not take place during acid adaptation and therefore this mechanism did not contribute toward acid tolerance in *L. plantarum* NCIMB 8826 cells.

In the case of fruit juices, no significant change in the concentration of citric acid was observed during storage, whereas lactic acid was not detected in any of the samples (Table 3.2), indicating that citrate fermentation most likely did not take place

during storage in fruit juices as well. In order to confirm this, the concentrations of citric and lactic acid were also measured inside the cells, primarily to evaluate whether small amounts of citric acid were transported inside the cells and whether glycolysis was taking place, which potentially could have resulted in the intracellular production of lactic acid. No citric and lactic acid were detected inside the cells, although a number of additional unknown peaks were detected in the case of acid adapted cells (data not shown), suggesting that the cells could have responded to acid adaptation through the accumulation of some small organic compounds. These were most likely not formic acid, butyric acid, caproic acid, oxalic acid and malic acid as the retention times of the unknown peaks were different to the retention times expected for these compounds with this particular column. It was not possible however to identify the compounds with the existing HPLC system. Liquid chromatography mass spectrometry would be required for identification, which was not available. Nevertheless, based on qualitative analysis of the chromatograms and comparisons of the retention times with the literature, it is likely that one of these compounds was pyruvic acid, which suggests that possibly some low metabolic activity was taking place during storage of the cells.

The analysis of the citric acid content of the three juices indicated that the lemon & lime juice contained the highest amount of citric acid (~30 mM) followed by pomegranate (~29 mM) and then cranberry juice (~15 mM) (Table. 3.2), while no lactic acid was detected in all cases (data not shown). Besides citric acid, other organic acids are likely to be present, such as malic and ascorbic acid, as shown by previous analysis of the same fruit juices including cranberry and pomegranate (Nualkaekul and Charalampopoulos, 2011). Previous work by Nualkaekul and Charalampopoulos (2011) demonstrated that increasing the concentration of citric acid in the synthetic media enhanced the survival of *L. plantarum* NCIMB 8826 during storage, although other

factors played a role as well, such as the pH and the sugar concentration. It is interesting to note that the citric acid concentration did not decrease during storage in those experiments either. Taking into account the survival data from the three juices, it seems that the higher the citric acid concentration in the juice the better the cell survival. This suggests a protective effect of citric acid which is even more evident when comparing the cell survival in cranberry and lemon & lime juices, as both juices had similar pH (~ 2.5) yet distinctly different citric acid concentration.

In summary, the citrate fermentation pathway was not implicated in the acid tolerance of *L. plantarum* NCIMB 8826 cells, as no changes were observed in the concentrations of lactic and citric acids after adapting the cells with either acidified solution. Nonetheless, high amounts of citric acid in fruit juices help improve the survival of *L. plantarum* NCIMB 8826 during storage in fruit juices, which is in accordance with the literature, indicating that citric acid plays a role in enhancing the acid tolerance of the cells; however, currently there is limited understanding of the mechanism.

Table 3.2 Extracellular concentrations of citric acid during refrigerated storage at 4 °C in three different juices. Results are expressed in mean ± SD (n=3). Significant differences of citric concentration among each fruit juice were determined by one-way Anova and Tukey's post-hoc tests. *indicates significant differences among fruit juices.

Juices	**Time**	**Extracellular citric acid concentration (mM)**		
		Without cells	**With control cells**	**With adapted cells**
Cranberry*	0 h	15.23±0.27	15.09±0.25	15.02±0.20
	72 h	14.84±0.23	15.33±0.34	15.22±0.15
Pomegranate*	Week 0	28.84±0.16	28.53±0.18	28.76±0.17
	Week 5	28.75±0.13	28.71±0.15	28.69±0.11
Lemon & lime*	Week 0	30.30±0.31	30.41±0.17	30.34±0.36
	Week 6	30.42±0.16	30.37±0.08	30.34±0.07

3.3.2. Morphological investigation of the cell surface of acid adapted and control cells

After acid adaptation in acidified MRS (pH 3, 25 ^{o}C, 1 h), the surface of adapted cells became rougher and thicker while the control cells exhibited a smooth surface (Figure 3.2).

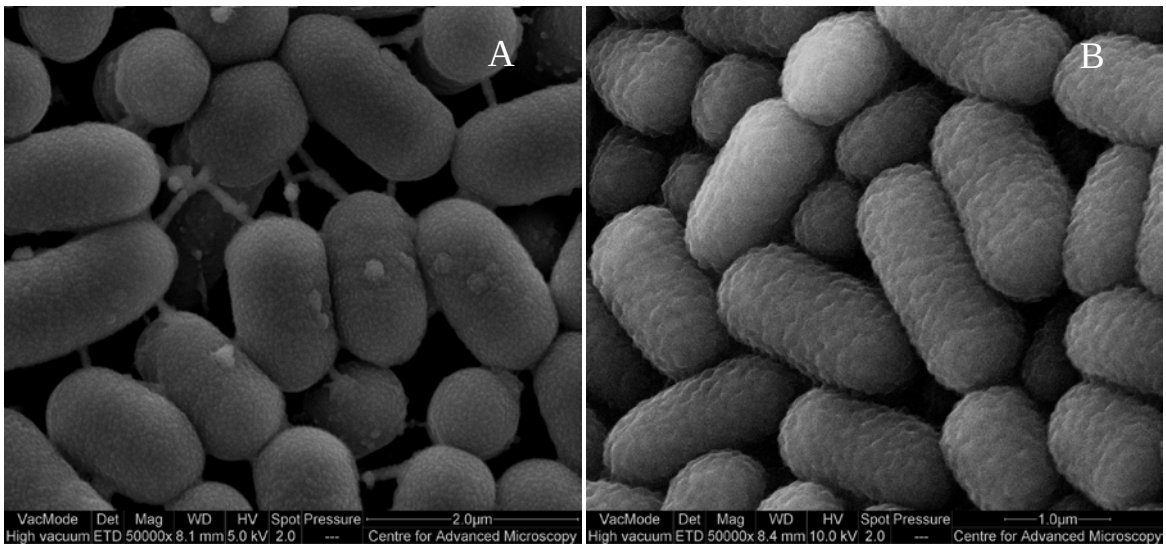

Figure 3.2 Cryo-SEM images of *L. plantarum* after incubation in MRS at pH 6.4, 25 °C for 1 h (control cells, A) and MRS at pH 3, 25 °C for 1 h (acid adapted cells, B).

Interestingly, the rough surface of *L. plantarum* WCFS1 due to acid stress were previously reported in the studies of Pieterse *et al*. (2005) and Ingham *et al*. (2008), which showed that after lactic acid stress, the surface of *L. plantarum* WCFS1 became rougher. In the case of the former study, acid stress was performed using steady state cells obtained during a continuous fermentation set up; the medium used was MRS (pH 4.8, 37 °C) and the dilution rate was 0.3/h. In the latter study the acid adapted cells were obtained from microcolonies after transferring mid-log phase cells from liquid broth at pH 5 to MRS gel (pH 3, 37 °C, 3 days). Based on Pieterse *et al*. (2005), the rough surface observed in acid stressed cells was associtated with the upregulation of three operons/genes encoding for cell surface proteins but their functions were not identified.

Although the functions of these cell surface proteins are unknown, the majority of them are likely to be attached to the cell wall surfaces, i.e. as s-layer, peptidoglycan, and cell membrane, through several mechanisms (Siezen *et al*., 2006). These include: i) covalent binding to amino- or carboxyl-terminus membrane-spanning anchors, ii) covalent binding to the LPxTG motif (Leucine-Proline-any-Threonine-Glycine), iii) covalent binding to lipid-bound anchors and iv) non-covalently bound to a variety of domains linked to peptidoglycan, teichoic acids, s-layer, surface polysaccharides and cell membrane. From all the mechanisms mentioned above, the non-covalent binding to cell wall glycopolymer (CWG), particularly wall teichoic acid, seems to be a likely mechanism leading to acid protection as teichoic acid plays an essential role in controlling the influx of positively-charged substances through the complex peptidoglycan network (Kovacs *et al*., 2006, Perea Velez *et al*., 2007, Giaouris *et al*., 2008, Weidenmaier and Peschel, 2008, Swoboda *et al*., 2010) and it also controls cell morphogenesis and particularly surface roughness, as shown for *L. plantarum* (Andre *et al*., 2011).

According to the image of the acid adapted cells (Figure 3.2 B) a rough coat-like structure is visible; besides surface proteins, this might be an EPS, particularly a capsular exopolysaccharide (CPS) which is secreted outside the cells but is able to form a tightly adherent cohesive layer surrounding the cell surface. According to Ruas-Madiedo *et al*. (2002) and Nwodo *et al*. (2012), EPS and CPS can potentially protect the cells against a variety of harsh environments such as desiccation, influx of antibiotics and toxins, and osmotic stress. Although there are several reports about EPS production by *L. plantarum* (Desai *et al*., 2006, Mostafa *et al*., 2006, Nagata *et al*., 2009, Tsuda *et al*., 2008, Wang *et al*., 2010), none of these have investigated their function against acid stress. However, it is important to note that despite the visible effects of citric acid on

the cell surface, no membrane injury or damage was observed due to citric acid adaptation. In contrast, this was reported for the cell membrane of *L. casei* strain Zhang, which became thinner and impaired after exposure of the cells to lactic acid at pH 3.5 for 1 h (Wu *et al*., 2012).

A possible hypothesis for the improved survival of acid adapted cells in fruit juices is that their thicker surface, potentially as a result of the binding of overexpressed surface proteins at the cell wall and particularly the teichoic acid component or due to the production of EPS and CPS, decreased the influx of hydrogen protons inside the cells. This may also explain why in the case of prolonged storage (after 3 weeks in pomegranate and lemon & lime juices) the survival profile was similar between acid adapted and control cells, indicating that the change in morphology of the cell surface was not permanent. This could be due to the fact that the binding of the surface proteins to the wall was becoming less strong or that the EPS and CPS were eroded and released into the juices. However, microscopic analysis of the cell surface during storage in the juices was not conducted and therefore this cannot be confirmed. Overall, more work, including compositional analysis of the cell surface, e.g. by X-ray photoelectron spectroscopy, nuclear magnetic resonance and X-ray crystallography, are needed to elucidate the potential changes at the surface of the cells and establish the link with acid resistance.

3.3.3. Modification of membrane fluidity by alteration of the cell membrane fatty acid composition

The total amount of saturated fatty acid increased by ~2.3% (cyclopropane is not included in the calculations) after acid adaptation whereas the unsaturated fatty acids decreased by ~1.3% (Table 3.3). However, these changes were not statistically

significant leading to the conclusion that the degree of saturation/unsaturation of membrane fatty acids did not change during acid adaptation and therefore did not influence the survival of *L. plantarum* NCIMB 8826 during storage in fruit juices.

Table 3.3 Relative percentages of cellular fatty acids after incubation of *L. plantarum* cells in control MRS (25 °C, pH 6.4, 1 h)[a] and acidified MRS (25 °C, pH 3, 1 h)[b].

Systematic nomenclature	Lipid number	Percentage of cellular fatty acids	
		Control cells[a]	Acid adapted cells[b]
Tetradecanoic acid methyl ester	14:0	4.82±0.62	3.63±0.02
cis-9-Hexadecenoic acid methyl ester	16:1w7c	8.10±1.31	6.33±0.10
Hexadecanoic acid methyl ester	16:0	25.86±1.39	24.74±0.58
cis-9-Heptadecenoic acid methyl ester	17:1w8c	0.83±0.06	0.71±0.01
cis-9-Octadecenoic acid methyl ester	18:1w9c	11.40±1.32	11.06±0.11
cis-11-Octadecenoic acid methyl ester	18:1w7c	15.46±2.43	13.73±0.01
Octadecanoic acid methyl ester	18:0	1.24±0.51	1.26±0.14
cis-13-Nonadecenoic acid methyl ester	19:1 w6c	25.65±0.21	27.32±1.06
cis-11,12-Methylene octadecanoic acid methyl ester	19:0 cycw7c	6.39±0.41	10.22±0.69*

*indicates significant difference to control cells ($p<0.05$)

The major fatty acids of *L. plantarum* NCIMB 8826 were myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1w7c}$), oleic acid ($C_{18:1w9c}$), cis-vaccenic acid ($C_{18:1w7c}$) and nonadecenoic acid ($C_{19:1}$). These acids constituted about 90% of the total fatty acids content, which is similar to the fatty acid composition previously reported for *L. plantarum* 2004, with the main exception being the presence of nonadecenoic acid ($C_{19:1}$) (Johnsson *et al*., 1995). The role of membrane fatty acid composition of LAB in acid stress response has been the subject of a number of studies; however, the results have been contradictory. The general hypothesis is that by modifying their membrane fatty acid composition the cells are able to adjust their membrane viscosity to respond to environmental stimuli (Guerzoni *et al*., 2001, Streit *et al*., 2008, Montanari *et al*., 2010, Wu *et al*., 2012). More specifically, in the case of acid stress, the cells should reduce

their membrane fluidity to protect the cytoplasm from the influx of hydrogen protons by either increasing the degree of saturation or decreasing the degree of unsaturation of the membrane fatty acids. The reasoning is that saturated fatty acids are linear and tightly pack together resulting in the production of a bilayer which has a high phase transition and low permeability, while the cis-double bond unsaturated fatty acids introduce a twist in the chain, which disrupts the order of the bilayer and leads to lower transition temperatures and higher permeability (Zhang and Rock, 2008). However, as mentioned above, the expected changes in the membrane fatty acid composition were not observed in this study (although a trend was observed suggesting an increase in the total saturated and a decrease in the total unsaturated fatty acids). The discrepancies observed in the published work could be attributed to differences in the intrinsic properties of the strains used, low accuracy and reproducibility in fatty acid analysis, as well as considerable differences in the experimental designs. For example some studies carried out acid stress experiments under growth conditions (at low pH or non-controlled pH), whereas others under non growth conditions in buffer solutions or media.

Interestingly, the fatty acid analysis indicates that the relative concentration of the cyclopropane fatty acid, cis-11,12-methylene octadecanoic acid ($C_{19cyclow7c}$) which was generally known as lactobacillic acid, significantly increased (from ~ 6% to ~ 10%) in the acid adapted cells compared to the control cells (Table 3.3). Although there are two cyclopropane fatty acids, dehydrosterculic acid (cis-9,10-methylene octadecanoic acid, $C_{19cyclow9c}$) and lactobacillic acid, which are normally found at high percentages in LAB after acid stress and cold stress (Montanari *et al*., 2010), only lactobacillic acid was detected in this study. According to Broadbent *et al*. (2010) and Wu *et al*. (2014), cyclopropane fatty acids are very important in controlling the biophysical properties of the cell membrane. An increase in cyclopropane due to acid stress was also observed

previously in *L. casei* strain Zhang (Wu *et al*., 2012). More specifically, the survival of *L. casei* strain Zhang was compared with mutants (obtained by serial sub-culturing the *L. casei* strain Zhang cells in MRS medium at pH of 4.3 for 70 days) in MRS acidified with HCl (pH 3), MRS acidified with lactic acid (pH 3.5), and simulated gastric juice (pH 2.5) for 0 to 2.5 h. The mutant strains showed better survival in the acidified solutions than the parental strain due to a higher amount of cyclopropane (octadecanoic acid, $C_{19cyclo}$) (3% to 9% higher at pH 3.5, from 0 to 1 h). Considering the results of this study in conjunction with the previous work with lactobacilli, it can be assumed that the increase in cyclopropane fatty acid in *L. plantarum* NCIMB 8826 improved cell survival during storage in the fruit juices by lowering the cell membrane fluidity which helps reducing the proton influx into the cells. Moreover, an increase in the cyclopropane content of *L. plantarum* DSM 10492 during growth at pH 5.5 in the presence of some phenolic compounds, such as caffeic acid, ferulic acid and tannin in the growth medium was observed in previous work (Rozes and Peres, 1998). It is possible to deduce that the increased levels of cyclopropane in the cell membrane not only reduce proton influx but also protect the cells from the phenolic compounds present in the fruit juices.

In the case of the combined acid and cold adaptation, the content of unsaturated and saturated fatty acids between the two treatments was very similar (Table 3.4), with the exception of a statistically significant increase in cyclopropane (lactobacillic acid, $C_{19cyclow9c}$) for acid adapted cells at 4 °C, although the absolute values were similar (9.8% versus 10.1%). A likely hypothesis that has been suggested to explain the influence of temperature is that in order for the cell membrane to function efficiently at low temperatures, the cells can potentially decrease the membrane fluidity by increasing the amount of unsaturated fatty acids (Aguilar *et al*., 1998, Deshnium *et al*., 2000). On

the other hand, it was seen in this and other studies that in acidic conditions, the cells can reduce the membrane fluidity by increasing the levels of saturated fatty acids and cyclopropane, in order to circumvent the influx of hydrogen protons inside the cells (Streit *et al*., 2008, Álvarez-Ordóñez *et al*., 2008, Alonso-Hernando *et al*., 2010). It appears that under highly acidic conditions, as those used in this study, the acid adaptation response is more important than cold adaptation, as overall the fatty acid compositions of acid adapted cells at 4 and 37 °C were very similar. This is consistent with the results presented in section 2.3.5 showing the survival of acid adapted cells (at the two temperatures) in fruit juices with similar patterns.

Table 3.4 Relative percentages of cellular fatty acids after incubation of *L. plantarum* cells in acidified citrate buffer (pH 3, 3 h) at 4 and 37 °C.

Systematic nomenclature	Lipid numbers	Percentage of cellular fatty acids	
		Acid adapted cells at 37 °C	Acid adapted cells at 4 °C
Tetradecanoic acid methyl ester	14:0	3.03±0.01	3.06±0.04
cis-9-Hexadecenoic acid methyl ester	16:1w7c	5.39±0.14	5.53±0.12
Hexadecanoic acid methyl ester	16:0	24.02±0.07	24.04±0.43
cis-9-Heptadecenoic acid methyl ester	17:1w8c	0.55±0.01	0.58±0.12
cis-9-Octadecenoic acid methyl ester	18:1w9c	8.36±0.02	8.48±0.02
cis-11-Octadecenoic acid methyl ester	18:1w7c	15.90±0.15	15.60±0.26
Octadecanoic acid methyl ester	18:0	1.52±0.01	1.45±0.02
cis-13-Nonadecenoic acid methyl ester	19:1 w6c	28.76±0.42	28.52±0.08
cis-11,12-Methylene octadecanoic acid methyl ester	19:0 cycw7c	9.76±0.03	10.05±0.03*

*indicates significant difference to control cells (p<0.05)

3.3.4. Gene expression analysis by RT-PCR

The c*fa* mRNA expression levels increased by approximately 12 fold after the cells were adapted in acidified MRS (pH 3, 25 °C, 1 h), relative to the control MRS (pH 6.4, 25 °C, 1 h). This increase in c*fa* mRNA is consistent with the results in table 3.3, which showed that in the acid adapted cells the concentration of cis-11,12-

methyleneoctadecanoic acid methyl ester (lactobacillic acid) increased from 6.4% to 10.2% and that of cis-11-octadecenoic acid methyl ester (cis-vaccenic acid) decreased from 15.46% to 13.73% compared with the control cells. These results demonstrate the significant role of *cfa* in the process of acid adaptation. The induction of the *cfa* gene under low pH was also observed in previous work with *Lactococcus lactis* MG 1363 (Budin-Verneuil *et al*., 2005) and *L. plantarum* KR6 (Seme *et al*., 2015), although a different experimental set up was used. More specifically, the amount of *cfa* mRNA in *Lactococcus lactis* MG 1363 determined by Northern blot analysis was higher when the cells were grown in HCl acidified M17 medium (pH 5, 30 min) compared to cells grown in control M17 medium (pH 7, 30 min). Upregulation of the *cfa* gene was shown for *L. plantarum* KR6 cells exposed to HCl acidified MRS (pH 4.5 and 2.5, 30 min) compared to control MRS (pH 7, 30 min).

In respect to the *fabH* gene, it was found that the levels of *fabH* mRNA increased 6 fold after acid adaptation, although this difference was not statistically significant. This increase is in accordance to the study by Fernandez *et al*. (2008), who reported that the level of *fabH* in *L. bulgaricus* cells increased 15 fold after being exposed to MRS acidified with lactic acid to pH 3.8, and subsequently incubated in MRS (pH 4.9 with lactic acid, 40 min). These suggest that an increase in straight chain saturated fatty acids should take place during acid adaptation in lactobacilli; however, the upregulation of *fabH* gene did not coincide with the saturated fatty acid profiles data between acid adapted and control cells (Table 3.3) as no significant differences were observed. This discrepancy might be due to the overexpression of *cfa* gene which utilised newly synthesised unsaturated fatty acids for cyclopropane production as mentioned above. Overall, the straight chain saturated fatty acids were synthesized in

L. plantarum NCIMB 8826 during acid adaptation but were converted to cyclopropane by *cfa* gene.

3.4. Conclusions

In summary, the results from this part of the work indicate that the response of *L. plantarum* NCIMB 8826 cells to short acid adaptation with citric acid did not involve the citrate fermentation pathway, which has been reported for *Lactococcus lactis*, as the citric acid was not metabolized and no lactic acid production was observed. In terms of cell morphology, the cell surface became rougher and thicker after exposure to acidic conditions, which indicates a possible overexpression of surface proteins or the production of EPS at the cell surface, resulting in a stronger cell wall with increased ability to inhibit the influx of hydrogen protons inside the cells during storage in fruit juices. Membrane fatty acid analysis indicated that the cyclopropane fatty acid cis-11,12-methylene octadecanoic acid ($C_{19cycw7c}$) content (expressed as percentage of total fatty acids) increased significantly ($p<0.05$) by about 1.67 fold during acid adaptation; this coincided with a significant upregulation (12 fold) of the *cfa* gene. No statistically significant ($p>0.05$) change in the degree saturation/unsaturation of the membrane fatty acids was observed in response to acid adaptation, although a trend showing an increase in total saturated fatty acids and a decrease in total unsaturated fatty acids was noted. Considering these results, it is likely that changes in the membrane fatty acid composition, particularly the increase in cyclopropane, in combination with changes in the call wall structure (potentially linked to the overexpression of surface proteins) enhanced the survival of acid adapted cells in the fruit juices. In order to investigate further the possible contribution of these mechanisms to the acid tolerance of the cells, proteomic analysis conducted by 2D-gel electrophoresis and mass spectrometry, as well

as intracellular analysis of amino acids was conducted. These experiments are presented and discussed in chapter 4.

3.5. References

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CHAPTER 4

Amino acid, GABA, and proteomic analysis of *L. plantarum* NCIMB 8826 cells in response to acid adaptation

4.1. Introduction

It has been demonstrated that amino acid metabolism is involved in the ΔpH homeostasis systems used by LAB during acid stress. For example, the accumulation of a basic amino acid, such as histidine, intracellularly was reported to protect *L. casei* ATCC 334 during acid stress (Broadbent *et al*., 2010), whereas *L. casei* strain Zhang accumulated aspartate and arginine intracellularly during acid stress and metabolised these to ammonia via the ADI system; this resulted in the generation of ammonia and thus the alkalization of the cytoplasm that led to enhanced survival at pH 3.5 for 3 h compared to unstressed cells (Wu *et al*., 2012, Wu *et al*., 2013). Moreover, the conversion of intracellular L-glutamate to GABA, with the concomitant consumption of a proton via the GAD system has been shown to contribute to the protection of a number of bacterial cells during acid stress including *E. coli* (Lin *et al*., 1995, Castanie-Cornet *et al*., 1999), *Listeria monocytogenes* (Cotter *et al*., 2001), *Lactococcus lactis* (Sanders *et al*., 1998) and *L. reuteri* (Su *et al*., 2011). In order to evaluate the potential involvement of amino acids and/or GABA in acid adaptation in this study, the intracellular and extracellular amino acid composition and the concentration of GABA after acid adaptation were analysed.

Proteomics is a powerful tool that can be used to monitor the overall changes of proteins throughout the cellular protein network during acid adaptation, including proteins involved in carbohydrate, lipid and protein metabolisms and stress responses. Based on 2D-gel electrophoresis, many studies have reported changes in the level of

expression of various proteins in lactobacilli during acid stress conditions including *L. acidophilus* (Lorca and de Valdez, 2001), *L. sanfranciscensis* (De Angelis *et al*., 2001), *L. casei* (Wu *et al*., 2011), *L. reuteri* (Lee *et al*., 2008) and *L. rhamnosus* (Koponen *et al*., 2012), although to our knowledge no such studies have been conducted with *L. plantarum*. The above proteomic studies have identified a large number of proteins changing during acid stress including molecular chaperone proteins or stress proteins, and proteins involved in fatty acid biosynthesis, nucleotide and amino acid biosynthesis, and peptidoglycan biosynthesis. Among these, the most commonly identified proteins are stress proteins, particularly the 70-kDa family (DnaK) and the 60-kDa family (GroEL) which play an important role in the folding and/or assembly of proteins (Hartke *et al*., 1996, Lim *et al*., 2000, Wu *et al*., 2014). On the other hand, MurA, MurD, and MurG, which are involved in the formation of the peptidoglycan backbone GlcNAc and MurNAc, and D-alanine-D-ligase which catalysed the assembly of D-alanyl-D-alanine dipeptide, have been recently reported by Wu *et al*. (2012) and Koponen *et al*. (2012) to be overexpressed in *L. casei* and *L. rhamnosus*, respectively.

As the cell walls of Gram-positive bacteria lack the outer membrane structure of Gram-negative bacteria, the thick layer of peptidoglycan in Gram positive bacteria, which contains CWGs, is essential for stabilizing and protecting the cell membrane from harsh environments (Chapot-Chartier and Kulakauskas, 2014). The major component of CWGs is a group of molecules called teichoic acids which can be sub-classified into two groups: WTA which is covalently linked to peptidoglycan, and lipoteichoic acid (LTA) which is anchored to the cytoplasmic membrane (Swoboda *et al*., 2010). More specifically, WTA is covalently linked to the MurNAc component of peptidoglycan by a disaccharide, while LTA is anchored to the cytoplasmic membrane

by a glycolipid. Besides binding to peptidoglycan and cytoplasmic membrane through these binding methods, the glycerol-phosphate chain structure of both teichoic acids can also bind to D-alanine via teichoic acid alanylation or D-alanylation, which is catalysed by the *dltABCD* operon (Weidenmaier and Peschel, 2008, Brown *et al*., 2013). The phosphodiester linkage between the D-alanyl ester residue and *sn* glycerol 1-phosphate of teichoic acid results in reducing the negative charge of glycerol 1-phosphate (PO_3^{2-}) in the cell envelope, as the protonated amino group of the D-alanyl ester residue can serve as counterions to the negatively charged phosphate group of the glycerol 1-phosphate unit of teichoic acid (Neuhaus and Baddiley, 2003). It seems likely that the more D-alanine is added the more positive charge the cell envelope has. Many cellular functional roles have been previously suggested for teichoic acid, such as maintenance cation homeostasis, transportation of ions, nutrients, proteins, and antibiotics, as shown for *Bacillus subtilis* (Neuhaus and Baddiley, 2003), control of divalent cation binding (Mg^{2+} and Ca^{2+}), as shown for *L. buchneri* and *L. plantarum* (Baddiley *et al*., 1973, Neuhaus and Baddiley, 2003), regulation of bacterial autolysis by preventing cationic autolysins binding on the bacterial surface, as shown for *Staphylococcus aureus* (Schlag *et al*., 2010), control of cell morphogenesis, particularly surface roughness, as shown for *L. plantarum* (Andre *et al*., 2011), and adhesion of abiotic surfaces and biofilm formation via surface-exposed polysaccharide of CWGs as shown *L. rhamnosus* (Lebeer *et al*., 2009). However, the most important function during acid stress is probably its control of cation binding, as this might help to protect the cells from the influx of high concentrations of protons. More specifically, this could be attributed to the binding between D-alanine and teichoic acid, which has been shown in a number of bacteria (such as *Bacillus subtilis*, *Staphylococcus aureus, L. buchneri*, *L. plantarum* and *L. rhamnosus*) to play an essential role in controlling the influx of positively

charged substances through the complicated peptidoglycan network (Kovacs *et al*., 2006, Perea Velez *et al*., 2007, Giaouris *et al*., 2008, Swoboda *et al*., 2010). The aim of this chapter was to evaluate the occurrence and potential contribution of the above mentioned mechanisms toward acid resistance of *L. plantarum* NCIMB 8826 following acid adaptation.

4.2. Materials and methods

4.2.1. Amino acid analysis

In order to investigate the potential role of amino acids in protecting the cells against acidic environment, the concentration of extracellular and intracellular amino acids were evaluated using the EZ faast™ amino acid analysis kit (Phenomenex, USA) which does not require any additional treatment for removing the interfering compounds (as traditional methods do). This method consists of a solid phase extraction step followed by derivatization and liquid/liquid extraction. After that, the derivatized amino acids were analysed by Gas Chromatography-Mass Spectrometry (GC-MS).

4.2.1.1. Amino acid analysis of supernatant samples

The supernatants of the adapted (pH 3, 25 ^{o}C, 1 h) and control cells (pH 6.4, 25 ^{o}C, 1 h) prepared as described in section 3.2.3 were collected for GC-MS analysis. A 100 µl aliquot of each supernatant including non-inoculated MRS was mixed with 200 µl of solution 2 (sodium carbonate). A 25 µl aliquot of the mixed samples and a 25 µl aliquot of an amino acid standard solution provided with the kit (over 50 amino acids) were separately loaded into a sample preparation vial and then 100 µl of 200 nM/ml norvaline was added into each sample as an internal standard. For solid-phase extraction, the samples were slowly filled up and passed through sorbent tips. After that,

the samples were washed with 200 µl of HPLC grade water and eluted with 200 µl of eluting solution (sodium hydroxide and N-propanol; ratio: 3:2). Then, the derivatized amino acids were extracted by 150 µl of isooctane/chloroform (ratio 1:2) and analysed by a Clarus 500 GC-MS system (Perkin Elmer, USA). An aliquot (2 µl) of the derivatized amino acid solution was injected at 250 °C in split mode (5:1) onto a 0.25 mm x 10 m Zebron ZB-AAA capillary column (Phenomenex, USA). The oven temperature was 110 °C for 1 min, then increased at 30 °C/min to 320 °C, and was then held at 320 °C for 2 min. The transfer line was held at 320 °C and the carrier gas flow rate was kept constant throughout the run at 1.1 ml/min. The ion source was maintained at 220 °C. Samples and mixtures of amino acid standards were analysed in triplicate. A calibration curve was plotted for each amino acid and used to calculate the amount of the amino acids in the samples. In order to confirm the accuracy of the results, a specific mass spectrum fragment ion for each amino acid described in the manual was selected. The area of the fragment ion of each amino acid was computed relative to the area of the fragment ion (158 m/z) of the internal standard (norvaline) to obtain the amount of each amino acid in the sample.

4.2.1.2. Intracellular amino acid analysis

The adapted and control cells derived from section 4.2.1.1 were rapidly quenched with 40 ml of pre-chilled 0.5 mM tricine in 60% (v/v) methanol (Sigma Aldrich, UK) in dry ice. The cell pellets were then collected by centrifugation at 12,000 g for 5 min, re-suspended in 4 ml of sterile distilled water, and disrupted with a Mini-Beadbeater as described in section 3.2.2. A 100 µl aliquot of a 1:10 dilution of the extract was loaded into a sample preparation vial and the amino acid composition was analysed as described in section 4.2.1.1.

4.2.2. Quantification of extracellular and intracellular GABA

In order to evaluate the effect of GAD system toward acid adaptation in *L. plantarum* NCIMB 8826, the concentrations of extracellular and intracellular GABA of acid adapted (pH 3) and control cells (pH 6.4) were determined according to the method of Tsukatani *et al*. (2005) following the modifications suggested by O'Byrne *et al*. (2011). Samples (90 μl) of reaction mixture containing 80 mM of Tris–HCl buffer (pH 9), 750 mM of sodium sulfate, 10 mM of dithiothreitol, 1.4 mM of NADP+, 2 mM of α-ketoglutarate, and 30 μg of GABase (Sigma-Aldrich, UK) were added into a 96-well microplate. Subsequently, 10 μl of the supernatants prepared as described in section 4.2.1.1 and 10 μl of disrupted cells prepared as described in section 4.2.1.2 were added into the 96-well microplate. The reactions were monitored for 3 h at 30 °C; non-inoculated MRS was used as a control. The formation of Nicotinamide adenine dinucleotide phosphate was measured as the absorbance at 340 nm using a microplate reader (Sunrise™, Tecan, UK). The concentration of GABA in the samples was calculated from a calibration curve using standard solutions. All samples and standards were analysed in triplicate.

4.2.3. Proteomic analysis of acid adapted and control cells

The expression of proteins in acid adapted and control cells was analysed by 2-D gel electrophoresis in order to monitor the overall changes in the proteome during acid adaptation. This method separates proteins based on their isoelectric points (pI) and their molecular weights. Differences between the adapted and control cells were identified taking into account the pI, size, and volume of the spots on the gels. Selected protein spots were digested using the in-gel trypsin digestion method and identified by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-

MS/MS). All proteomic analyses were performed at the Faculty of Medical Technology, Mahidol University, Thailand, while the differential protein expression analysis and spot selection (described in section 4.2.3.3) were carried out at the University of Reading.

4.2.3.1. Protein extraction

The pellets of acid adapted and control cells prepared as described in section 3.2.3 were collected and resuspended in 1 ml of lysis buffer consisting of 8 M urea (Sigma-Aldrich, UK) and 2% (v/v) CHAPS (GE Healthcare, UK) plus 10 µl/ml of protease inhibitor (GE Healthcare, UK). The cells were disrupted thrice by a Mini-Beadbeater as described in section 3.2.2. The supernatants were transferred to sterile 1.5 ml microtubes and stored at -80 °C until further analysis. The protein concentration in the supernatant was measured by the Bradford assay using Bradford solution (Sigma-Aldrich, UK). Bovine serum albumin (Sigma-Aldrich, UK), at various concentrations (0 to 1 mg/ml), was used to construct the calibration curve.

4.2.3.2. Protein separation

A 100 µl aliquot of extracted proteins were cleaned up using the 2-D Clean-Up Kit (GE Healthcare, Thailand). A 300 µl aliquot of precipitant solution was added into a microtube containing 100 µl of extracted proteins. The sample was mixed by inversion and incubated on ice for 15 min. A 300 µl aliquot of co-precipitant solution was then added into the mixture. After mixing for 5 sec by vortex mixer (vortex genie 2, Scientific Industries, USA), the mixture was centrifuged at 12,000 g for 5 min (Eppendorf® Minispin Plus Microcentrifuge, GE Healthcare, Thailand). The supernatant was removed by pipetting prior to adding 40 µl of co-precipitant solution on top of the pellet. The sample was kept on ice for 5 min prior to centrifugation (as above) and the

supernatant removed. A 25 µl aliquot of distilled water was added to cover the pellet. The sample was vortexed for 5 sec, followed by addition of 1 ml of pre-chilled wash buffer (-20 °C) and 5 µl of wash additive. The mixture was vortexed until the pellet was fully dispersed, then incubated at -20 °C for 30 min, and centrifuged at 8,000 g for 10 min. The pellet was collected, allowed to air dry for 5 min, and resuspended in 5 µl of rehydration buffer which consisted of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer with pH range 3–10 (GE Healthcare, Thailand), 0.002% (w/v) bromophenol blue and 12 µl/ml of DeStreak reagent (GE Healthcare, Thailand), with the aim to obtain a high enough protein concentration for first-dimension isoelectric focusing (IEF). An 18 cm Immobiline DryStrip precast gel (nonlinear gradient from pH 3 to 10) was rehydrated with 350 µl of the same rehydration buffer overnight on a reswelling tray, as recommended by the manufacturer (GE Healthcare, Thailand). A total amount of 80 µg (silver stain gel) and 300 µg (Coommasie blue gel) of each extracted protein was loaded into Immobiline DryStrip gel ran on the Ettan IPGphor 3 isoelectric focusing system equipped with a cup with a manifold loading system (GE Healthcare, Thailand). Additionally, pre-stained molecular weight markers (NEB, New England Biolabs Inc., Thailand) were loaded onto the gel. During the gel run, the voltage was increased from 500 to 1,000 V during the first 2 h and then increased to 8,000 V and kept at that value for 8 h. The temperature was maintained at 20 °C. After IEF, the strips were frozen at -80 °C for at least 1 day, thawed at room temperature and placed in the equilibration solution, consisting of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue, twice for 15 min, first in the presence of 10 mg/ml dithioreitol (Sigma-Aldrich, Thailand) and then in the presence of 25 mg/ml iodoacetamide (Sigma-Aldrich, Thailand). The second-dimension gel was performed using ExcelGel SDS Homogeneous 12.5 precast gels. For

the gel loaded with 80 µg of extracted protein the proteins were stained with silver strain (PlusOne Silver Staining Kit, GE Healthcare Biosciences, Thailand), while the gel loaded with 300 µg of extracted protein was stained by Coomassie blue (PlusOne Coomassie Tablets, PhastGel Blue R-350, GE Healthcare, Thailand).

4.2.3.3. Differential protein expression analysis

After staining, the gel images were digitized using an Image scanner III (GE Healthcare, Thailand). Two-dimensional gel analysis was performed by the ImageMaster 2D-platinum version 7.0 (GE Healthcare, Thailand) software tool. Images acquired from 6 independent gels were grouped in two classes according to the two treatments (acid adaptation and control). Each class contained 3 reference gels from the same environmental condition. After spot detection and landmark registration, each protein spot on the first set (control cells) were matched with its corresponding spot on the second set (acid adapted cells). Mismatches were checked and edited. The differential protein expression levels on the gels were quantified and computed by pixel intensity, area, and volume of the spots. A Student t-test was applied to validate the significance of the detected differences between the spot shapes and sizes. Only spots with Student t-values higher than 1.4 (P value set at <0.01) and relative expression ratio higher than 2 were selected and analysed by LC-ESI-MS/MS.

4.2.3.4. In-gel trypsin digestion for protein identification

The selected spots were manually excised from the gel and stored in 1.5 ml microtubes at −80 °C until further use. The proteins of interest were in-gel digested using the trypsin digestion method. Initially, the gel pieces were washed twice with 100 µl of 25 mM ammonium bicarbonate (Sigma-Aldrich, Thailand) in 50% (v/v) acetonitrile (Merck, Thailand) at room temperature for 30 min. Once the solvent was

removed, 50 µl of 100% acetonitrile were added and kept until the gel became white. After that, the acetonitrile was discarded and 10 µl of 0.01 mg/ml trypsin (Promega, Thailand) in 25 mM ammonium bicarbonate were added into each tube. The reaction was carried out at 37 °C for 24 h and the supernatant was centrifuged and collected into a new 1.5 ml microtube. This extraction process was repeated twice using 15 µl solution of 50% (v/v) acetonitrile and 5% (v/v) trifluoroacetic acid (Sigmal-Aldrich, Thailand) for 15 min in order to obtain high amounts of peptides inside the gel piece. All supernatants from the extraction processes were pooled together in the same microtube. Finally, the extracted peptides were dried in a vacuum centrifuge concentrator (CentriVap Benchtop Vacuum Concentrator, Labconco, USA) and reconstituted in 15 µl of 0.1% (v/v) formic acid (Sigma-Aldrich, Thailand) prior to analysis by LC-ESI-MS/MS.

4.2.3.5. LC-ESI-MS/MS analysis of extracted peptides

The extracted peptides were analysed by a LC-ESI-MS/MS system consisting of a liquid chromatography system (Dionex Ultimate 3000, Thermo Scientific, Thailand) in combination with an electrospray ionization (ESI) and Quadrupole ion trap mass spectrometer (Model amazon SL, Bruker, Germany). The peptide separation was performed on a 50 x 0.5 mm x 5 µm C18 reversed phase column (Hypersil GOLD, Thermo Scientific, Thailand) protected by a 30 x 0.5 mm x 5 µm C18 guard column (Hypersil GOLD, Thermo Scientific, Thailand). 0.1% (v/v) formic acid was used as mobile phase A and 100% (v/v) acetonitrile as mobile phase B. The flow rate was eluted at 100 µl/min under a gradient condition ranging from 5% B to 80% B in 50 min. Mass spectral data from 300 to 1500 m/z were collected in the positive ionization mode.

4.2.3.6. Protein identification

For the identification of proteins, all the MS/MS spectra recorded on the tryptic peptides present in the obtained gel spots were searched against the protein sequences from the NCBI and SwissProt databases using the MASCOT search program (www.matrixscience.com). The searching criteria were the following: complete carbamidomethylation of cysteine and partial methionine oxidation; an initial mass tolerance of ± 200 ppm was applied for all searches; the number of missed cleavage sites was allowed up to 1. The MASCOT search scores that were greater than 67 were considered to be significantly different ($p<0.05$) to decrease the possibility of false matches between the peptide sequences from the experimental data and the reference databases. Moreover, the similarity of the experimental pI and molecular weight data of the identified proteins compared to the theoretical ones, obtained from reference databases, were also taken into account.

4.3. Results and discussion

4.3.1. Extracellular and intracellular amino acid composition

All amino acids in acidified MRS (pH 3) and unmodified MRS (pH 6.4) decreased after adding the cells for 1 h at 25 ^{o}C compared to non-inoculated MRS (pH 6.4) (Figure 4.1). The levels of decrease ranged from 5% to around 75% depending on the amino acid. The greatest decreases were observed for glutamic acid, phenylalanine, lysine and proline (all between 60% and 75% decreases) for both acid adapted and control cells. It is interesting to note that glutamic acid is one of the essential amino acids along with valine, leucine, isoleucine, methionine, phenylalanine and tryptophan, and as such is required for the growth of *L. plantarum* WCFS1; lack of these amino acids in the media significantly affects its growth (Teusink *et al*., 2005). Moreover,

these amino acids can be used by the bacterial cells for ATP generation through the tricarboxylic acid cycle; for example lysine can be converted to the acetyl-coA while proline and glutamic acid can be converted to α-ketoglutarate. It is therefore likely that the decrease that was observed for these particular amino acids might be associated with cell maintenance activities rather than acid resistance. On the other hand, a significant decrease in certain extracellular amino acids, in particular alanine (~10% difference), glycine (~20% difference), leucine (~15% difference), aspartic acid (~10% difference) and tryptophan (~5% difference), was observed between acid adapted and control cells, suggesting that these amino acids might be involved in acid adaptation. The particular case of alanine and its potential contribution is discussed later on in this chapter in detail.

Alanine was the most abundant intracellular amino acid found in both acid adapted and control cells (~47% and ~67%, respectively, of the total amino acid content) followed by glycine, glutamic acid, ornithine, and lysine (Table 4.1). In response to acid adaptation, only alanine was significantly reduced (by around 1.4 times), whereas the other amino acids tended to increase in most cases, except for aspartic acid and ornithine. Although there is no previous study investigating the changes in intracellular amino acid profile of *L. plantarum* due to acidic environments, the role of amino acids in response of acid stress has been previously reported for *L. casei* (Broadbent *et al*., 2010, Wu *et al*., 2012, Wu *et al*., 2013). These studies have indicated that the intracellular accumulation of basic amino acids, particularly histidine, protected *L. casei* ATCC 334 during acid stress (Broadbent *et al*., 2010), whereas the accumulation of arginine (a basic amino acid) and to a lesser extent aspartate during acid stress of *L. casei* strain Zhang was able to enhance survival at pH 3.5 for 3 h (Wu *et al*., 2012, Wu *et al*., 2013). More specifically, intracellular aspartate can be converted

to arginine by Ass and Asl at the expense of one molecule of ATP, resulting in the intracellular accumulation of arginine. Arginine can be then used to reduce the pH_i due to the production of ammonia by the ADI pathway. In addition to the above amino acids the conversion of intracellular L-glutamate to GABA by the GAD system can lead to protection from acid stress in many bacteria (Lin *et al*., 1995, Sanders *et al*., 1998, Castanie-Cornet *et al*., 1999, Cotter *et al*., 2001, Su *et al*., 2011).

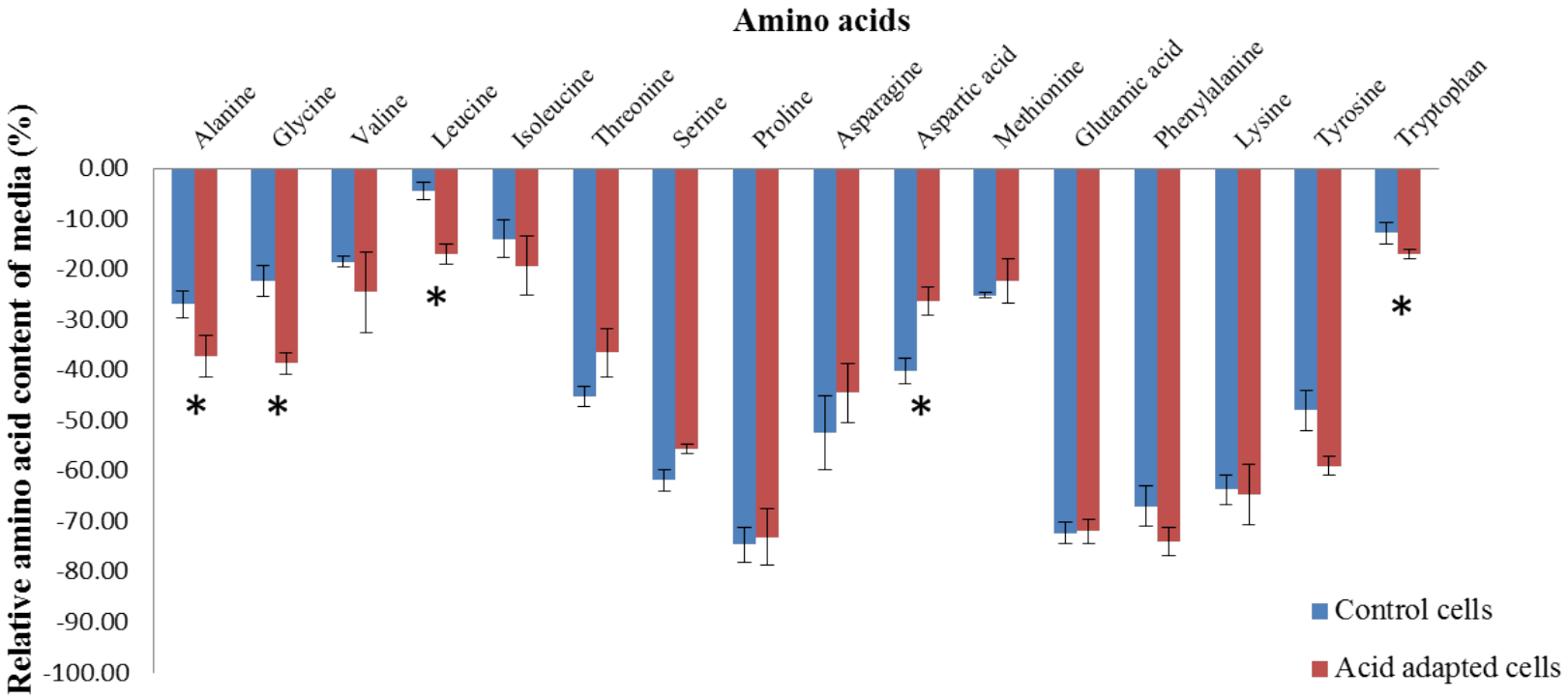

Figure 4.1 Relative amino acid content of MRS media (relative to non-inoculated MRS) derived from acid adapted cells (acidified MRS, pH 3, 25 °C, 1 h) and control cells (unmodified MRS, pH 6.4, 25 °C, 1 h). * indicates significant differences for each amino acid between acid adapted and control cells determined by paired t-test.

Histidine and arginine, both basic amino acids, were not observed in either acid adapted or control cells whereas the % intracellular aspartate was not significantly different between acid adapted and control cells (Table 4.1), indicating that the ADI pathway was most likely not involved in enhancing the acid tolerance of the cells. Among the other basic amino acids, lysine significantly increased in acid adapted cells, although the increase was relatively low (~1.6%). The above suggested that *L. plantarum* NCIMB 8826 most likely did not accumulate significant amounts of basic amino acids as the means for increasing its pH_i.

The intracellular glutamic acid increased significantly in acid adapted cells compared to control cells (~7.5%), which also coincided with the high consumption of extracellular glutamic acid. This suggests that a possible mechanism for the cells to maintain their pH_i in the case of acid stress is through the conversion of intracellular L-glutamate to GABA, with the concomitant consumption of a proton, via the GAD system. To test this hypothesis the concentration of GABA produced by the GAD system was examined (see next section).

It is very interesting to note that alanine, which represented the most abundant intracellular amino acid, was significantly reduced in the case of acid adapted cells by ~20% (Table 4.1); this coincided with a significant decrease in extracellular alanine (~10%) (Figure 4.1). These decreases suggest that alanine might be utilised by the cells as a response to acid adaptation. Furthermore, it has been previously suggested that increased D-alanylation of teichoic acid (an esterification reaction), which in the case of LAB is covalently linked to peptidoglycan, can potentially protect the cells from the influx of hydrogen protons and cationic antimicrobials (e.g. nisin), although the mechanisms have not been elucidated (Weidenmaier and Peschel, 2008, Giaouris *et al*.,

2008, Swoboda *et al*., 2010). However, it was reported by MacArthur and Archibald (1984) that the degree of ester linkage (binding) between D-alanine and teichoic acid in *Staphylococcus aureus*, a Gram positive bacterium, decreased when the pH of the medium increased (degree of binding 0.75 at pH 6.1 to 0.07 at pH 8.1). A similar result was shown for *Bacillus subtilis* when the pH of the growth medium decreased from pH 7 to pH 5 (a higher degree of binding was observed in the latter case) (Ellwood and Tempest, 1972). In this study, determination of degree of ester linkage for acid adapted and control *L. plantarum* NCIMB 8826 cells was not conducted and therefore this hypothesis cannot be confirmed.

Table 4.1 Intracellular amino acid profiles (expressed as % of total amino acids) in cells exposed to acidified MRS (pH 3, 25 °C, 1 h) and unmodified MRS (pH 6.4, 25 °C, 1 h).

Name	**Fragmention (m/z)**	**Retention time (min)**	**Percentage of intracellular amino acid**		**P value for Paired t-test**
			Control cells	**Acid adapted cells**	
Alanine	130	1.49	66.71±2.09	47.32±3.50	0.020
Glycine	116	1.61	5.91±0.31	10.13±0.54	0.010
Leucine	172	2.04	0.90±0.90	2.34±0.27	0.200
Isoleucine	172	2.10	0.45±0.10	1.30±0.23	0.039
Serine	146	2.36	3.25±0.61	3.74±1.26	0.555
Proline	156	2.44	0.74±0.09	1.31±0.06	0.023
Asparagine	155	2.54	0.79±0.22	3.42±0.51	0.020
Aspartic acid	216	3.11	0.85±0.21	0.77±0.09	0.690
Glutamic acid	84	3.47	4.03±1.03	11.55±1.39	0.027
Phenylalanine	148	3.51	0.00±0.00	1.24±0.34	0.024
Ornithine	156	4.54	11.81±2.86	11.86±1.79	0.988
Lysine	170	4.80	4.57±0.41	6.14±0.53	0.022

Results are expressed in mean ± SD where n=3. Significant differences in the content of each amino acid between acid adapted and control cells was determined by paired t-test.

4.3.2. Extracellular and intracellular GABA content

Extracellular GABA increased significantly from 1.5 mM in non-inoculated MRS to 2.4 mM in unmodified MRS (supernatant from control cells), but no GABA was detected in acidified MRS (supernatant from acid adapted cells). This suggests that for acid adapted cells GABA were reduced to zero whereas for control cells it was produced to about 0.9 mM. This was an unexpected finding, particularly for LAB as previous research has shown that the wild type of *L. reuteri* 100-23 produced GABA when incubated for 24 h in acidified phosphate buffer containing 10 mM glutamate (pH 2.5 using HCl, 37 °C), whereas a *gadB* mutant strain did not (Su *et al*., 2011). The mechanism proposed in that study was that cellular response to acid stress induced GAD which catalysed the conversion of L-glutamate by consumption of a proton, thus yielding CO_2 and GABA; the latter was secreted out of the cells via its antiporter in order to avoid significant drop in pH_i. However, the absence of GABA in acid adapted *L. plantarum* NCIMB 8826 cells in this study might be due to pH 3 which is most likely lower than the optimum pH for GAD activity. The optimum pH reported for GAD activity was various depending on the LAB species, e.g. a pH of ~ 4.2 has been reported for *L. brevis* (Ueno *et al*., 1997) and a pH of ~ 5.0 for *L. sakei* (Sa *et al*., 2015). Additionally, the production of extracellular GABA by *L. paracasei* grown in MRS containing 500 mM glutamate at 37 °C for 5 days, was considerably lower at pH 4 compared to pH 5 and 6 (Komatsuzaki *et al*., 2005) indicating a negative effect of low pH on GAD activity. Furthermore, glutamate is potentially more beneficial for the cells than converting it to GABA, as it has a higher buffering capacity than GABA at pH values lower than 3 (due to the differences in the pKa of their α-carboxyl groups

[glutamate pKa =2.1, GABA pKa =4.2]; (Feehily and Karatzas, 2013), which may also explain the absence of GABA in acid adapted cells.

Taking the above into account, it would be expected that after acid adaptation GABA would be similar to non-inoculated MRS (~ 1.5 mM). The fact that no GABA was detected indicates that acid adapted cells most likely used GABA as substrate for other metabolic activities; more techniques such as Phenotype MicroArrays (Biolog) is needed in order to identify the potential biochemical reactions involved or changed. Furthermore, as no GABA was produced, and considering the significant decrease in glutamic acid (~70% decrease) in acid adapted cells (Figure 4.1), a major substrate for GAD, this raises the question on the fate of glutamic acid. The proposed hypothesis is that in the case of acid adapted cells glutamic acid was used as a component for peptidoglycan biosynthesis; this will be evaluated based on the results from the proteomic analysis and discussed in the following section of this chapter. On the other hand, in the case of control cells, glutamic acid seemed to be partly used for the production of GABA by GAD as normally found in LAB at pH around 5 to 6.

No GABA was detected intracellularly for either acid adapted cells or control cells. This was expected for acid adapted cells as there was no production of GABA as shown and discussed above, but not for the control cells. Interestingly, this result is in agreement with the study of Komatsuzaki *et al*. (2005) who demonstrated that the intracellular GABA of *L. plantarum, L. brevis* and *L. paracasei* grown in MRS (pH 6.5, 30 °C, 6 days) was extremely low compared with the extracellular concentration, indicating that the GABA produced by control cells was rapidly secreted after its synthesis inside the cells. Overall, the results from this part of the study indicated that the GAD system did not contribute toward the enhanced survival of acid adapted

L. plantarum NCIMB 8826 cells in fruit juices but it seems likely that the glutamic acid present in acidified MRS was used for peptidoglycan biosynthesis rather than in ADI pathway in order to protect the cells from the low pH of the fruit juices during storage.

4.3.3. Proteomic analysis of acid adapted and control cells

Proteomic analysis of *L. plantarum* NCIMB 8826 cells in acidified MRS (pH 3, 25°C 1 h) and unmodified MRS (pH 6.4, 25°C, 1 h) was conducted and the obtained protein profiles were compared. In the initial experiments, silver stain was used to stain the gels (Figure 4.2). Approximately 1,500 protein spots were detected after acid adaptation. Most proteins were located between pH 4 to 7 in the gradient gel, which ranged from pH 3 to 10. Upon analysis of the data set using the ImageMaster 2D-platinum software, 18 proteins were selected which had at least 2 fold difference (range ratio > 2) in % volume of spots between acid adapted and control cells; these are listed in table 4.2 and are pinpointed in figure 4.2. More specifically, after acid adaptation at pH 3 for 1 h, 2 proteins were upregulated, 12 proteins were downregulated, and 4 proteins were only found in the gel of the control cells. Overall, from this initial analysis it was demonstrated that short exposure to citric acid affected the expression of several proteins with a pH ranging between 4 and 7. However, these proteins were unable to be identified as silver stained proteins were difficult to be further analysed with the available identification system. Therefore, Coomassie blue staining was thereafter used to analyse and evaluate the expression of proteins in acid adapted and control cells.

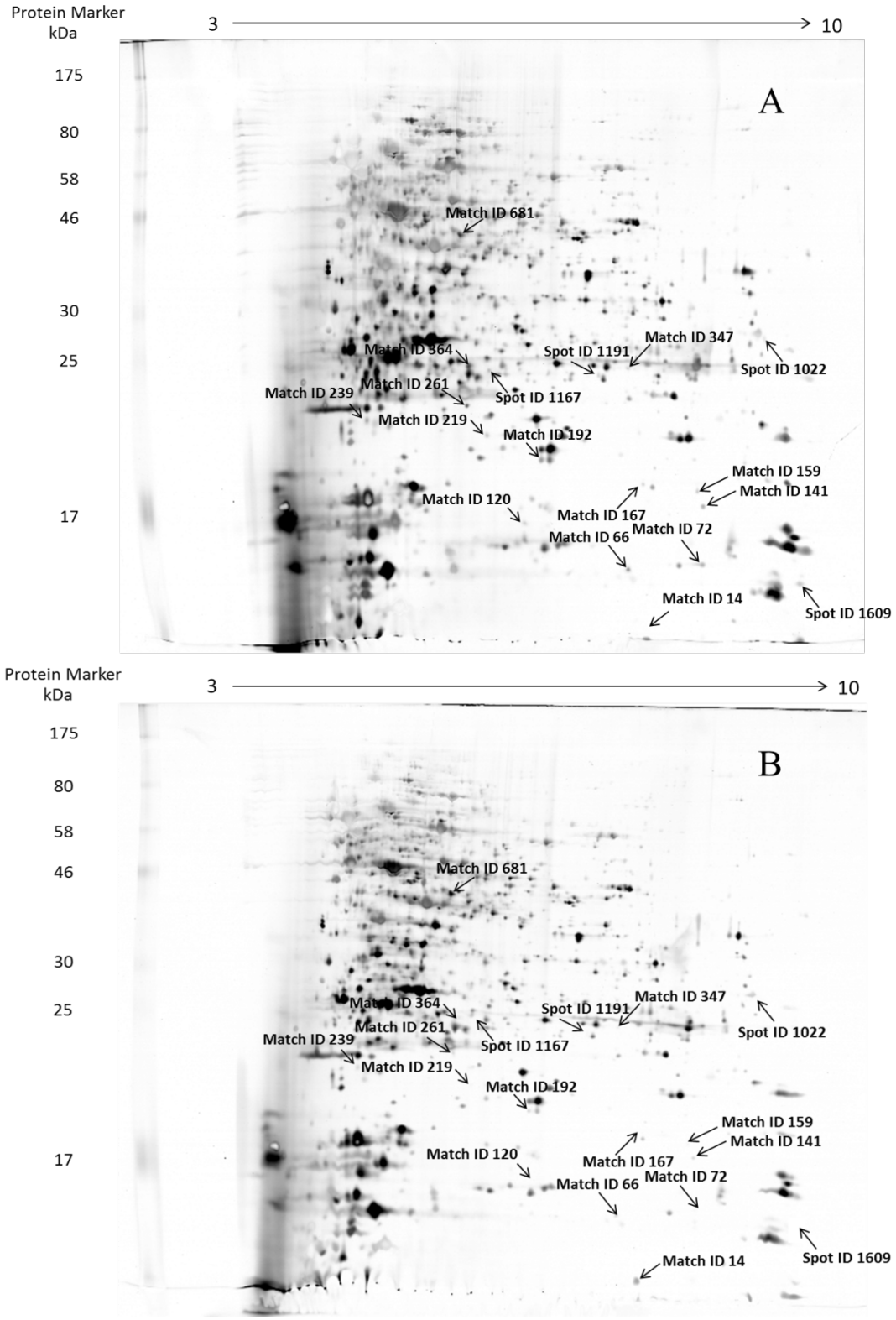

Figure 4.2 Silver stain gels depicting the total protein spots for *L. plantarum* NCIMB 8826 after incubation in: (A) MRS at pH 6.4, 25 °C for 1 h (control cells), and (B) acidified MRS at pH 3, 25 °C for 1 h (acid adapted cells). The arrows pinpoint the spots which were significant differences in shapes and sizes between acid adapted and control cells.

Table 4.2 Expressed proteins showing at least a range ratio > 2 between acid adapted and control cells. The gel was stained with silver stain and analysed using the ImageMaster 2D-platinum software. The Match/Spot ID refer to figure 4.2.

Match/Spot ID	Range Ratio	%Volume of Control cells spot	%Volume of acid adapted cells spot
14	17.16	0.006	0.098
72	-10.16	0.055	0.005
159	-5.74	0.020	0.004
192	-3.93	0.079	0.020
261	-3.76	0.055	0.015
120	-3.60	0.024	0.007
347	-3.01	0.022	0.007
66	-2.83	0.043	0.015
219	-2.74	0.026	0.010
141	-2.65	0.033	0.013
364	-2.63	0.137	0.052
681	2.61	0.068	0.177
167	-2.37	0.013	0.005
239	-2.24	0.046	0.020
1022	-	0.01	-
1609	-	0.01	-
1191	-	0.02	-
1167	-	0.03	-

The expression level was determined by the relative volume of each spot in the gel and expressed as %Volume (%Vol = spot volume/Σvolumes of all spots resolved in the gel). The range ratio was calculated as the ratio of average values of %Vol between non-stressed and acid stressed cells. Only spots with a range ratio greater than 2 (with significance set at 2-fold change, $p<0.05$) in the ImageMaster 2D-platinum report were considered relevant. (– indicates downregulation)

With Coomassie blue staining, approximately 750 protein spots were detected in each gel (Figure 4.3). Out of these, 8 proteins were selected, as their % volume of spots changed by about 1.4 fold (range ratio > 1.4) or more; they were then identified by mass spectrometry and were compared with the reference proteins obtained by the MASCOT search programme. The proteins and their predicted properties and functions are listed in table 4.3; they include molecular chaperone GroEL, aminopeptidase C, 30S ribosomal protein S1 and S2, aspartate semialdehyde dehydrogenase, D-alanine-D-

ligase, and two proteins with unknown function which were UPF0356 protein Ip_2157 and the hypothetical protein HMPREF0531_11643. It needs to be noted that *cfa*, a ~45 kDa protein with a pI of 6.03, was not one of the selected proteins even though it was upregulated (12 fold) during acid adaptation as previously discussed. This might be due to the fact that a wide range IPG strip (pH 3 to 10) was used in this study, which might not be suitable for separating this particular protein; *cfa* from several *L. plantarum* strains (LC56, LC804, and 299V) was identified using a middle range IPG strip (pH 4 to 7) in the study of Hamon *et al*. (2011). In summary, 5 proteins were upregulated and 3 proteins were downregulated in the case of acid adapted cells compared to control cells.

The molecular chaperones GroEL is a part of the two main groups of molecular chaperones which consist of the DnaK chaperone family (DnaK, DnaJ and GrpE) and the GroEL chaperone family (GroEL and GroES) (De Angelis *et al*., 2004). These proteins play an important role in the folding and/or assembly of proteins under normal and stress conditions (Georgopoulos and Welch, 1993, Ryabova *et al*., 2013). Desmond *et al*. (2004) indicated that overexpression of GroEL in stress-adapted cells (e.g. heat, salt, solvent) had the potential to improve the survival of *L. plantarum* NFBC 338 under stress conditions compared with non-adapted cells. Moreover, this protein was previously shown to be upregulated in *Lactococcus lactis* and *L. bulgaricus* during acid stress along with other related acid shock proteins including GroES, GrpE and DnaK (Champomier-Verges *et al*., 2002). Overall, GroEL was found to be upregulated in several lactobacilli due to acid adaptation but there is no proposed mechanism how GroEL improves cell viability under acidic conditions. It is likely that the

overexpression of GroEL in acid adapted cells might help to prevent the denaturation of cellular proteins and to repair them.

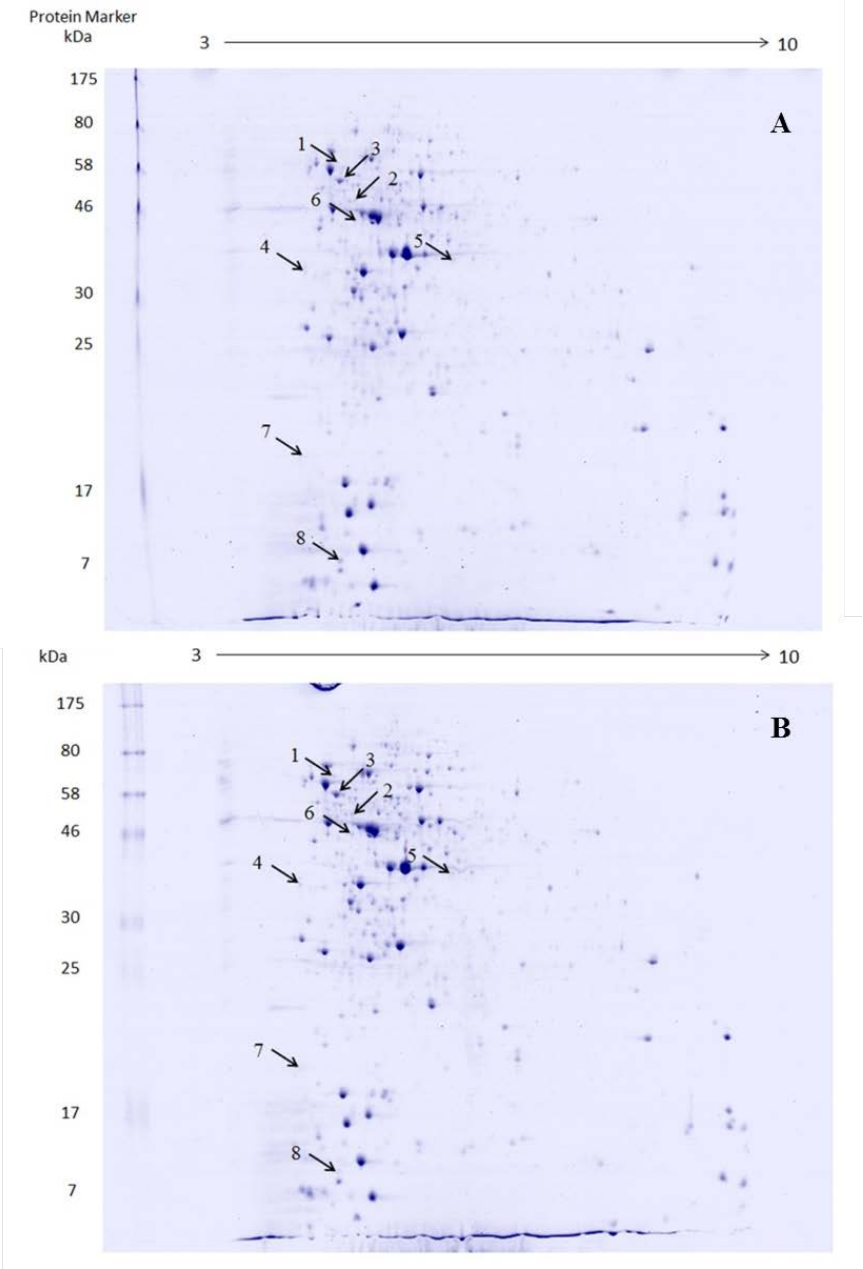

Figure 4.3 One of the three Coomassie blue staining gels depicting the total protein spots for *L. plantarum* NCIMB 8826 after incubation in: (A) MRS at pH 6.4, 25 °C for 1 h (control cells), and (B) MRS at pH 3, 25 °C for 1 h (acid adapted cells). The arrows pinpoint the spots which were significant differences in shapes and sizes between acid adapted and control cells. 1) Molecular chaperone GroEL, 2) Aminopeptidase C, 3) 30S ribosomal protein S1, 4) 30S ribosomal protein S2, 5) Aspartate semialdehyde dehydrogenase, 6) D-alanine-D-ligase, 7) UPF0356 protein Ip_2157, and 8) Hypothetical protein HMPREF0531_11643.

Table 4.3 Expressed proteins showing at least a range ratio > 1.4 between acid adapted and control cells. The gel was stained with Coomassie blue and analysed using the ImageMaster 2D-platinum software. The spot number refers to figure 4.3.

Spot No	Database Accession no.	Putative function	Gene	Mass (Da)	pI	Protein score	Fold change
Acid stress							
1	gi|489736891	Molecular chaperone GroEL	*groEL*	57402	4.69	329	1.55
Protein degradation							
2	gi|489739850	Aminopeptidase C	*pepC*	50413	4.89	136	1.60
Protein synthesis							
3	gi|489736494	30S ribosomal protein S1	*rpsA*	47131	4.79	330	1.46
4	gi|489734874	30S ribosomal protein S2	*rpsB*	30208	5.15	157	-1.51
Amino acid biosynthesis							
5	gi|334880771	Aspartate semialdehyde dehydrogenase	*asd*	38422	5.48	91	-1.40
Peptidoglycan biosynthesis							
6	DDL_LACPL	D-alanine-D-ligase	*ddl*	41378	4.86	297	1.52
Unknown function							
7	Y2157_LACPL	UPF0356 protein Ip_2157	-	8713	4.49	88	1.47
8	gi|300494317	Hypothetical protein HMPREF0531_11643	-	15898	5.33	103	-4.45

Protein score: the probability whether an observed match between experimental data and peptide sequences found in a reference database has occurred by change.

Aminopeptidase C is one of the enzymes that catalyze the cleavage of amino acids from the amino terminus (N-terminus) of proteins or peptides. The specificity of aminopeptidase C has been studied in many LAB using a variety of synthesized amino acid β-naphthilamide as substrates, and the results have shown that this peptidase acts on specific basic amino acids (arginine, histidine lysine), acidic amino acids (glutamic acid and aspartic acid), hydrophobic/uncharged amino acids (alanine and leucine) and the aromatic amino acid (phenylalanine) (Christensen *et al*., 1999). Wu *et al*. (2011) demonstrated that several peptidases, namely, aminopeptidase P, aminopeptidase N, endopeptidase, dipeptidase and tripeptidase were induced after acid stress of *L. casei* strain Zhang. However, *L. plantarum* WCFS1 is known to contain more than 19 peptidases (Kleerebezem *et al*., 2003), but only aminopeptidase C was overexpressed in this experiment with *L. plantarum* NCIMB 8826 during acid adaptation compared to the control conditions. It is likely that the overexpression of aminopeptidase C provided specific amino acids to the cells, particularly alanine, lysine, and glutamic acid, which were accumulated inside the cells after acid adaptation (Table 4.1). The hypothesis that the overexpression of aminopeptidase C might improve the survival of *L. plantarum* NCIMB 8826 in acidic condition will be discussed further below.

The expression of the ribosomal protein S1 increased by about 1.5 fold while ribosomal protein S2 decreased by about 1.5 fold after acid adaptation. This result is contradictory with the fact that ribosomal proteins S1 and S2 contribute toward the translation of mRNA by stimulating the binding of the 30S ribosomal subunit to the ribosomal binding site of mRNA, the Shine-Dalgarno sequence (Nikolay *et al*., 2015); therefore, the expression patterns of both proteins should be in same direction. There is no clear explanation for this phenomenon; however, it is interesting to note that it has

been previously suggested that the overexpression of protein S1 resulted in the inhibition of general protein synthesis in *E. coli* (McGinness and Sauer, 2004). Based on this, it is likely that the translation of proteins from mRNA might have decreased instead of increased during acid adaptation, which is in accordance with the observed decrease in the total protein concentration, measured by Bradford's method, of acid adapted cells compared with control cells (data not shown).

Aspartate semialdehyde dehydrogenase generally catalyses the conversion of *β*-aspartyl phosphate to aspartate *β*-semialdehyde, which is an important intermediate in the biosynthesis of some amino acids including lysine, methionine, leucine and isoleucine from aspartate (Biellmann *et al*., 1980). It is also involved in the production of DAP, an essential component for peptidoglycan synthesis of Gram-positive bacterial cell walls (Pavelka and Jacobs, 1996). The downregulation of this protein might have led to the accumulation of *β*-aspartyl phosphate or aspartic acid. In that case, aspartic acid might have been used to alkalinize the cytoplasm due to ammonia produced via the ADI pathway as reported for *L. casei* strain Zhang during acid stress (Wu *et al*., 2013) and discussed previously in section 4.3.1. However, the % of intracellular aspartic acid of acid adapted cells (0.77%) was not significantly different to that of control cells (0.85%) (Table 4.1), and therefore it is difficult to pinpoint the potential role, if any, of aspartate semialdehyde dehydrogenase towards the acid tolerance of *L. plantarum* NCIMB 8826 cells.

D-alanine-D-ligase is an enzyme participating in D-alanine metabolism and peptidoglycan biosynthesis by catalysing the ligation of two D-alanine molecules to form the D-alanyl-D-alanine dipeptide, a key building block in peptidoglycan biosynthesis (Bruning *et al*., 2011). This role of D-alanine-D-ligase is in accordance

with the observed significant reduction in the % of intracellular alanine of acid adapted cells compared to control cells (Table 4.1) and the decrease in the extracellular alanine observed for acid adapted cells (Figure 4.1). Based on these changes, it is likely that acid adapted cells utilised alanine to increase the thickness the peptidoglycan or modify its structure, for example through increased bonding of alanine and WTA leading to tighter cell wall, which was also indicated by electron microscopy in chapter 3. As discussed previously, teichoic acid has been shown to play an essential role in controlling the influx of positively-charged substances through the peptidoglycan network (Kovacs *et al*., 2006, Perea Velez *et al*., 2007, Giaouris *et al*., 2008, Swoboda *et al*., 2010). However, it must be noted that D-alanine-D-ligase might compete for alanine which, as discussed previously, is likely to be used during acid adaptation for D-alanylation reactions, catalysed by the *dltABCD* operon. Interestingly, the upregulation of D-alanine-D-ligase gene and other genes involved in peptidoglycan biosynthesis were also previously reported in *L. casei* strain Zhang during acid stress at pH 3.5 (Wu *et al*., 2012). In order to synthesize peptidoglycan, not only D-alanine is required but other amino acids are also needed, such as glutamic acid, lysine and ornithine (Chapot-Chartier and Kulakauskas, 2014). More specifically, the sugar component of bacterial peptidoglycan consists of alternating residues of GlcNAc and MurNAc linked by β-(1,4) glycosidic bonds, while the amino acid component, a peptide chain containing 3 to 5 amino acids, is attached to MurNAc. Typically, L-alanine is bound to MurNAc, followed by D-glutamic acid, which is linked by an interpeptide cross-bridge using lysine, ornithine or DAP, depending on the bacterial species. Finally, the D-alanyl-D-alanine dipeptide is attached to this bridge (Chapot-Chartier and Kulakauskas, 2014). However, in contrast to alanine, in the case of glutamic acid and

lysine an increase in their intracellular content (expressed as % of total amino acids) was observed for acid adapted cells compared to control cells (Table 4.1), which contradicts the above hypothesis. This might be explained by the higher ratio of alanine used typically for peptidoglycan biosynthesis and binding to teichoic acid, the likely rerouting of GABA by GAD system as discussed in section 4.3.2, the likelihood that the interpeptide cross-bridge was replaced by other amino acids such as DAP, and the possibility that lysine and glutamic acid were accumulated as a result of the hydrolytic activity of aminopeptidase C, which was upregulated as discussed previously. In summary, the observed changes in the proteome and the amino acid composition taking place during acid adaptation of *L. plantarum* NCIMB 8826 cells suggest that the enhanced survival of acid adapted cells in fruit juices was associated with the modification of the peptidoglycan matrix during acid adaptation, and highlighted the key role of D-alanine in this change.

4.4. Conclusion

The results from the analysis of amino acid and GABA after acid stress indicated that the response of *L. plantarum* cells to acid adaptation did not involve the ADI pathway as indicated by the fact that aspartic acid and arginine did not accumulate inside the cells. Moreover GAD, which has been reported to be active in LAB under growth conditions at pH between 4.2 and 5.0, most likely was not involved during acid adaptation as GABA was not produced. Proteomics analysis of acid adapted and control cells demonstrated that: i) the overexpression of molecular chaperone GroEL, which plays an important role in the folding and/or assembly of proteins, most likely has an active role toward acid tolerance which helps to improve the survival of *L. plantarum* NCIMB 8826 in fruit juices although the exact mechanism cannot be elucidated without

further investigation; ii) the upregulation of D-alanine D-ligase and the considerable reduction in intracellular and extracellular alanine indicate that structural and compositional modifications of the peptidoglycan took place during acid adaptation. These most likely resulted to the formation of a thicker and rougher cell wall, as observed by SEM, potentially through increased bonding of alanine and cell wall teichoic acid, thus protecting the cells from the influx of hydrogen protons and enhancing their survival during subsequent storage in the fruit juices.

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CHAPTER 5

General discussion

5.1. General discussion

The extensive demand for new probiotic products encourages the development of novel non-dairy products containing probiotics, particularly fruit juices because they are perceived by consumers as being healthy and refreshing foods; have sensory profiles which are acceptable to all age groups; and are suitable for people with lactose intolerance (Luckow and Delahunty, 2004). However, from a new product development point of view, it is necessary that the probiotic strain has to survive well during storage, in order to ensure that at the time of consumption a high concentration of viable cells is present in the food product; this is required for its efficacy in the GIT. Previous research studies have shown that cell survival during storage is significantly affected by the type of juice, in particular, some very acidic juices (Champagne *et al*., 2005, Sheehan *et al*., 2007, Mousavi *et al*., 2011, Nualkaekul and Charalampopoulos, 2011). The aim of this work was to evaluate whether exposing early stationary phase *L. plantarum* NCMIB 8826 cells (a potential probiotic strain) to acid for a short period of time would improve their subsequent survival during refrigerated storage in fruit juices. Although using the probiotic strains that are able to maintain their viability and activity during production (fermentation) and selecting the suitable secondary processing (e.g. drying, dried storage, food processing and storage) are very important parts of probiotic product development, implementing an acid adaptation step at some point in the manufacturing process (e.g. after harvesting of the probiotic cells from the fermenter before subsequent

drying or incorporation into fruit juices) could potentially increase the robustness of the strains and lead to the development of a range of low pH fruit juice products containing probiotics. The rationale for this work was that such a strategy, if successful, could be relatively easy to transfer to a commercial process without a need for extensive capital investment. It can also be used in combination with other technologies, such as encapsulation in order to increase probiotic survival during processing and storage. A considerable number of foods containing probiotic bacteria are acidic foods, and therefore if developed, such technology could find significant applications within the food industry and possibly other sectors as well, such as industrial and environmental biotechnology.

The results of the present study demonstrated that when *L. plantarum* NCMIB 8826 cells were adapted for a short period of time in acidic solutions (HCl or citric acid) at various pH, particularly pH 3 and 4, their subsequent survival during refrigerated storage in three fruit juices including cranberry (pH 2.7), pomegranate (pH 3.5), and lemon & lime juices (pH 2.8) were improved. However, even under the best acid adaptation conditions, (i.e. incubation of up to 3 h at 25 °C in MRS pH 3 and 4 °C in citrate pH 3), the survival of *L. plantarum* NCMIB 8826 cells in cranberry juice increased by only 1 to 2 days compared to control cells. Whilst, in pomegranate and lemon & lime juices, the rate of cell death was significantly decreased for weeks; however, after prolonged storage no significant differences were observed between acid adapted and control cells indicating that the alteration process by acid adaptation was not permanent. These findings indicate that acid adaptation is most likely not a suitable commercialisation strategy to be used on its own for ensuring that a sufficient number of viable probiotic cells (more specifically for *L. plantarum* NCMIB 8826 cells),

necessary to exert their beneficial health effects *in vivo* (> 10^7 CFU/ml) (Corcoran *et al*., 2007), is maintained during refrigerated storage of fruit juices for 6 weeks. However, given the positive effects that were observed on cell survival, it is possible that this strategy can be used in conjunction with other technologies, such as encapsulation. Moreover, in order to improve the cell survival of *L. plantarum* NCMIB 8826 in these particular fruit juices, it would be possible to investigate whether increasing the pH of the fruit juices could have a positive effect. A previous study using this approach showed that the cell viability of *L. paracasei* NFBC43338 (measured after 9 days of storage) was significantly improved (by about 8 logs) when the pH of cranberry juice was increased from 2.5 to 5.5 (Sheehan *et al*., 2007). Serial sub-culturing of *L. plantarum* NCMIB 8826 cells in acidic growth media (e.g. MRS supplemented with fruit juices) might be another approach to develop an appropriately adapted which has the ability to withstand the harsh environment of these fruit juices as shown in the study by Wu *et al*. (2012). Serial sub-culturing of *L. casei* strain Zhang in MRS at pH of 4.3 for 70 days led to cells that had increased in acid tolerance. It is important to note that a mutant strain obtained by such a method is not considered a genetically modified microorganism because it is a phenotypic change which can occur naturally while other genetic engineering techniques, in particular gene cloning, are unacceptable to be recently used for food-grade microorganisms. The investigation into the mechanisms for enhancing acid tolerance of *L. plantarum* NCMIB 8826 cells, revealed three potential mechanisms likely to be involved: i) modification of the membrane fatty acid composition, particularly that of CFA (lactobacillic acid) which increased significantly during acid adaptation leading to decreased membrane fluidity; ii) modification of the peptidoglycan by either D-alanylation or EPS production leading to a thicker/denser cell

wall which was able to inhibit the influx of protons; and iii) upregulation of molecular chaperone GroEL leading to the prevention of protein denaturation from low pH environment. A key finding of this study was the observation that the intracellular alanine, which represented the most abundant amino acid (> 45%), was significantly reduced in the case of acid adapted cells (by ~20%) compared with control cells, which coincided with a significant decrease in the extracellular alanine (~10%). The extensive utilization of intracellular and extracellular alanine seems to support the hypothesis of a denser and thicker peptidoglycan cell wall in the case of acid adapted cells, which could possibly be due to the binding between D-alanine and wall teichoic acid; this process is called D-alanylation and has been shown previously to reduce the negative charge of the cell envelope and the influx of positively-charged molecules into the cells (Neuhaus and Baddiley, 2003, Giaouris *et al*., 2008, Weidenmaier and Peschel, 2008, Swoboda *et al*., 2010). It seems likely ,therefore, that acid adaptation leads to cells utilising the available alanine to increase the D-alanylation of the wall teichoic acid, resulting in a cell wall with enhanced ability to reduce the influx of hydrogen protons during subsequent storage in fruit juices; and hence to better cell survival. Biochemical analysis of the cell wall composition or other type of analysis such as fluorescence imaging was not conducted in this study to confirm the above hypothesis. However, the work of Ellwood and Tempest (1972) has shown that the degree of D-alanylation in *Bacillus subtilis* was increased when the pH of the growth medium was decreased from pH 7 to pH 5. To our knowledge there is no information on such aspects for lactobacilli. An understanding on the potential mechanisms utilized by the cells to increase their acid resistance is important for identifying new probiotic strains which have the ability to survive in harsh acidic conditions. For instance, a probiotic strain which has a high content of

cyclopropane or the ability to produce EPS could be selected specifically for incorporation in selected low pH food products. Moreover, the information generated from this research contributes considerably to the growing body of knowledge on the possible acid adaptation mechanisms employed by lactobacilli, in particular *L. plantarum* NCMIB 8826.

One limitation of this work was that the cells used for the adaptation experiments were already pre-exposed to acidic pH as they were grown with MRS in flasks, with no pH control. As a result, the pH of the medium, when the early stationary phase cells were harvested, was ~4.6. One way to partly circumvent this would be to grow the cells in a bioreactor under controlled pH conditions, although even in that case the cells would be stressed due to the high amounts of lactate being produced. For future work, in an effort to obtain non-stressed cells as control cells, it is recommended to use steady state cells from continuous culture at pH ~6 which is (optimal pH for culturing *L. plantarum* NCMIB 8826). This approach has not been explored for these types of experiments and could potentially provide more accurate information on the physiology of non-stressed cells.

Finally, comparative analysis of *L. plantarum* NCMIB 8826 genes and proteins of acid adapted and control cells should be further investigated by DNA microarray and 2D-liquid chromatography-mass spectrometry to complement the results generated from this work. Such techniques are more powerful than the techniques used in this study and will help to clearly elucidate the genes and proteins involved in acid adaptation of *L. plantarum* NCMIB 8826.

5.2. References

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