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Degree: M.Sc. Dept/Institute/School: IMBS

Thesis Title: THE IMMUNOMODULATORY ROLE OF LIPOTEICHOIC ACID IN PROBIOTIC BACTERIA

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The Immunomodulatory Role of Lipoteichoic Acid from Probiotic Bacteria

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science
in
Biochemistry

Institute of Molecular BioSciences
Massey University
Palmerston North, New Zealand.

Carel Michael Hutchings Jöbsis

2011

Abstract

Different probiotics provide different health benefits, and some of these may be explained by immunomodulatory activity. These immunomodulatory effects can vary between different probiotic strains and microbial-associated molecular patterns (MAMPs) may be responsible for this variation. One MAMP, lipoteichoic acid (LTA), is a macroamphiphile associated with the cell surface of gram positive bacteria. LTAs from different strains of bacteria have been shown to induce different immunomodulatory profiles.

LTA was purified from three strains of lactic acid bacteria (LAB) that are known to elicit different immune responses, then analysed for immunomodulatory activity using human cell based assays. The activity of each LTA was shown to reflect elements of the immunomodulatory profile of the original strain. The structure of each LTA was determined using NMR (nuclear magnetic resonance spectroscopy). Structural differences found between the LTAs were compared to the differences in their immunomodulatory behaviours, showing that the differing structures may be responsible for strain-specific immune profiles. It has been previously shown that inactivation of the *dlt*D gene in an established probiotic strain of LAB results in changes to the immune effects induced by the mutant bacterial cell compared to the wild type. This study has shown using NMR analysis that the structure of LTA isolated from this mutant strain is altered, reflecting the distinct immune profile of the mutant bacteria.

LTAs from the three strains in this study were found to contain N-acetyl-glucosamine substituents, which have previously been found only on highly pro-inflammatory LTAs, e.g., those from *Staphylococcus aureus*. LTAs from the three strains were also shown to contain unsaturated fatty acids, which have so far been found in the LTAs of only LAB, including three other probiotic strains. These structural features may explain some of the immunomodulatory effects observed for these strains. It was found that isolated LTA may not be as effective at inducing immune responses as LTA on cells. Further exploration of potential interactions of LTA with other MAMPs, and other factors that may alter the presentation of LTA to immune cells in the case of intact cells is necessary to fully understand the role of LTA in immunomodulation.

For Carel Otto Jöbsis
1925 - 2011

Acknowledgements

No man is an island, which is especially true in science. There are many people I'd like to thank for connecting me to the mainland over the course of this project:

All the members of the S113 lab and the DIH team at FRC and the X-Lab and Centre for Structural Biology at IMBS for their assistance and camaraderie over my time there. Also all the workshop and administrative support staff that keep things happening behind the scenes.

Michael Bian, Merie Cannon and James Dekker for advice on immune cell assays and sharing in my trials and tribulations with the FACS analyser. Jean Garman, Caroline Chambers, Paul McJarrow, Dinuk Peiris, Kevin Ma, Yvonne van der Does and Bernadette Guthrie for assistance with a variety of equipment and lab procedures. Mark Reynolds for advice on phosphate quantitation, and Paul Plieger in IFS for assistance with microwave equipment. Trevor Loo, jack of all trades and master of many, for useful advice about lab techniques and all things chromatographical. The academic staff at IMBS, particularly Peter Farley, Mark Patchett and Kathryn Stowell. Pat Edwards, for carrying out the NMR analysis, and helping me understand the mystery wrapped in an enigma that is NMR.

The Foundation for Research Science and Technology (FRST, now MSI) for financial assistance through a Technology Fellowship. The opportunity to work in a commercial research lab in collaboration with the university was invaluable.

My co-supervisor Mike Collett, for introducing me to the fascinating field of probiotics and cultivating my interest in immunology. Always encouraging, I've appreciated his help with finding the big picture, and his straight-forward approach to problem solving.

My supervisor Gill Norris, for sharing her wisdom on purification and structure, and providing the guidance and encouragement to get me through. She helped me to see the light at the end of the tunnel, when at times it seemed a long way away.

All my family for support and encouragement, especially Mum, who (along with MacGyver) really got me interested in science in the first place. Also all the friends who've kept me sane and in touch with the real world outside of the lab. Special thanks to Matt Bennett and Simon Oakley for encouraging me to take on this project in the first place.

Finally Kelly, for always being there, helping me see the bright side behind the broken machines and disappearing thesis figures. I couldn't have got through it without your love and support.

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Abbreviations

In addition to *le Système international d'unités* (SI) and the derived SI units, the chemical symbols from the Periodic Table of Elements, the standard amino acid abbreviations and the cytokine abbreviations as given by Delves *et al.* (2011), the following abbreviations were used :

~	Approximately
1D	One dimensional
2D	Two dimensional
A, Abs	Absorbance
AcO^-	Acetate ion
amt	Amount
APC	Antigen presenting cell
CBA	Cytometric bead array
CD4	Cluster of Differentiation 4
CD14	Cluster of Differentiation 14
CD36	Cluster of Differentiation 36
CFA	Cyclopropane fatty acid
cfu	Colony forming units
CMC	Critical micelle concentration
concn	Concentration
COSY	Correlation Spectroscopy
CV	Column volumes
D_2O	Deuterium oxide (heavy water)
DAG	Diacylglycerol
D-ala	D-alanine
DC	Dendritic cell
DNA	2'-deoxyribonucleic acid
*dlt*A to E	The operon responsible for D-alanylation of LTA
DQF-COSY	Double Quantum Filtered Correlation Spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccharide
ESI FT-MS	Electro-spray ionisation Fourier transform-mass spectrometry
EU	Endotoxin units
FA	Fatty acid
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal bovine serum

FID	Free Induction Decay
FPLC	Fast Protein Liquid Chromatography
g	Gravity
Gal	Galactose
GBS	Group B *Streptococcus*
GC-MS	Gas Chromatography –Mass Spectrometry
GlcNAc, GNAc	N-acetyl-glucosamine
Gro	Glycerol
GroP	Glycerol-phosphate
HIC	Hydrophobic Interaction Chromatography
HOD	Hydrogen-Oxygen-Deuterium (semi-heavy water)
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HSQC	Heteronuclear Single Quantum Correlation
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
IL	Interleukin
LAB	Lactic acid bacteria
LAL	Limulus Amoebocyte Lysate
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAMP	Microbe-associated molecular pattern
MDP	Muramyl dipeptide
MQ	Milli-Q highly purified water
MS	Mass Spectrometry
N	Normal (for an acid, the concn of H+ ions in mol/L)
n.d.	Not determined
NK	Natural Killer lymphocyte
NMR	Nuclear magnetic resonance spectroscopy
NOD	Nuclear oligeromisation domain
NOESY	Nuclear Overhauser effect spectroscopy
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PGN	Peptidoglycan
PGP	Poly(glycerol-phosphate)
pH	Activity of the H^+ ion, where pH = -log[H^+]
PNH	Phosphate non-hydrolysed (measured with rapid method)
ppm	Parts per million

PRR	Pattern recognition receptor
PtdG	Phosphatidyl-glycerol
rDNA	Ribosomal DNA
RFU	Relative fluorescence units
rpm	Revolutions per minute
RPMI	A media for human cell culture (named for Roswell Park Memorial Institute)
SEM	Standard error of the mean
SFA	Saturated fatty acid
S/N	Signal to noise ratio
TA	Teichoic acid (encompassing LTA and WTA)
Th cell	Helper CD4+ T lymphocyte
TNF	Tumour Necrosis Factor
TLR	Toll-like receptor
TOCSY	Total Correlation Spectroscopy
Treg	Regulatory CD4+ T lymphocyte
UFA	Unsaturated Fatty Acid
UV	Ultraviolet (light)
WT	Wild type
WTA	Wall teichoic acid
w/v	Weight per volume
v/v	Volume per volume

Chapter 1 - Introduction

1.1 Probiotic bacteria

Probiotics were defined by an expert report from the Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) in 2001 as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." (2001). This remains the most widely used definition of probiotics.

Probiotic bacteria are usually lactic acid bacteria (LAB), chosen from non-pathogenic microorganisms found in human or food sources, and are most commonly delivered in foods traditionally containing live cultures, such as yoghurt and fermented soy products. Probiotics were initially thought to improve health by competing with and/or inhibiting growth of pathogenic bacteria in the digestive system. It is now recognised that probiotic bacteria may have a wide range of specific beneficial effects and these are currently being investigated. This introduction is focused on probiotics with immunomodulatory activity. There is, however, a vast literature on the use of probiotics in other areas, and this is reviewed elsewhere (Parvez *et al.* 2006; de Vrese and Schrezenmeir 2008; Masood *et al.* 2011).

1.1.1 Mechanism of Immune Response to Probiotics

Responses to probiotic bacteria are highly strain-dependent. It has been demonstrated that different strains of LAB used as probiotic bacteria, sometimes strains from the same species, trigger different immune responses both *in vitro* and *in vivo* (Grangette *et al.* 2005; Foligne *et al.* 2007b; Shida and Nanno 2008; Lomax and Calder 2009; Mileti *et al.* 2009). This strain-specific response is a result of interaction of whole bacteria, or components from them, with immune cells; particular types of immune cells are thought to be responsible for the resultant immunomodulatory responses.

In mammals, dendritic cells (DCs) are important for the initial sampling of the bacterial population. Activation of DCs by bacteria leads to interaction with naïve CD4+ T cells; the interaction and signalling between DCs and CD4+ T cells results in polarisation of the T cells into various lineages, as illustrated in Figures 1 and 2. This differentiation is dependent in part on the specific bacteria encountered by the DCs, and is modified by immune cross talk (Kapsenberg 2003).

CD4+ T cells are central regulators of immune responses. Modulation of their populations by appropriate probiotic bacteria may offer a means of influencing many inflammatory conditions. For example, strains of probiotic bacteria which are anti-inflammatory may provide a way to treat or prevent disorders such as inflammatory bowel disease (IBD), which is characterised by an abnormally high inflammatory response (Foligne *et al.* 2007a). It has been shown that strains of bacteria with strong anti-inflammatory activity are able to induce the formation of tolerogenic dendritic

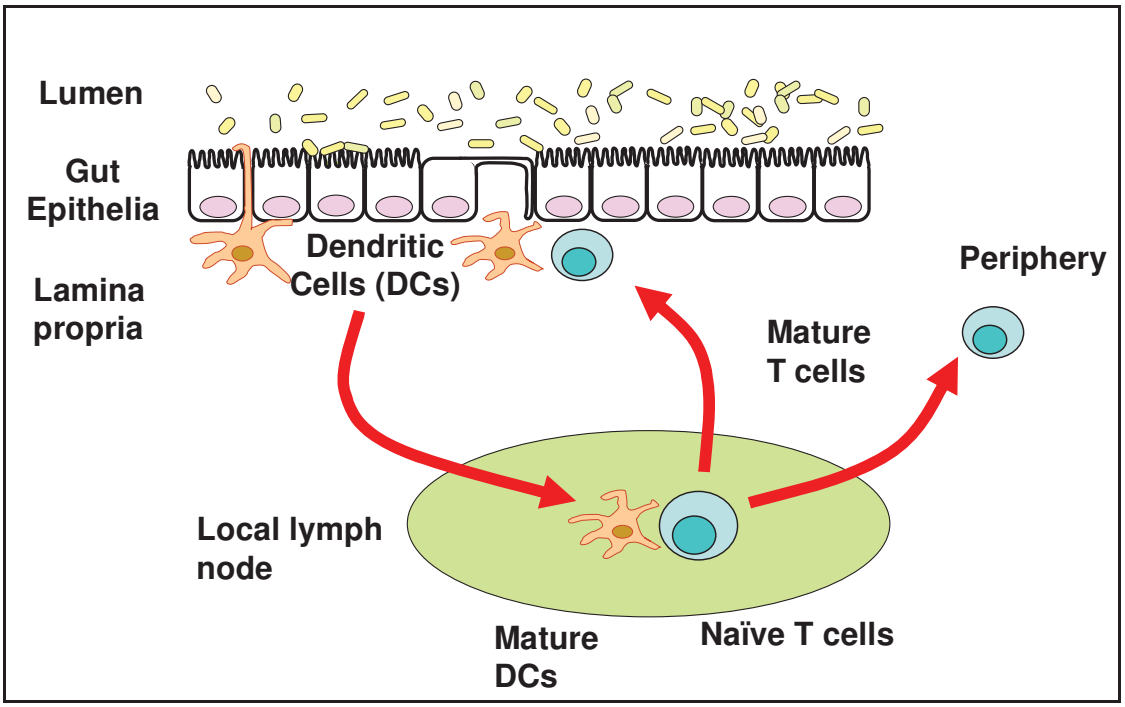

Figure 1: Antigen Sampling in the Gut.
Dendritic cells sample bacteria from the intestinal lumen, leading to their maturation and migration to the lymph node and subsequent polarisation of naive CD4+ T cells into special classes of mature T cells. These mature T cells then travel to the gut and other locations to carry out their specialised immune functions. Figure by M. Collett (personal communication), adapted with permission.

cells, which go on to polarise CD4+ cells into regulatory T cells (Treg) (Reiff and Kelly 2009; Reading and Kasper 2011). These Treg cells can modulate both pro- and anti-inflammatory T cell responses, allowing control of the aberrant response in IBD and other disorders (Kapsenberg 2003; Foligne *et al.* 2007a; Foligne *et al.* 2007b; Kullberg 2008; Mazmanian *et al.* 2008; O'Mahony *et al.* 2008; Roselli *et al.* 2009). The role of dendritic cells in probiotic-mediated protection against IBD was investigated by transferring mature DCs from mice treated with probiotic lactic acid bacteria into genetically identical mice. The mice receiving these DCs were protected against

chemically-induced IBD when compared to mice that did not receive this treatment. This response has also been shown to be dependent on both the TLR2 (Toll-like receptor 2) and NOD2 (Nucleotide-binding oligomerisation domain containing 2) receptors (Foligne *et al.* 2007b). TLR2 and NOD2 are pattern recognition receptors (PRRs), a class of innate immune receptors that play key roles in activating dendritic cells as well as other immune responses.

PRRs recognise microbial-associated molecular patterns (MAMPs) on bacteria and this interaction initiates an immune response. MAMPs (occasionally still referred to as PAMPs, for pathogen-associated molecular patterns) are bacterial molecular motifs present on a wide range of bacteria, including many probiotic bacteria. The interaction of MAMPs with the PRRs on dendritic cells is a key factor in the maturation of DCs and the subsequent polarisation of CD4+ T cells. A number of surface molecules on gram positive bacteria, including peptidoglycan (PGN), lipopeptides and lipoteichoic acid (LTA) have been identified as the MAMPs responsible for these immune responses. The highly strain-dependent immune responses of probiotic bacteria may be explained by differences in the combination of MAMPs possessed by the bacteria, along with any associated tissue signals generated as a result of the presence or activities of the bacteria (Kapsenberg 2003; Artis 2008; Kimbrell *et al.* 2008; Kawai and Akira 2010).

1.1.2 Cytokines

Cytokines are small signalling proteins that mediate the communications between the numerous types of cells involved in the immune response. Although cytokines are not limited to the immune system, they are essential to the regulation of both the innate and adaptive immune responses. Each cytokine has a specific receptor, and each immune cell type has a different distribution of cytokine receptors. Thus a given cytokine is capable of inducing different responses from various immune cells. As the interaction of a cytokine with its target cell often leads to increased expression of cytokine receptors and further cytokines, an immune response can be amplified to produce a large response from a relatively small initial stimulus (Delves *et al.* 2011).

The polarisation of CD4+ T cells into different subsets of T helper (Th) cells after interaction with mature DCs is described in Figure 2. Different combinations of signals involved in this interaction promote the development of particular types of CD4+ T cell, which have different roles in the immune response, including the production of different classes of cytokines. Th1 cells promote cell-mediated immunity, while Th2 cells

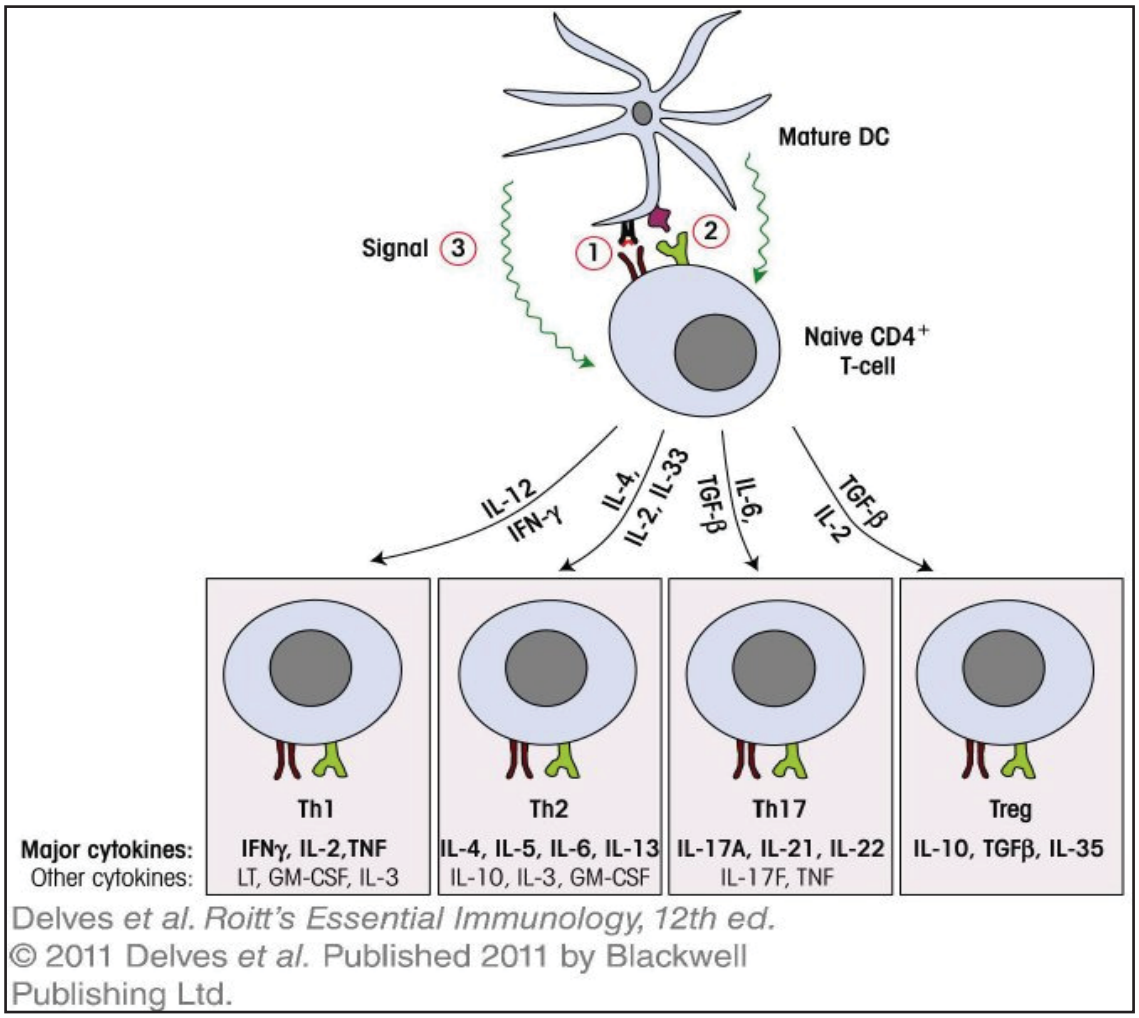

Figure 2: Cytokines are Involved in the Generation of CD4+ Cell Subsets.
Signals 1 and 2 are direct interactions with specific molecules on the surfaces of the mature DC and the naïve CD4+ T cell. Signal 3 represents cytokines produced by the mature DCs. The CD4+ T cell produces different cytokines dependent on all of these interactions, which go on to promote the development of naïve T cells into specific classes, including Th1, Th2, Th17 and Treg. Reproduced with permission (Delves *et al.* 2011).

support the humoral immune response. Interferon-γ (IFN-γ), a cytokine produced by Th1 cells, inhibits the proliferation and action of Th2 cells; and similarly, interleukin-4 (IL-4) which is produced by Th2 cells inhibits the Th1 response. This may skew the immune response towards either a Th1 or Th2 biased response, respectively. Treg cells are involved in modulating the effects of both Th1 and Th2 cells to prevent excessive

immune responses, often by secreting the anti-inflammatory cytokine interleukin-10 (IL-10). Th17 cells are thought to be important in controlling chronic inflammation and autoimmune diseases, and produce the cytokine interleukin-17 (IL-17) (Delves *et al.* 2011).

Pro-inflammatory cytokines produced by Th1 cells include interleukin-12 (IL-12), tumour necrosis factor (TNF), and IFN-γ. IL-12 is produced by antigen presenting cells (APCs) in response to certain classes of microorganisms, and plays a key role in the initial triggering of the Th1 response. As IL-12 can begin a signal cascade of different cytokines, a low level of IL-12 is often able to produce a large immune response. IFN-γ is produced by T cells and is involved in triggering the Th1 response, is important for antiviral responses and can also activate macrophages. IFN-γ also inhibits Th2 proliferation and the actions of IL-4, potentially altering the Th1/Th2 balance. TNF is produced by Th1 cells and APCs and has important tumour-killing activity. It plays a number of roles in generating pro-inflammatory responses, including activating monocytes and polymorphonuclear cells (PMN), and promoting cytokine secretion. TNF can act in synergy with IFN-γ to enhance cytotoxic activity. IL-1 (interleukin-1) is a pro-inflammatory cytokine produced by many immune cell types. There are two IL-1 genes, and each encodes a protein that is similar, but not identical, to the other. IL-1α and IL-1β are both recognised by same receptor and give the same biological response; however, they differ in the regulation of their genes and the cell types that produce them. IL-1α associates with the membrane of the producing cell, while IL-1β is secreted (Sims and Smith 2010). IL-1 promotes the production of IL-2 (interleukin 2), which in turn contributes to the pro-inflammatory Th1 response, enhancing killing by monocytes, macrophages and NK (Natural Killer) cells. IL-1 also induces production of many other pro-inflammatory cytokines including itself, TNF, interleukin-6 (IL-6) and interleukin-8 (IL-8). The pro-inflammatory cytokine IL-8 is a chemokine, which is a special class of cytokines that act as chemoattractants. Their signals direct other immune cells, such as neutrophils, to migrate to the site of infection (Delves *et al.* 2011).

The interleukins IL-4, IL-5 and IL-6 are involved in the Th2 response, and stimulate B cells to produce antibodies, which is particularly important for defence against extra-cellular pathogens. IL-4 can down-regulate IL-12 production to inhibit the

differentiation of CD4+ cells into Th1 cells, which may result in a bias towards the Th2 response (Delves *et al.* 2011).

IL-10 is made by many immune cell types, including Treg cells, is important for the regulation of cytokine responses, and inhibits the release of pro-inflammatory cytokines, particularly TNF and IL-1. The anti-inflammatory action of IL-10 can dampen both Th1 and Th2 responses, preventing excessive inflammation to protect the host, and may restore the Th1/Th2 balance to overcome a Th1 or Th2 bias (O'Garra *et al.* 2004; Saraiva and O'Garra 2010).

In examining the ability of a bacteria or molecule to induce an immune response, the measurement of secreted cytokines often provides a convenient way of quantitating the response. Immunoassay techniques such as ELISA (enzyme-linked immunosorbent assay) are commonly used to determine amounts of cytokine secreted in blood or cell culture supernatants. There are, however, more advanced methods available including CBA (cytometric bead array), which allow higher throughput analysis of several cytokines simultaneously (Morgan *et al.* 2004).

1.1.3 *Lactobacillus rhamnosus* HN001 (DR20™)

Lactobacillus rhamnosus HN001 (DR20™) is a probiotic developed and marketed by Fonterra. It was initially isolated from cheese, and was selected by a systematic process for development as a probiotic from a culture collection of more than 2000 strains. Two hundred strains thought to have potential as probiotics were initially screened for ability to survive acid and bile salts, indicating a tolerance for the environment in parts of the digestive system. A subset of strains was then tested in an *in vitro* model for adherence to human intestinal cells, and in animal models for immune impacts. Four promising strains were selected from this process, including *L. rhamnosus* HN001 (Prasad *et al.* 1998; Gopal *et al.* 2005). Feeding HN001 to healthy mice was experimentally shown to enhance several components of both the innate and acquired immune systems (Gill *et al.* 2000). Both blood and peritoneal leukocytes were demonstrated to have increased phagocytic activity in mice that were fed either live or heat-killed HN001; the enhancement in blood leukocytes was shown to be dose-dependent for the live probiotic. Mice that consumed viable HN001 also exhibited enhanced gut mucosal

protection against cholera toxin vaccine, but mice fed killed HN001 did not (Gill and Rutherfurd 2001b). Mice undergoing an induced antigen-specific Th2 (T helper 2 cell) response that were fed HN001 demonstrated enhanced production of both Th1 and Th2 cytokines when compared to a control group (Cross *et al.* 2002). HN001 was also shown to have a protective effect against the pathogenic bacteria *Escherichia coli* O157:H7 and *Salmonella typhimurium* in mouse infection models (Gill *et al.* 2001b; Shu and Gill 2002).

A recent study using a pig allergy model demonstrated the capacity of HN001 to decrease the severity of both skin and lung allergic responses, compared to a control group. These improvements were correlated with increased IFN-γ and IL-10 expression (Thomas *et al.* 2011).

Following these demonstrations of immune impacts by HN001, the safety of HN001 was studied first by feeding to mice, resulting in no clinically adverse effects (Shu *et al.* 1999; Zhou *et al.* 2000a; Zhou *et al.* 2000b; Zhou *et al.* 2001). This was subsequently confirmed to be true for human subjects (Tannock *et al.* 2000).

The animal efficacy and safety trials were followed by clinical trials in humans to show the probiotic efficacy of HN001. Studies where milk containing HN001 was consumed by healthy middle aged or elderly adults showed that HN001 led to increased *in vitro* phagocytic activity of peripheral blood polymorphonuclear cells and improved *in vitro* tumour killing by NK cells (Gill and Rutherfurd 2001a; Gill *et al.* 2001a; Sheih *et al.* 2001). In a recent study, consumption of cheese containing HN001 and another probiotic, *Lactobacillus acidophilus* NCFM, was also shown to enhance the phagocytic and NK cell activity in elderly subjects (Ibrahim *et al.* 2010).

Newborn infants whose mothers had been fed HN001 during pregnancy were shown to have higher protective interferon-γ levels in their cord blood, and the mothers' breast milk contained more of the immune factors TGF-β (transforming growth factor beta) and immunoglobulin A, when compared with a placebo group (Prescott *et al.* 2008).

The potential of HN001 to prevent eczema was investigated in a clinical trial carried out by University of Otago's Wellington School of Medicine and Auckland University. At-

risk infants and their mothers were given either *L. rhamnosus* HN001, *Bifidobacterium animalis* subsp. *lactis* HN019, or a placebo. The prevalence of eczema was reduced by half in the group taking HN001 (as seen in Figure 3), and symptoms were less severe in those that were still affected in this group (Wickens *et al.* 2008). This suggests that HN001 may have efficacy in preventing or attenuating the effects of eczema in infants. Other probiotic strains have also been studied for their effect on eczema (Johannsen and Prescott 2009; Kalliomaki *et al.* 2010).

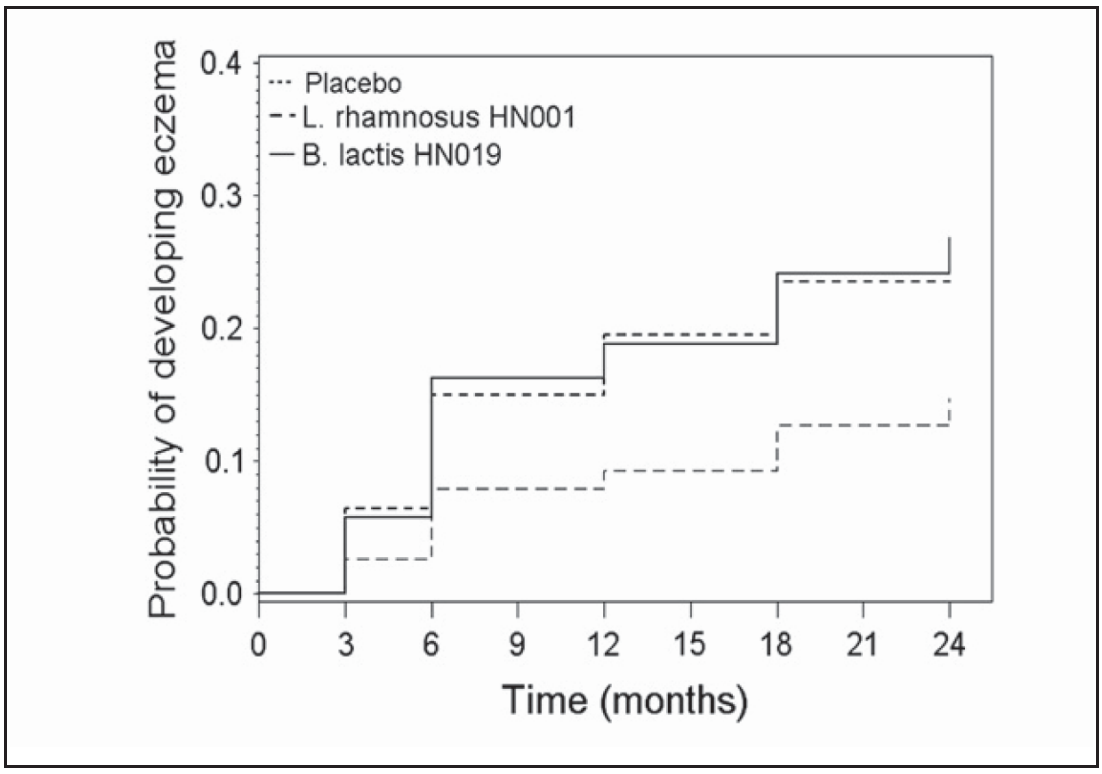

Figure 3: Clinical Trial of *L. rhamnosus* HN001 in Infants At-Risk of Eczema.
The prevalence of eczema was reduced over a two-year study when at-risk infants were given *L. rhamnosus* HN001, when compared to another probiotic (*Bifidobacterium animalis* subsp. *lactis* HN019) or a placebo. Reproduced with permission (Wickens *et al.* 2008).

A possible explanation for the impact of HN001 on such diverse responses as anti-pathogen defence, tumour killing and protection from eczema/allergy is the immunomodulation effect of HN001 in animals and humans.

1.2 Lipoteichoic Acid

Lipoteichoic acid (LTA) is a macroamphiphile associated with the cell surface of gram positive bacteria. Teichoic acids (including LTA and wall teichoic acids (WTA),

section 1.2.1) are the second largest component of the cell wall, after peptidoglycan (Delcour *et al.* 1999; Neuhaus and Baddiley 2003; Weidenmaier and Peschel 2008; Xia *et al.* 2010). LTA is a known ligand for TLR2 (Schwandner *et al.* 1999), and is thus a candidate for the induction of immune signalling by probiotic bacteria.

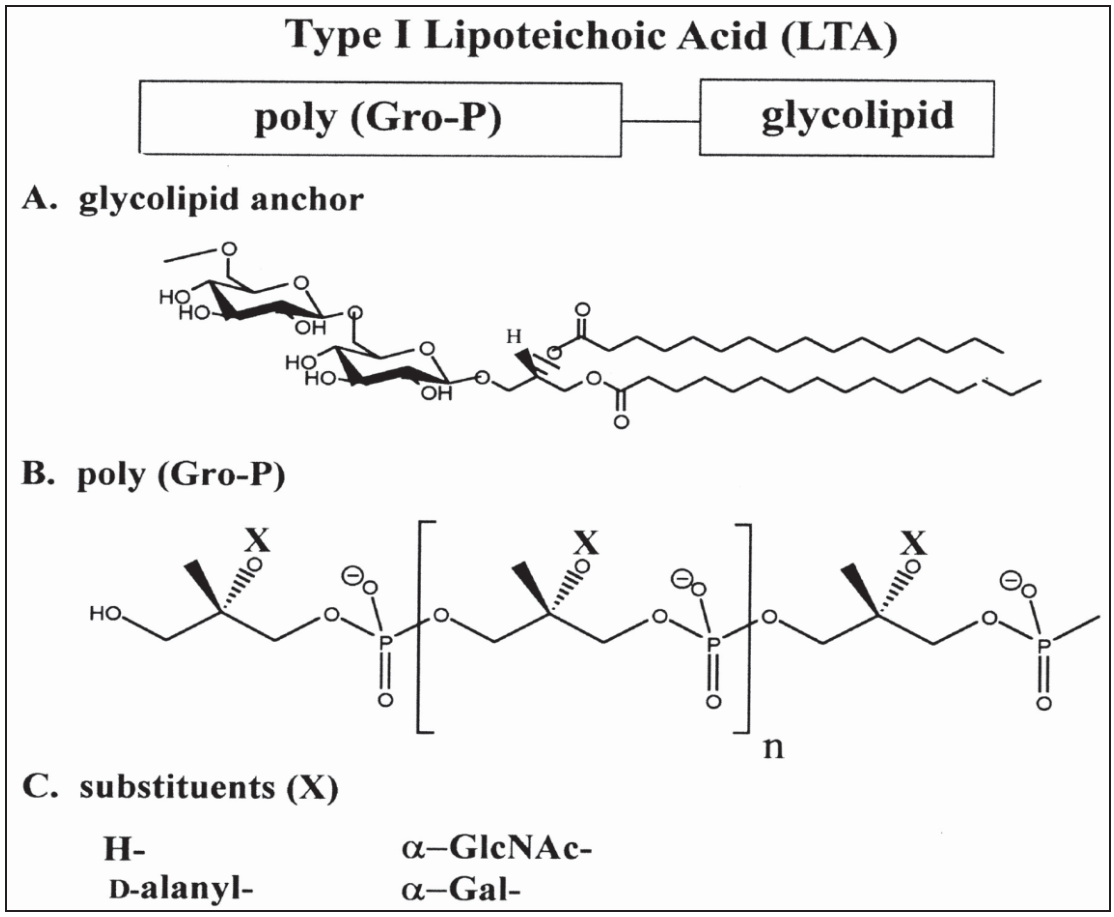

Figure 4: Structure of Type I Lipoteichoic Acid
The general structure of poly-glycerol lipoteichoic acid. A: Glycolipid anchor. B: Poly(glycerol-phosphate) (poly(Gro-P)) backbone, with positions of substituents indicated by X. C: Possible substituents to the GroP units: H, proton; D-alanyl; α-GlcNAc, α-N-acetyl glucosamine; α-Gal-, α-galactose. Reproduced with permission (Neuhaus and Baddiley 2003).

LTA consists of a hydrophilic alditol- (generally glycerol- or ribitol-) phosphate polymer chain that is anchored in the cell membrane by a glycolipid tail (Figures 4 and 5). LTA can associate with the membrane in a coiled configuration, or extend perpendicular to the membrane through the peptidoglycan (Figure 5). The most frequently occurring is Type I LTA, consisting of glycerol-phosphate (GroP) repeating units; while the less common Types II and III have polymers containing galactose (Gal), with GalGal-GroP and Gal-GroP repeating units, respectively. The polymer chain head

group contains between five and fifty or more repeating units, and is commonly substituted with D-alanine (D-ala) and/or monosaccharides such as N-acetyl-glucosamine (GlcNAc). The lengths of both the poly(glycerol-phosphate) (PGP) chain and the lipid tail vary considerably between species and strains of gram positive bacteria, as do the type and degree of substitutions and the sugar linker present. There is also some microheterogeneity of the LTA population within a cell. Not all gram positive bacteria have LTA; however, many species that lack traditional LTA have similar macroamphiphiles with different repeating units in their hydrophilic chain, such as teichuronic acid, in their membrane (Fischer 1994; Delcour *et al.* 1999; Neuhaus and Baddiley 2003; Weidenmaier and Peschel 2008).

1.2.1 Wall Teichoic Acid (WTA)

Wall teichoic acid (WTA) is another polymer of glycerol- or ribitol- phosphate found on the cell surface of many gram positive bacteria. It was originally referred to as simply teichoic acid (TA); however, the discovery of LTA necessitated a distinction between the two types. It is dissimilar to LTA, in that WTA is covalently attached to cell wall peptidoglycan through a phosphodiester linkage, rather than being anchored in the membrane by a glycolipid (Figure 5). WTA is initially linked to the peptidoglycan near the inner surface of the cell wall and migrates with the peptidoglycan layer towards the outer cell wall as the cell grows. The alditol-phosphate polymers of WTA extend into the cell wall, and as with LTA, can be projected through the outer surface of the cell wall to varying degrees. Synthesis of WTA seems to occur separately to LTA synthesis, and the types of alditol-phosphate polymers are not necessarily the same in the two types of TA within a cell. Some gram positive bacteria have both LTA and WTA, while others possess only one type of teichoic acid (Fischer 1994; Delcour *et al.* 1999; Neuhaus and Baddiley 2003; Bhavsar and Brown 2006). *Lactobacillus rhamnosus* has been shown to possess LTA but not WTA (Baddiley and Davison 1961; Kelemen and Baddiley 1961; Valyasevi *et al.* 1990).

1.2.2 Biosynthesis of LTA

The pathways for synthesis of LTA and WTA are independent, apart from D-alanylation. The D-alanine esters of WTA are derived from D-alanyl-LTA, but

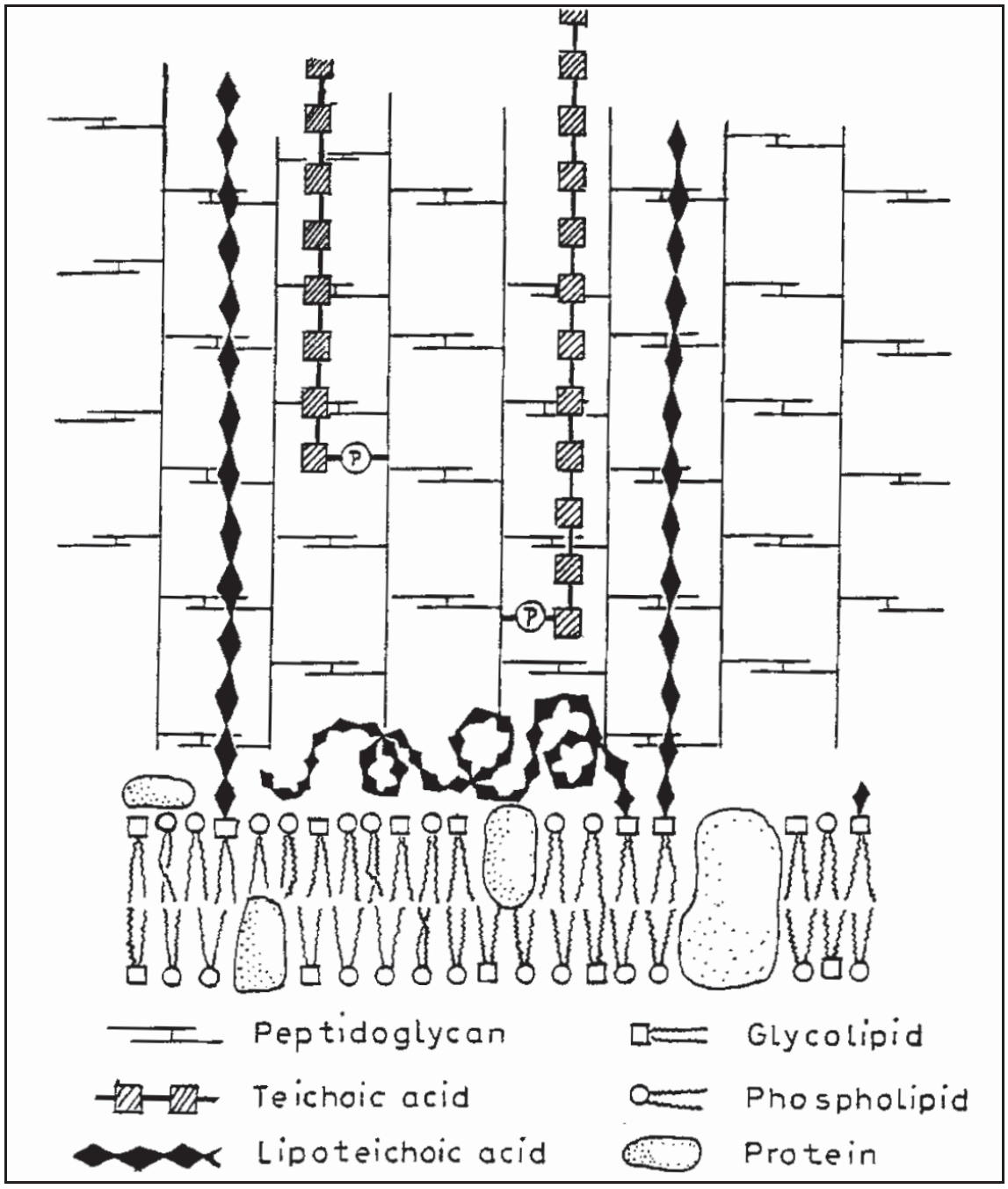

Figure 5: Location of Teichoic Acids in Gram Positive Bacteria
Schematic diagram of the cell wall of a gram positive bacterium, reproduced with permission (Fischer 1994). The two types of teichoic acids are major components of the gram positive cell wall. Lipoteichoic acid (LTA) is anchored in the plasma membrane, while Teichoic acid (WTA) is covalently linked to the peptidoglycan. LTA may associate with the membrane in a coiled configuration, or extend perpendicular to the membrane. Other commonly occurring cell surface components, including various polysaccharides and surface proteins, are not shown here.

otherwise WTA and LTA syntheses use source molecules of opposite chirality (Neuhaus and Baddiley 2003).

The biosynthesis of LTA is summarised in Figure 6. In *Bacillus subtilis* and *Staphylococcus aureus* the glycolipid is synthesised by the diglucosyldiacylglycerol synthase enzyme YpfP, which transfers two glucose molecules to diacylglycerol, using UDP-glucose as a source. The glycolipid used varies between species, but is usually specifically chosen from a number of glycolipids produced by the organism. The glycolipid is translocated from the inner to the outer leaflet of the membrane bilayer by the membrane protein LtaA, proposed to have flippase activity. In bacilli, the *sn*-glycerol-1-phosphate moiety of phosphatidylglycerol is transferred to a free membrane glycolipid, and the diacylglycerol component is released. (Delcour *et al.* 1999; Neuhaus and Baddiley 2003; Grundling and Schneewind 2007; Xia *et al.* 2010).

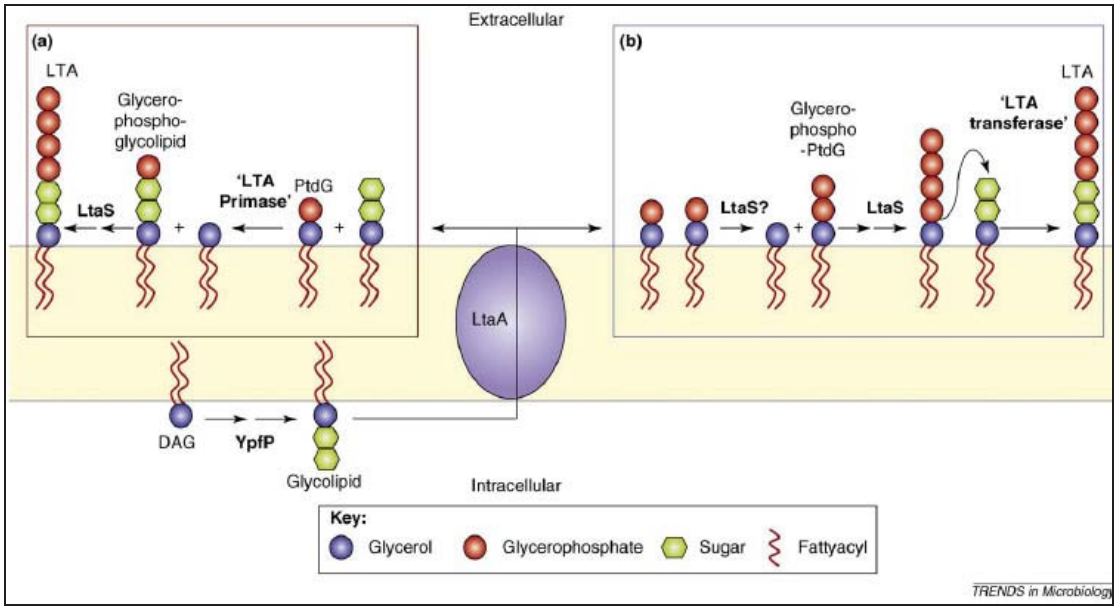

Figure 6: Biosynthesis of LTA
Two possible models for biosynthesis of LTA in *S. aureus* (Rahman *et al.* 2009), figure reproduced with permission. After assembly by the YpfP enzyme the glycolipid is translocated to the outer leaflet of the membrane by the LtaA 'flippase'. It is then possible that either: (a) LtaP primase attaches the first glycerol phosphate from phosphatidylglycerol (PtdG) then the chain is extended one residue at a time by the synthase LtaS; or (b) the chain is preformed on diacylgycerol (DAG) by LtaS and then transferred intact to the glycolipid by an LTA transferase.

Two possible models have been hypothesised for assembly of the head group polymer. In the first, the chain is elongated by addition of individual glycerophosphate residues by LTA synthase (LtaS) enzymes after an initial priming reaction, as shown in Figure 6(a). In the second model, a preformed poly(glycerophosphate) chain is transferred to the glycolipid by an as yet unidentified LTA transferase enzyme, illustrated in Figure 6(b) (Rahman *et al.* 2009). Evidence from recent studies supports the first model, as LTA primase (LtaP) enzymes have now been identified in *Listeria*

monocytogenes (Webb *et al.* 2009), and in *Bacillus subtilis* (Wormann *et al.* 2011). *B. subtilis* has been reported to have multiple LtaS enzymes, and it has been suggested that this proliferation of synthases may allow switching between synthesis of different types of LTA as required under specific growing conditions (Schirner *et al.* 2009; Sutcliffe 2011; Wormann *et al.* 2011).

Addition of D-alanyl and/or glucosyl substituents begins during elongation of the poly-(glycerophosphate) chain, but may also continue once the chain is complete. The sugar substituents are added by glycosyl transferases; in several strains of *B. subtilis*, GlcNAc subtituents are added to GroP units by GlcNAc transferases using β-GlcNAc-P-polyprenol as a substrate. A cluster of five *dlt* genes labelled A to E, responsible for D-alanyl esterification of teichoic acids was first identified in *Bacillus subtilis* (Neuhaus and Baddiley 2003). The proteins of the *dlt* mechanism utilise free D-alanine from the cytosol as the substrate. These *dlt* genes have been disrupted to investigate the importance of D-alanyl substituents in several gram positive species, as described in section 1.2.6. It has been shown that D-ala substituents may be redistributed by transacylation after addition to LTA, either along the GroP chain, or between separate chains (Childs *et al.* 1985).

The growth environment can have a large impact on the content of LTA in bacteria. Several strains of *Lactobacillus* and *Streptococcus* were shown to vary the total amount of LTA, as well as the quantity of LTA released into the media when grown in media containing different amounts of glucose or fructose (Hardy *et al.* 1981; Wicken *et al.* 1982). The pH at which cultures are grown is also influential on LTA structure. Experiments by MacArthur and Archibald (1984) with cultures of *Staphylococcus aureus* grown at a range of pH values demonstrated a reduction in D-alanine substitution at alkaline pH, although there was very little change in the total amount of LTA. The D-alanine content of purified LTA from *S. aureus* grown chemostatically at pH 6 is 0.75 moles of D-ala per mole of phosphate, compared to the 0.54 moles of D-ala per mole of phosphate produced when the culture is maintained at pH 7. Growth at pH 8 reduced this even further to 0.07 moles per mole of phosphate (MacArthur and Archibald 1984). It is possible that bacterial growth under these conditions alters *dlt* gene locus activity, resulting in variations in the addition of D-ala to LTA. High salt concentrations in the growth medium can also lead to reduction in D-alanine

substitution (Fischer and Rosel 1980). Growth of *S. aureus* on solid culture media by one group yielded LTA that was completely devoid of the D-alanine or GlcNAc substitutions that are normally found when the same strain is grown in liquid culture (Zahringer *et al.* 2008). Research suggesting that LTA helps to regulate charge across the surface of the cell supports the idea that bacteria may make changes to their LTA in response to changes in the environment (Neuhaus and Baddiley 2003). Thus the impact of growth conditions on the structure of LTA is complex, and the possible mechanisms by which the alternative LTA structures might be synthesised by bacteria requires further investigation.

1.2.3 Structural Analysis of LTA

The composition of the LTA molecule was initially established using chemical methods. A number of different techniques have been used to determine the phosphorus content, and this is the moiety most often exploited for initial detection and quantitation of LTA (Fischer *et al.* 1983; Josephson *et al.* 1986). The phenol sulphuric acid assay for total carbohydrate was originally used to measure the total hexose content of LTA (Dubois *et al.* 1956; Josephson *et al.* 1986). More recently, sugars have been quantified using gas chromatography-mass spectrometry (GC-MS) (Josephson *et al.* 1986). The glycerol content of acid-hydrolysed LTA was determined using enzymatic methods originally developed to determine blood triglycerides (Fossati and Prencipe 1982; McGowan *et al.* 1983; Nealon and Mattingly 1983).

Amino acid analyses were initially used to quantitate the substitution of D-alanine on LTA (Josephson *et al.* 1986). This method has been superseded by modern NMR (nuclear magnetic resonance spectroscopy) techniques, which are now the preferred methods used determine the degree of D-alanine substituents on LTA (Batley *et al.* 1987; Morath *et al.* 2001).

Separation of the acyl chain from LTA by either hydrofluoric acid (HF) hydrolysis (Roethlisberger *et al.* 2000), methanolysis in hydrochloric acid (Morath *et al.* 2001; Fedtke *et al.* 2007) or alkaline hydrolysis (Vidal *et al.* 2002) allows the glycolipid anchor to be characterised using either silica gel chromatography or GC-MS (Roethlisberger *et al.* 2000; Morath *et al.* 2001; Vidal *et al.* 2002). The first use of mass

spectrometry (MS) to analyse a complete native LTA was reported by Roethlisberger *et al.* (2000). MS has been used to resolve some discrepancies in the previously accepted model of the lipid anchor of pneumococcal LTA (Seo *et al.* 2008). Zahringer *et al.* (2008) used high resolution electro-spray ionisation Fourier transform-mass spectrometry (ESI FT-MS) to structurally characterise LTA from *S. aureus*. They were able to determine the number of units in the PGP polymer, and showed the relative amounts of subpopulations of the LTA with different polymer lengths. Intriguingly, this study identified LTA from *S. aureus* that had no D-alanine or GlcNAc substituents on the GroP repeating units by ESI FT-MS analysis, in contrast to previous findings by a group using NMR to characterise LTA from this species (see section 1.2.3.1). The PGP polymer lengths were also dissimilar, and these differences may be due to the different culture conditions used in each study, or to fragmentation of the polymer in the mass spectrometer. The development of more powerful mass spectrometers will lead to greater use of MS in the structural analysis of macromolecules such as LTA in the future.

1.2.3.1 NMR

Analysis by NMR has replaced many of the chemical methods previously used to obtain molecular information about LTA, and provides information that cannot be obtained using those methods. Variable and damaging purification methods and the lack of highly powered instruments limited the usefulness of NMR in early analyses of LTA, when structural assignments could only be made based on comparison of signals to those of known structures (Batley *et al.* 1987; Fischer *et al.* 1987). NMR has only been used to study LTA structure relatively recently, but modern NMR technology provides a powerful, non-destructive means of determining the absolute structure of these molecules (Morath *et al.* 2005; Perea Velez *et al.* 2007). While chemical methods commonly require separation of components of LTA, NMR is performed on the intact molecule, thus providing better information about the structure of, and the linkages between, the different components that make up the LTA molecule. However, as with any technique there are also problems. The inherent microheterogeneity of purified LTA samples is responsible for the band broadening that is characteristic of LTA NMR spectra (Morath *et al.* 2001). Furthermore, formation of micelles in water has been shown to cause band broadening in NMR spectra of LTA, due to the limiting of

molecular tumbling (Batley *et al.* 1987). ^{1}H, ^{13}C and ^{31}P NMR have all been used to analyse LTA, with ^{1}H NMR the most common in recent studies.

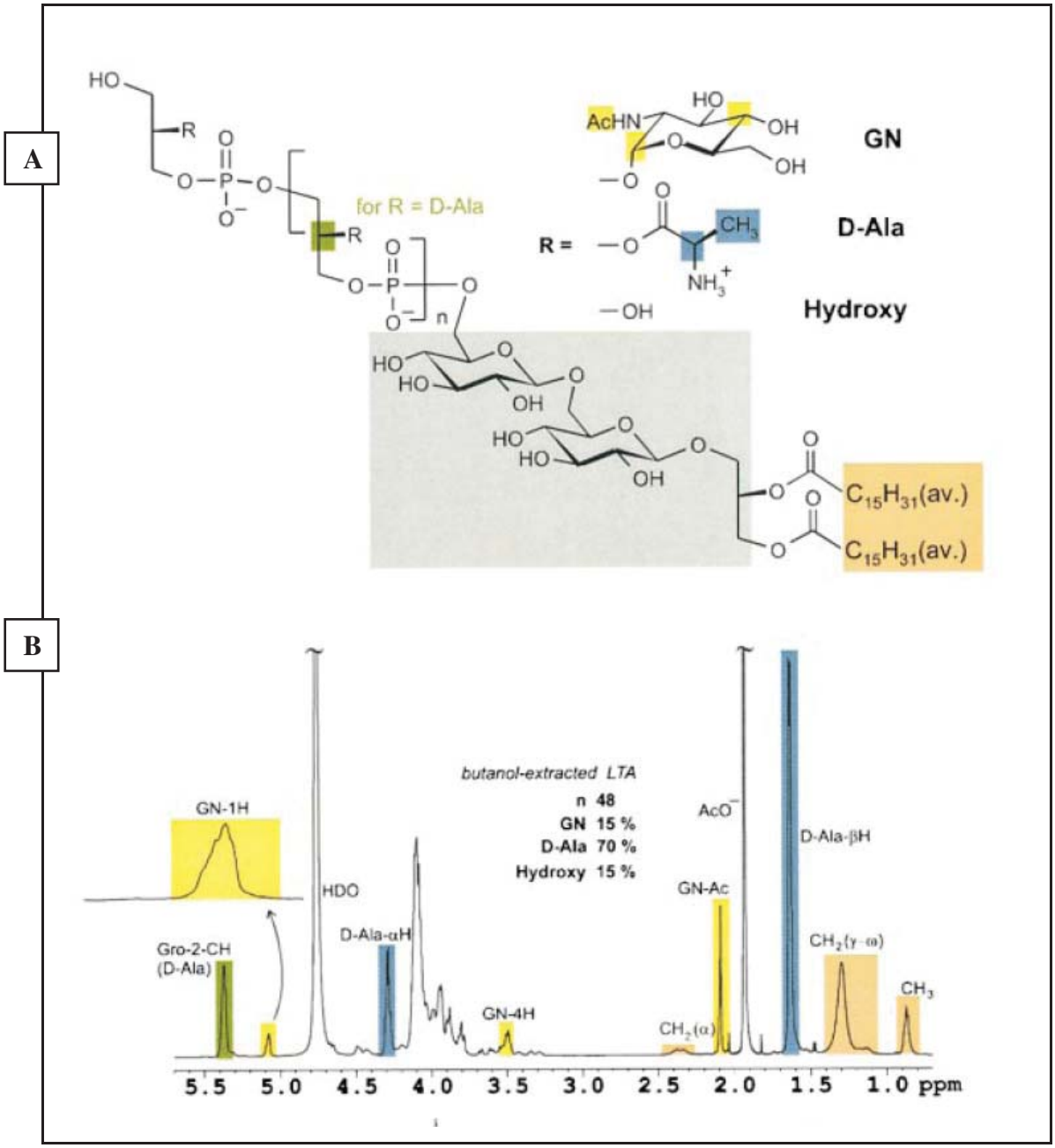

Figure 7: ^{1}H NMR Spectra of *S. aureus* LTA.
Structural components and substituents of *S. aureus* LTA were identified and the relative quantities determined by analysis of the ^{1}H NMR spectrum (Morath *et al.* 2001). The shaded colours of the structure elements in (A) correspond to the colours in the ^{1}H NMR spectrum in (B). Figure reproduced with permission.

Morath and co-workers carried out a complete ^{1}H NMR (600 MHz, 300 K) analysis of highly purified butanol extracted LTA from *S. aureus* (Morath *et al.* 2001). They were able to determine that the average GroP chain length was ~ 45 - 50 repeating units, and

that the degrees of substitution of the chain with N-acetyl glucosamine and D-alanine were 15 and 70 %, respectively, as illustrated in Figure 7. The D-alanine residues were then hydrolysed in the NMR tube at pH 8.5, which enhanced the resolution of the signals corresponding to the saccharide groups attached to GroP units. The anomeric stereochemistry of the sugar substituents could also be characterised. D-alanine bound to the LTA could be differentiated from free hydrolysed D-alanine. The structure of the gentiobiosyl-diacylglycerol glycolipid membrane anchor could be assigned after digestion with hydrofluoric acid (HF), and the fatty acid composition was determined using GC-MS.

In 2005, a *dlt*B- mutant of *Lactobacillus plantarum* was constructed by Grangette *et al*., and NMR spectra generated for purified LTAs from both the mutant and WT (wild type) bacteria. LTA from the mutant was shown by NMR to have only 1 % of its glycerophosphate units substituted with D-alanine, compared with 42 % in the wild type. Interestingly, glucose substituents increased to 24 % in the mutant, whereas they were only barely detectable in the wild type. The majority of the LTA from this mutant was also determined by NMR to be three times longer than that of the wild type, and it was found that the longest LTA from the mutant also carried the smallest number of D-ala substituents (Grangette *et al.* 2005; Palumbo *et al.* 2006).

The NMR structures of purified LTAs from both a *dlt*D- mutant of *Lactobacillus rhamnosus* GG and the WT strain were characterised by Perea Velez *et al*. (2007) using ^{1}H and ^{13}C NMR spectroscopy. The WT LTA was shown to have a chain of 50 glycerophosphate units on average, and 74 % of these were substituted with D-alanine. Two different peaks of *dlt*D- LTA were evident after hydrophobic interaction chromatography (HIC) on octyl sepharose, and were analysed separately. A closer look at the elution profile of the WT LTA showed that this also had a similar secondary peak that was not as well resolved from the main peak, instead appearing as a shoulder on the major peak. The major fraction of *dlt*D- LTA had an average of 29 glycerophosphate residues, while the minor peak had 7. While, as expected, D-alanine substituents were not detected in the mutant LTA, it was also shown to have two extra carbons in the lipid anchor compared to the wild type LTA.

It seems therefore that for a number of strains, altering the D-alanylation of LTA is accompanied by structural changes to other parts of the molecule. Although the mechanism behind this is not understood, it is important to be cautious when comparing the characteristics of mutant LTA with altered D-ala substitution with those of their respective wild type molecules. The PGP and acyl chain lengths and the saccharide substitution may also be altered in LTAs from Dlt- mutants, so that any differences in immune activity between the mutant and WT cannot necessarily be attributed to the changes in D-alanylation alone.

1.2.4 Functions of LTA

The functions of LTA are still unclear, but roles in regulation of cell surface charge and the subsequent regulation of enzymes have been suggested. They are also likely to be involved in adhesion to surfaces or other cells. The network of interactions between WTAs, LTAs and peptidoglycan constitute a matrix that is responsible for many functions of the cell envelope. The makeup of this network influences many cell wall properties such as packing density, hydrophobicity, the ability to traffic various molecules, and the provision of resistance to host defences (Delcour *et al.* 1999; Neuhaus and Baddiley 2003; Weidenmaier and Peschel 2008; Xia *et al.* 2010).

Lipoteichoic acid is considered to be a high energy intermediate, and is thought to provide a reservoir of energy for reactions occurring within the cell wall. Teichoic acids are involved in cation homeostasis, e.g., they bind divalent calcium and magnesium cations, providing a buffering system for the control of the concentration of these metal ions in the cell wall (Neuhaus and Baddiley 2003).

Shielding of the anionic charge on the glycerol phosphate backbone of LTA results in localised variations in cell surface charge. Proteins that are regulated by ionic charge, such as autolysins involved in peptidoglycan synthesis, may be regulated by these environments, and in this way TAs may be responsible for targeting the activity of such proteins (Neuhaus and Baddiley 2003). LTA has been shown to protect against the action of cationic antimicrobial peptides (CAMPs) in a number of species, presumably through regulation of the surface charge (Xia *et al.* 2010).

1.2.5 Mechanism of Immune Response to LTA

LTA has been proposed to be the MAMP responsible for gram positive septic shock, analogous to the gram negative endotoxin lipopolysaccharide (LPS). In fact, purified LTAs from different strains of bacteria have been shown to induce quite different immune cell responses, as shown in Figure 8 (Bhakdi *et al.* 1991; Ryu *et al.* 2009).

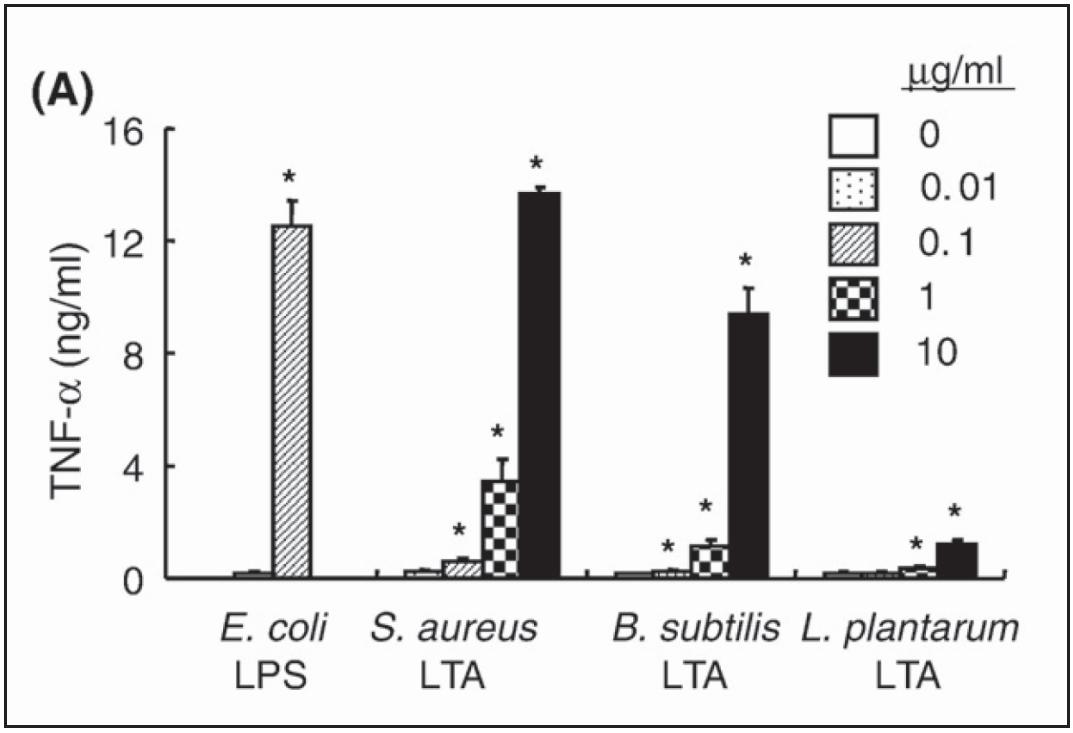

Figure 8: TNF Cytokine Induction by Purified LTAs.
RAW 264.7 cells were stimulated with LTAs from *Staphylococcus aureus*, *Bacillus subtilis* and *Lactobacillus plantarum* at the range of concentrations indicated in the key, or LPS from *Escherichia coli* at 0.1 μg/mL, and concentrations of secreted TNF cytokine were measured by ELISA. Reproduced with permission (Ryu *et al.* 2009).

The PRR, Toll-like receptor 2 (TLR2) has been shown to be required for triggering immune responses to LTA (Schwandner *et al.* 1999; Michelsen *et al.* 2001; Opitz *et al.* 2001; Matsuguchi *et al.* 2003; Travassos *et al.* 2004; Chan *et al.* 2007). Some researchers have reported LTA-induced immune responses that were independent of TLR2 (Hattar *et al.* 2006; Zahringer *et al.* 2008; Rockel *et al.* 2010; Fischer *et al.* 2011). Some of these studies, however, may have been done in conditions where not all of the PRRs and co-receptors required for TLR2-dependent recognition of LTA were present; for instance, using TLR2-transfected cell lines, which may not be representative of the *in vivo* response. Binding of LTA to TLR2 is dependent on CD36 as a co-receptor, as shown in homozygous mutant mice with inactive *CD36* genes. Negatively charged

diacylglycerol (DAG) is a ligand for CD36, which suggests a model where CD36 binds the DAG moiety of LTA, then transfers the LTA to TLR2 via CD14 (Hoebe *et al.* 2005; Jimenez-Dalmaroni *et al.* 2009). The binding of LTA to TLR2 begins a signal transduction cascade via the NF-κB pathway that results in a transcription response in the nucleus (Schwandner *et al.* 1999; Opitz *et al.* 2001). The resulting immune response varies depending on the source of the LTA. It has been shown that LTAs altered by mutation or chemical modification elicit different responses to those elicited by the respective wild type LTAs for a range of both pathogenic and probiotic bacteria (Morath *et al.* 2001; Deininger *et al.* 2003; Schroder *et al.* 2003; Grangette *et al.* 2005; Palumbo *et al.* 2006; Chan *et al.* 2007; Perea Velez *et al.* 2007; Walter *et al.* 2007; Fittipaldi *et al.* 2008; Cox *et al.* 2009). It has also been shown that the formation of tolerogenic DCs requires signalling through both TLR2 and NOD2; NOD2 is one of the PRRs responsible for the recognition of peptidoglycan (Girardin *et al.* 2003; Strober *et al.* 2006; Foligne *et al.* 2007b; Le Bourhis *et al.* 2007).

Morath *et al.* (2002b) used chemically modified *Staphylococcus aureus* LTA and synthetic LTA to explore the structural requirements for cytokine induction by this molecule. The chemically separated lipid anchor alone brought about only low levels of cytokine release from whole blood when compared with the strong cytokine response to intact native LTA. A competition experiment demonstrated that the hydrophilic chain alone (after removal of the lipid anchor) did not compete with native LTA, indicating that the lipid anchor may be required for recognition in whole blood. This is consistent with CD36 binding LTA via DAG. It should be noted, however, that the lipid anchor was removed by alkali treatment, which is also likely to have altered the D-alanine ester content, which the authors do not mention. A synthetic LTA that had six glycerophosphate residues, and was substituted with D-alanine and N-acetyl-glucosamine in approximately the same distribution as that seen in native *S. aureus* LTA elicited a strong TNF cytokine response, at comparable levels to that seen with native LTA. A synthetic LTA with L-alanine substituents was shown to be one hundred fold less effective at stimulating TNF production, demonstrating the importance of the stereochemistry of the amino acid substituent in recognition of LTA (Morath *et al.* 2002b; Deininger *et al.* 2003; Deininger *et al.* 2007). Perhaps surprisingly, no immune analyses of synthetic Type I LTAs lacking fatty acids have been reported; however, a synthetic derivative with a glycolipid anchor moiety at each

end of the PGP polymer was shown to have increased immune activity compared to the normally anchored synthetic molecule. Type IV LTA from *Streptococcus pneumoniae* has a hydrophilic chain that is very different to that of the Type I LTA found in *S. aureus.* A synthetic LTA based on *S. pneumoniae* LTA lacking the fatty acid anchor was not able to induce an immune response (Schmidt *et al.* 2011).

1.2.6 Importance of D-Alanine

Historically, hot aqueous phenol was used in the extraction of the macroamphiphilic lipopolysaccharide (LPS) from gram negative bacteria. This method was subsequently employed in isolating macroamphiphiles, such as LTA, from gram positive bacteria. It was subsequently discovered that use of phenol leads to loss of the D-alanine and sugar substituents of LTA, and can result in shortening of the polyglycerophosphate chain (Morath *et al.* 2005; Finney *et al.* 2007). Unfortunately, a great deal of work had already been done using LTA extracted by means of the phenol method, including a number of studies using commercially produced LTA from *Staphylococcus aureus* (Morath *et al.* 2002a). This prevented researchers from gaining accurate structural information and perhaps more importantly, the functional analyses of phenol-extracted LTA were compromised, as these D-alanines have since been shown to be important for the immune activity of many LTAs (Deininger *et al.* 2003; Palumbo *et al.* 2006; Perea Velez *et al.* 2007). Extraction protocols were therefore modified by replacing phenol with butanol. Comparisons between the two methods revealed the significant depletion of D-alanine substituents when the phenol extraction method was used (Morath *et al.* 2001; Morath *et al.* 2002a). Room temperature butanol extraction is now generally accepted as the initial extraction procedure used for LTA. The studies carried out using the old phenol method should therefore be re-evaluated.

The new non-destructive butanol method was first used to isolate LTA from the pathogen *Staphylococcus aureus*. The structure of the butanol-extracted LTA was determined by NMR, along with LTA purified by the old phenol method. Significant (~ 50 %) loss of D-alanine esters was observed where hot phenol had been used. Next, the D-alanines were deliberately hydrolysed from the butanol-extracted LTA by mild alkaline treatment. While intact D-alanyl LTA induced a strong TNF cytokine (pro-inflammatory) response from whole blood, the response for de-alanylated LTA was

reduced by two orders of magnitude (Morath *et al.* 2001). A similar reduction in cytokine induction was seen for LTAs from *S. aureus*, *B. subtilis* and *L. plantarum*, after mild alkaline hydrolysis with 0.2 M NaOH (Ryu *et al.* 2009). It is important to note, however, that this alkaline treatment may also hydrolyse the lipid anchor, which may be important for immune activity (Morath *et al.* 2002b).

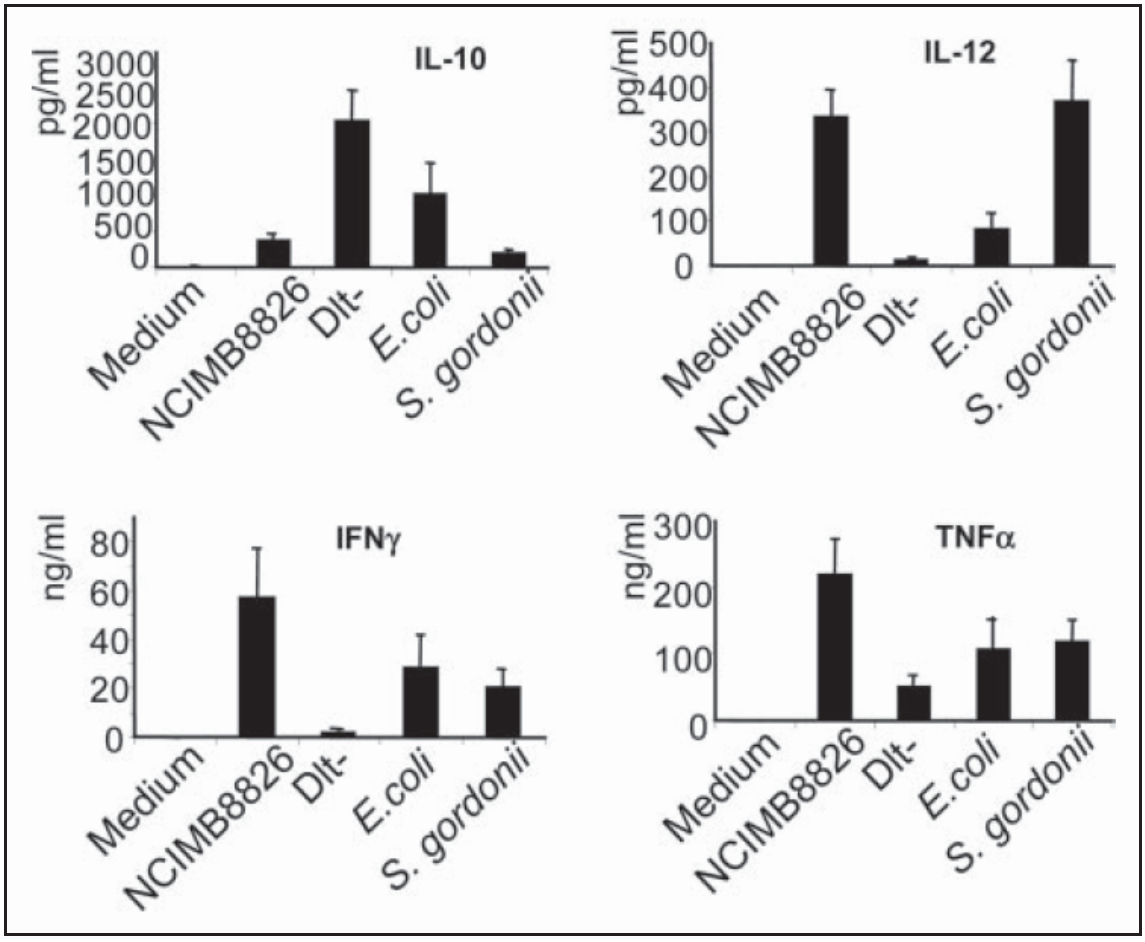

Figure 9: Cytokine Response Induced by Bacteria: WT and D-Ala Deficient LTA
Human PBMC were incubated for 24 hrs with either: culture medium as a control, or preparations of whole bacteria from WT *Lactobacillus plantarum* NCIMB8826, or Dlt-, the D-ala deficient mutant. *Escherichia coli* and *Streptococcus gordonii* were included as controls, representative of gram negative and gram positive bacteria, respectively. The WT NCIMB8826 induced a strong pro-inflammatory response. The Dlt- mutant was a weak inducer of the pro-inflammatory IL-12, IFN-γ and TNF, compared to the WT, but induced higher levels of anti-inflammatory IL-10. Reproduced with permission (Grangette *et al.* 2005).

To investigate the impact of D-alanylation of LTA from lactic acid bacteria, Grangette and co-workers (2005) constructed a *dlt*B- mutant of *Lactobacillus plantarum* in which D-alanine ester substitution of its LTA was reduced to ~ 1 % of the GroP units. The WT bacteria, with ~ 42 % D-ala substitution on its LTA, strongly induced the pro-inflammatory cytokines IL-12, IFN-γ and TNF, but induced low levels of the anti-

inflammatory cytokine IL-10, in an *in vitro* immune cell assay (Figure 9). The mutant bacteria (with ~ 1 % D-ala on the LTA) brought about an anti-inflammatory response, that is, it induced much higher levels of IL-10, and weak induction of IL-12, IFN-γ and TNF. The purified LTA from the WT and mutant strains (Figure 10) each induced similar cytokine profiles to the parent cells, i.e., LTA from the *dlt*B- mutant induced a weaker cytokine response than the WT LTA. This illustrates the influence of the structure of LTA on the immunomodulatory profile of the bacteria. The authors did not, however, describe the amounts of IL-10 induced by either of the purified LTAs. The response to LTA was also shown to be TLR2 dependent, as no cytokine release was seen when cells derived from *TLR2* gene knock-out mice were challenged with purified LTAs. The mutant and WT bacteria were tested in a mouse model of colitis, and the mutant gave significantly enhanced protection against tissue damage. This *dlt*B- mutation resulted in LTA with triple the glycerol-phosphate chain length and increased glucose substitution (Palumbo *et al.* 2006). The mutant cells differed markedly from the wild type in length and growth rate, and displayed evidence of separation defects during cell division.

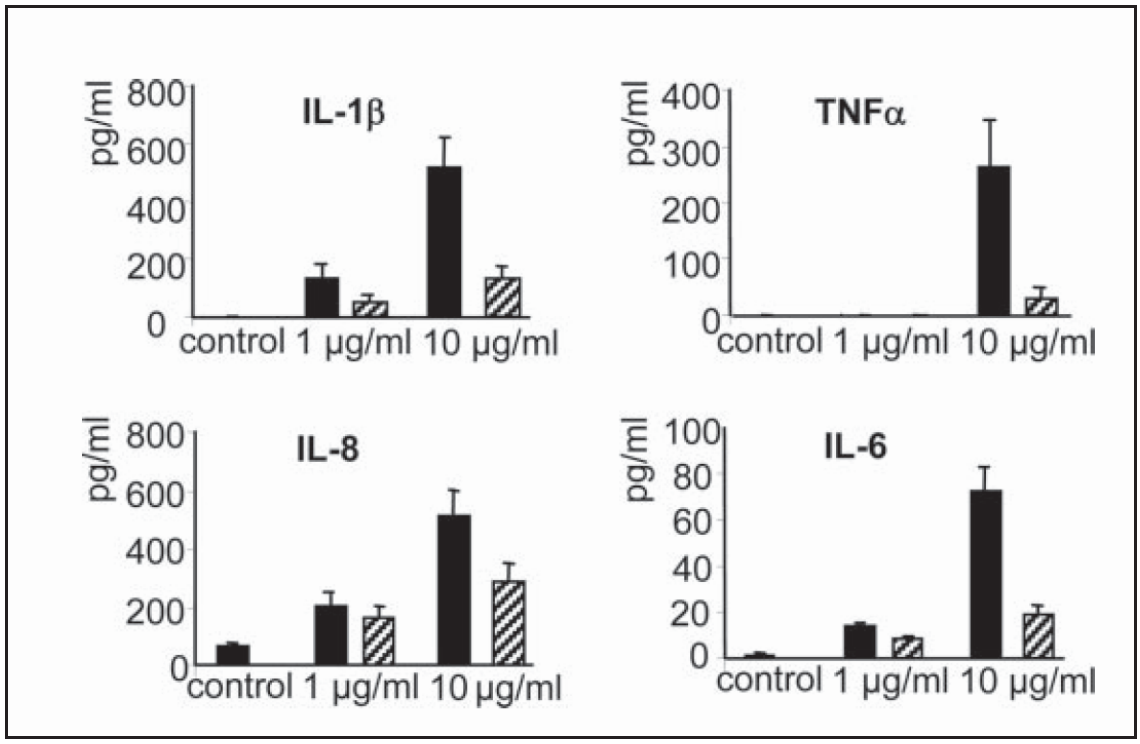

Figure 10: Cytokine Response to WT and D-Ala Deficient LTA.
Cytokine induction in human PBMC (peripheral blood mononuclear cells) by a negative control or LTAs purified from WT *Lactobacillus plantarum* NCIMB8826 (black bars) and a D-ala deficient mutant (hatched bars) of this strain (Grangette *et al.* 2005). The concentrations of the pro-inflammatory cytokines IL-1β, TNF, IL-8 and IL-6 were measured in response to LTA added at either 1 or 10 µg/mL final concentrations. Reproduced with permission.

The *dlt*D gene has been inactivated in the probiotic strain *Lactobacillus rhamnosus* GG, removing the ability to add D-alanine esters to LTA (Perea Velez *et al.* 2007). This mutation resulted in major changes of the cell surface properties of the bacteria, including cell morphology, reduced ability to survive in gastric juices and increased autolysis relative to the wild type. The purified LTA from this mutant has a shorter average glycerophosphate chain, and the lipid chain is two carbons longer. Surprisingly, elimination of the D-alanine from the LTA resulted in only minor changes in the secretion of several cytokines from human immune cells exposed to the mutant and wild type cells. Although the authors say that no significant differences were seen, their results do seem to show a measurable decrease in IL-10 induction for the mutant. The purified LTAs prepared from these bacteria were not tested in the immunomodulation assay in this study; the inclusion of such an experiment might have revealed differences between the wild type and the mutant that were not clear from the results for the intact bacteria. A subsequent study of this mutant in a mouse model of colitis revealed that the DltD- mutant bacteria improved the condition, while the wild type GG did not. The mutant was also shown to down-regulate the expression of both TLR2 and pro-inflammatory cytokines (Claes *et al.* 2010).

Complete depletion of D-alanine from LTA was achieved by insertional inactivation of the *dlt*A gene in *Lactobacillus reuteri* 100-23. This mutant had similar growth characteristics to the wild type in *in vitro* culture, but had a reduced ability to colonise the mouse gastrointestinal tract, probably because it was unable to form a biofilm during colonization (Walter *et al.* 2007).

In contrast to *S. aureus*, LTA purified from a D-alanine deficient LTA mutant of pathogenic *Streptococcus pyogenes* was shown to induce secretion of inflammatory cytokines at levels equivalent to those of the corresponding wild type with normal D-alanine content. The authors note that D-alanine is the only substituent on the glycerol phosphate chain of LTA in wild type *S. pyogenes*. They suggest that the presence or absence of N-acetyl glucosamine or other sugar substituents on LTA may influence the requirement for D-alanine to induce cytokines. This is consistent with the findings for group B streptococci which also lack monosaccharides on the poly-glycerophosphate polymer of their LTA. In some cases the overall structure of LTA

may be as important as the individual substituents (Hasty *et al.* 2006). A later study of this mutant strain revealed that there was reduced expression of virulence factors by the mutant bacteria compared to the wild type, further illustrating the pleiotropic nature of such a mutation (Cox *et al.* 2009).

A *dlt*A knockout mutant of the swine pathogen *Streptococcus suis* had no D-alanine on its LTA and exhibited greatly reduced virulence compared to the wild type in pig and mouse models. Greater susceptibility to cationic antimicrobial peptides (CAMPs) and killing by neutrophils was seen in the mutant organisms, indicating that D-alanine is involved in resistance to these host defences (Fittipaldi *et al.* 2008). Similar sensitivity to CAMPs was seen when the same gene was deleted from *Enterococcus faecalis* (Fabretti *et al.* 2006).

LTA from *Streptococcus pneumoniae* is unique in that it has a repeating pentamer unit consisting of a ribitol linked to a tetrasaccharide containing phosphocoline and N-acetyl-D-galactosamine. NMR analysis of LTAs from two different serotypes of this species revealed that the LTA from the strain which exhibited a much higher cytokine induction response was substituted with D-alanine on the ribitol, while the other strain lacked this substituent (Draing *et al.* 2006).

A *dlt*D gene knockout mutant of *Lactobacillus rhamnosus* HN001 expected to lack the ability to add D-alanine substituents to the lipoteichoic acid has been created at Fonterra Research Centre. This mutant bacterium has been shown to induce greater amounts of the anti-inflammatory cytokine IL-10, as well as more of the pro-inflammatory cytokines TNF and IFN-γ, than the wild type HN001. This suggests that the LTA is important for the immune response; however, as with many of the *dlt* mutants mentioned here, this is a very pleiotropic mutant, and differs in morphology and growth characteristics to the wild type (M. Collett, Personal Communication). Analysis of the isolated LTA is needed to determine whether the modified LTA itself is responsible for eliciting these specific responses. The LTAs from both the DltD- mutant and its wild type parent strain HN001 are examined in this thesis.

1.2.7 *Lactobacillus rhamnosus* IM126

Lactobacillus rhamnosus IM126 is a single colony isolate of a strain originally isolated from faecal samples. It was identified as a *L. rhamnosus* strain initially by sequencing a portion of the 16s rDNA. This was confirmed by analysis of sugar fermentation patterns and by sequencing of the *dnaK* gene. IM126 was chosen for inclusion in this study as it was found to have a stronger anti-inflammatory IL-10-inducing ability in PBMC-CBA (peripheral blood mononuclear cells-cytometric bead array) assays, in comparison to HN001 and a collection of other *L. rhamnosus* strains (M. Collett, personal communication).

1.3 Aims of this Study

1.3.1 Hypothesis

The hypothesis for this study is that lipoteichoic acid influences the immunomodulatory properties of lactic acid bacteria, and that differences in LTA structure are responsible for strain dependent immunomodulatory properties.

1.3.2 Aims

- To isolate LTA from strains of lactobacilli with predetermined immunomodulatory profiles.
- To analyse the immunomodulatory properties of each isolated LTA.
- To determine the structure of each isolated LTA, and relate this to the immune responses.

1.3.3 Reasons for this Study

At the time of writing, lipoteichoic acid has been characterised from only a small number of strains, including a very few probiotic strains. Complete characterisation of LTA from a wide number and variety of strains will be required to develop a clear picture of the importance of LTA in immune recognition and response to this molecule. Elucidation of the relationship of the structure of LTA to its immune function will support the use of probiotic bacteria as prophylactics with specific immunomodulatory properties, and improve the understanding of the interactions of all gram positive bacteria with host immune systems.

Chapter 2 - Materials & Methods

2.1 Materials & Equipment

2.1.1 Materials

2.1.1.1 Bacterial Culture

Difco MRSB (Fort Richard 288130)
Bacto™ Agar (Fort Richard 214010)
Glycerol (Merck 104092.2500)
AnaeroPack® System (Mitsubishi Gas Company M101)

2.1.1.2 Bacterial Strains

Lactobacillus rhamnosus HN001
Lactobacillus rhamnosus DltD- mutant
Lactobacillus rhamnosus IM126

2.1.1.3 LTA Preparation/Chromatography

Acid-washed glass beads 106 µm (Sigma G4649)
Tri-Sodium citrate AnalaR® (Merck 106448.0500)
Citric Acid AnalaR® (VWR BDH 100813M)
N-butanol for analysis EMSURE® (Merck 101990)
HiPrep™ Octyl FF 16/10 column (GE Healthcare 28-9365-48)
Superloop™ 10 mL (GE Healthcare 18-1113-81)
N-propanol (1-Propanol) Unilab (Ajax 424-2.5L GL)
Ammonium acetate AnalaR® (VWR BDH 100134T)
Acetic acid 100 % GR, glacial (Merck 100063.2500)
Isopropanol (2-Propanol) gradient grade (Merck 101040.2500)
Sodium hydroxide AnalaR® (Merck 106498.0500)
Limulus Amebocyte Lysate (LAL) Kit QCL-1000® (Lonza 50-648U)
LAL Reagent Water (Lonza W50-500)
Sterile flat-bottomed 96-well plate (Nunc 167008)

2.1.1.4 LTA Quantitation

Ammonium heptamolybdate (J.T. Baker 1-0716)

L-ascorbic acid (Ajax Unilab A79-100G)

Sulfuric acid AnalaR® (Merck 100731.2500)

Hydrochloric acid 30 % Ultrapur (Merck 101514.025)

Pressure Vial 10 mL and Septa Accessory Kit for Discover® microwave (CEM 908310)

EnzyChrom™ Glycerol Assay Kit (BioAssay Systems BAS-EGLY-200)

Flat-bottom 96-well 96F Microwell plate, non-sterile (Nunc 269620)

MatriPlate™ 96-well, 0.17 mm thickness glass-bottom, black for fluorescence (GE Healthcare 28932399)

2.1.1.5 PBMC-CBA Assays

DPBS (Dulbecco's Phosphate Buffered Saline) sterile 1× (Gibco® 14190)

RPMI 1640 + GlutaMAX™ (Invitrogen™ 61870-036)

Heat inactivated FBS (Invitrogen™ 10093-144)

Penicillin/Streptomycin mixture (Invitrogen™ 15140-122)

Human Peripheral Blood Mononuclear Cells (hPBMC) (Lonza CC-2702)

Trypan Blue stain 0.4 % (Gibco® 15250061)

Sterile flat-bottomed 96-well plate (Nunc 167008)

Lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Sigma L4391)

Phorbol 12-myristate 13-acetate (PMA; Sigma P1585)

Ionomycin (Sigma I0634)

Phytohemagglutinin (PHA-P) lectin from *Phaseolus vulgaris* (Sigma L9017)

BD™ Human Soluble Protein Master Buffer Kit (BD™ 558265)

BD™ CBA Flex Sets: Human TNF C4 (560112), Human IL-1β B4 (558279), Human IL-4 A5 (558272), Human IL-8 A9 (558277), Human IL-10 B7 (558274), Human IL-12p70 E5 (558283), Human IL-17A B5 (560383), Human IFN-γ E7 (558269).

MultiScreen® HTA Filter Plate 1.2 µm (Millipore MSBVN1250)

2.1.1.6 NMR

Deuterium oxide (D_2O) Uvasol® 99.9 % (Merck 113366)

Shigemi 5mm Symmetrical NMR microtube (Sigma Z543349)

2.1.2 Equipment

Braun Cell Disintegrator

Millipore Milli-Q Gradient Water Purification System

Biochrom Libra S22 UV/Vis Spectrophotometer

GE Healthcare Life Sciences Äkta Explorer 10 FPLC system with GE Unicorn™ software

GE Healthcare Typhoon™ fluorescence scanner with ImageQuant TL™ software

Molecular Devices OPTImax™ tunable microplate reader

CEM Discover® microwave incubator

BD™ FACSArray™ Bioanalyser

Bruker 700 MHz NMR spectrometer with Bruker Cryoprobe

2.2 Methods

2.2.1 Precautions to Avoid Lipopolysaccharide Contamination

Lipopolysaccharide (LPS) endotoxins from gram positive bacteria are potent inducers of immune responses, and are ubiquitous in laboratory environments. The presence of LPS contaminants can cause high background signals in the immune cell assay used to analyse samples prepared for this project. For this reason, special precautions were taken to eliminate and/or reduce the LPS contamination in this work.

Aseptic techniques were used at each processing step to minimise the contamination by gram-negative bacteria.

All disposable plastic-ware used was certified as being endotoxin- or pyrogen-free by the manufacturer. All equipment not certified as such, was tested using the Limulus Amoebocyte Lysate (LAL) assay (see section 2.2.8.1) to ensure that there was no contamination. Reusable glassware was cleaned thoroughly then baked at 180 °C overnight, as autoclaving does not destroy LPS. Any laboratory equipment that could not be baked was soaked in 0.5 M NaOH for at least one hour, before rinsing with LPS-free Milli-Q (MQ) water until the pH returned to that of the water. Growth media used for bacterial culture was filter-sterilised, as autoclaves are a common source for the introduction of LPS. Similarly, all buffers and solutions that were required to be sterile were either purchased sealed and certified as sterile, and/or were filter-sterilised rather than autoclaved.

Unless specified otherwise, water from a Millipore Milli-Q water system was used to prepare all buffers and other reagents used in work upstream of the immune assays. This water was tested for endotoxin by LAL assay at regular intervals.

2.2.2 Culture of *Lactobacillus rhamnosus*

All three strains of *Lactobacillus rhamnosus* in this study were routinely grown in deMan-Rogosa-Sharpe (MRS) media prepared as specified by the supplier and sterilised by passing through a 0.22 µm filter membrane under vacuum, rather than autoclaving.

Liquid cultures were grown in sterile plastic tubes or Schott bottles, full nearly to the top with media, with caps screwed on loosely, creating a semi-anaerobic environment. Liquid cultures were incubated in a water bath at 37 °C to maintain consistent growth temperature. The agar plates used were standard MRS plates made with 1.5 % agar, and incubated in anaerobic jars, using the AnaeroPack® system. For long and medium term storage, freezer stocks containing 20 % v/v final concentration of sterile glycerol were prepared in 1 mL aliquots from overnight liquid cultures and stored at -80 ºC. Generally, seed cultures were inoculated at 1 % from glycerol stocks and grown overnight. These seed cultures were then used to inoculate the cultures used in experiments. Typically, a 24 hr culture was diluted 100-fold into 4.4 L (four one litre Schott bottles, close to brim full) of prewarmed MRS broth and incubated at 37 ºC until early stationary phase was reached: HN001 ~ 18 hours, the DltD- mutant ~ 14 hours and IM126 ~ 16 hours.

Numbers of bacteria in cultures were calculated after determining the relationship between optical density (OD) and cell concentration for each strain. An overnight culture was grown then serially diluted from 2- to 64-fold in MRS. The OD at either 600 or 650 nm was measured for each dilution. If necessary, further dilutions were made to obtain cell suspensions with ODs that are within the linear range of the spectrophotometer. The concentration of cells in the original culture was determined by dilution plating on MRS agar and counting of bacterial colonies. The concentrations of colony forming units (cfu) in each measured 2-fold serial dilution were calculated by dividing the concentration of the original culture by the appropriate dilution factor. The concentrations in cfu/mL were plotted against their respectively ODs, and the mathematical relationship between the two values was determined by regression for the linear portion of the curve. The cell concentration of a given culture was calculated from the OD using this linear relationship. The optical density of bacterial cells is determined by light scattering, thus it should be noted that the curve is specific to each strain of bacteria and the spectrophotometer used.

2.2.3 Extraction and Purification of LTA from Bacteria

The LTA purification protocol was adapted from the method of Morath *et al.* (2001). All solutions and labware used were pyrogen-free. Aliquots of partially purified LTA

were taken at several intermediate steps of the purification procedure for later analysis in the immune cell assays, and as a precaution to track potential sources of LPS contamination.

2.2.3.1 Butanol Extraction

Cells were harvested by centrifugation at 5500 ×g for 20 minutes, and washed once with cold 100 mM sodium citrate buffer, pH 4.7. Cell pellets were resuspended in cold 100 mM sodium citrate buffer, pH 4.7 at a concentration of 40 g wet weight/100 mL of buffer, before adding 50 mL of suspension at a time to a Braun Cell Disintegrator vial containing 25 mL acid washed and baked 0.1 mm glass beads. Cell suspensions were shaken at full speed (4000 rpm) for three minutes, cooling with a short burst of liquid CO_2 every 30 seconds, then placed on ice for ten minutes before being shaken again at full speed for three minutes. Cell homogenates were separated from the glass beads by centrifugation at 100 ×g for five minutes, then stirred with an equal volume of N-butanol for 30 minutes at room temperature. The two phases were separated by centrifugation at 13000 ×g for 20 minutes at room temperature, and the aqueous phase recovered by pipette, then lyophilised. Dried material was resuspended to a concentration of ~ 150 mg/mL in 15 % v/v N-propanol in 100 mM ammonium acetate buffer, pH 4.7 (chromatography equilibration buffer, see section 2.2.3.2) and centrifuged at 45000 ×g for 15 minutes at 4 ºC. The supernatant was collected and syringe-filtered through a 0.2 µm membrane. Where necessary this crude LTA was stored in the short-term in aliquots at -80 ºC, and was lyophilised for long-term storage.

2.2.3.2 Hydrophobic Interaction Chromatography of LTA

All samples and solutions for chromatography were pyrogen-free and filtered through a 0.2 µm membrane. All lines of the GE Health Life Sciences Äkta Explorer 10 FPLC (fast protein liquid chromatography) system were depyrogenated with 1.0 M sodium hydroxide and rinsed back to neutral pH before use with pyrogen-free water. Crude LTA equivalent to 15 µmoles of total phosphate was loaded at 0.5 mL/min via a 10 mL Superloop™ onto a GE Health Life Sciences HiPrep™ Octyl Sepharose Fast Flow 16/10 hydrophobic interaction chromatography (HIC) column (volume ~ 20 mL), equilibrated with 5 column volumes (CV) of 15 % v/v N-propanol in 100 mM

ammonium acetate buffer, pH 4.7. The column was washed with 7 CV of the equilibration buffer at a flow rate of 0.5 mL/min before applying a gradient from 15 to 60 % v/v N-propanol in 100 mM ammonium acetate buffer, pH 4.7 over 24 hours, at the same flow rate. The column was washed with 4 CV of 60 % v/v N-propanol in 100 mM ammonium acetate buffer, pH 4.7 once the gradient was complete. Fractions of 5 mL were automatically collected throughout, and fractions containing LTA were identified using the phosphate assay described in section 2.2.4.

Once the retention times/volumes of the phosphate containing fractions had been established, the elution program was optimised to be shorter. The final parameters used are shown in Table 1. The column was cleaned at regular intervals as per the manufacturer's instructions.

Segment	No. of Column Volumes	Flow rate (mL/min)	% B	% N-Propanol	Fractions Collected
1	1	0.5	0	15	N
2	6	3.0	0	15	N
3	1	3.0	3.2	16.4	N
4	8	0.5	28.8	28	Y
5	1	1.0	100	60	N
6	5	2.5	100	60	N
7	1	1.0	0	15	N

Table 1: Final HIC Program Used to Purify LTA
The stages of the optimised chromatography gradient program used to purify LTA for the structure/function analyses are listed in the table. The volume of the column was ~ 20 mL. The equilibration buffer contained 15 % N-propanol, and elution buffer (B) contained 60 % N-propanol. The actual percentage of N-propanol at each stage is also listed in the table. Where indicated in the table, fractions of 5 mL were collected.

2.2.4 Determination of Phosphate

As lipoteichoic acid does not appreciably absorb visible or UV light, it is difficult to monitor the progress of chromatography using traditional detectors. Because the LTA molecule contains a glycerol-phosphate repeating unit, phosphate or phosphorus concentration is commonly used to detect and quantify LTA. The phosphate concentration of samples was determined using the method of Chen *et al*. (1956), with some modifications. This method is based on formation of a coloured phosphomolybdate complex. Special care was required to ensure that all reagents and

glassware used were free of any phosphate containing contaminants. Phosphate standards were prepared in the range of 1 – 50 µM in volumetric flasks from disodium orthophosphate. The colour reagent was prepared daily as required by mixing one volume of 2.5 % w/v ammonium heptamolybdate with one volume of 6 N (3 M) sulphuric acid, one volume of 10 % w/v ascorbic acid and two volumes of MQ water. The assay was carried out in flat-bottom microplates as follows; 100 µL of standard or sample was mixed with 100 µL of colour reagent before briefly mixing by tapping the edge of the plate. The plate was then incubated at 37 ºC for 90 minutes, and the absorbance measured at 820 nm in a Molecular Devices OPTImax™ tuneable microplate spectrophotometer. Phosphate concentrations of samples were determined from a phosphate standard curve. Where samples were in solvents or buffers other than MQ water, a reagent blank was included in the phosphate assay and the absorbance value of this subtracted from each sample.

It must be stated that the above assay performed directly on LTA samples does not give the true phosphate concentration. LTA must be hydrolysed in acid before phosphate determination to provide an accurate result. The molybdate method without acid hydrolysis gives a rapid indication of phosphate concentration and as such is useful for detecting lipoteichoic acid containing fractions after chromatographic purification. As long as the method is carried out in a consistent manner, then the molybdate assay provides repeatable results on non-hydrolysed LTA samples and is useful for making comparisons between samples, rather than for quantitation. For comparison of the immune responses to LTA from the three different strains in this study, it was necessary to completely hydrolyse samples of LTA before determining the phosphate concentration, to ensure that equivalent amounts of LTA were being added to the immune cell assays. To distinguish where phosphate concentrations are for non-hydrolysed LTA, concentrations are noted as µM PNH (phosphate-non-hydrolysed) where the rapid method was used.

2.2.5 Acid Hydrolysis of LTA

Complete hydrolysis of LTA in acid was required before accurate determinations of phosphate and glycerol concentrations could be made. Acid hydrolysis trials were carried out to establish the optimum conditions for complete hydrolysis of LTA; the

following are the optimised conditions. 300 - 500 µL samples were lyophilised in microtubes then each sample was resuspended thoroughly by vortex and sonication in 1 mL of 2 M HCl. Each sample was then transferred to a 10 mL glass pressure- and temperature-resistant sample vial containing a magnetic stir bar and flushed with argon for 8 minutes before sealing with a Teflon® pressure cap. The pressure vessel containing the sample was placed into a CEM Discover® microwave incubator set to 160 ºC, with stirring, for 30 minutes. The sample was cooled to room temperature, before slowly opening the pressure cap in a fume hood to equalise any residual pressure difference. The bulk of the acid was removed by vacuum using a liquid nitrogen-cooled cold-trap system to condense and collect the acid followed by resuspension in MQ water and freeze-drying. The final step was repeated until the pH of the samples had returned to neutral.

To determine the concentration of any background glycerol or phosphate present, a control containing MQ water (no sample) plus HCl was processed alongside the samples. Another control containing both disodium orthophosphate and glycerol standards at 250 µM each was included to determine the losses of glycerol and phosphate during hydrolysis. The relative proportion of phosphate and glycerol lost due to the hydrolysis method was used to adjust the final concentrations of glycerol and phosphate in the LTA samples. Triplicate aliquots of each sample and control were processed.

2.2.6 Determination of Glycerol

Glycerol is a major component of and can be used to quantitate LTA. Its concentration was determined after acid hydrolysis (section 2.2.5) using the BioAssay Systems EnzyChrom™ Glycerol Assay Kit. This commercial kit uses linked enzyme assays with an end dye product that can be measured by both fluorescence and absorption. The assay was carried out according to the supplier's instructions, with some modifications, in a glass-bottomed, black-sided low background fluorescence microplate. A series of standards was constructed in duplicate using the glycerol standard provided, with concentrations of 0, 5, 15, 20, 30, 50, 300, 600 and 1000 µM glycerol. 100 µL of freshly prepared working reagent was added to 10 µL of samples in MQ water (diluted in MQ water if necessary) and standards, and mixed gently by tapping the edge of the plate.

The plate was then covered in foil to protect it from light, and incubated at room temperature for 20 minutes. Fluorescence was measured at an excitation wavelength of 532 nm and emission wavelength of 580 nm on a GE Typhoon™ fluorescence scanner at a resolution of 200 µm, with the sensitivity set to normal and the photomultiplier tube (PMT) voltage set to 400 V. Relative fluorescence values were determined from the scanner image using GE ImageQuant TL™ software without noise filtering. The glycerol concentrations of samples which fell within the fluorescent range of the assay (2 – 50 µM glycerol) were determined from a standard curve constructed in this range. If the glycerol concentrations of samples were above the fluorescent range, the absorbance of the plate was read at 570 nm on a Molecular Devices OPTImax™ tuneable microplate reader. In this case, a standard curve was constructed in the colourimetric range of 0 – 1000 µM.

2.2.7 Determination of the Critical Micelle Concentration (CMC) of LTA

The CMC of any molecule is often dependent on the solvent. For this reason the diluent used in the CMC determination experiment must be as close as possible to the solvent/buffer in which the CMC is required to be known. For the purposes of adding LTA into human cell culture for immune cell assays, the CMC value of LTA in the growth medium needs to be determined. As the CMC determination protocol used here employs Coomassie Brilliant Blue R-250 dye (CBB R-250), which binds to proteins, the closest approximation to the cell culture medium that could be used is RPMI 1640 + GlutaMAX™ without the addition of fetal bovine serum (see section 2.2.8.2).

The CMCs of LTAs from all strains in this study were determined by the method of Courtney *et al.* (1986), with some modifications. The method was tested on the detergent Triton X-100, and gave a CMC that agreed with the literature value of ~ 0.2 mM. An aliquot of purified LTA that had been analysed for phosphate was concentrated by freeze-drying and then resuspended in RPMI 1640 + GlutaMAX™ to prepare a 1000 µM (PNH) stock. The assay was carried out in duplicate wells of a flat-bottomed microplate. 160 µL of 50 µg/mL CBB R-250 dye solution (made up in the same diluent as the LTA) was added to 40 µL of each dilution to give a final dye concentration of 40 µg/mL in a total volume of 200 µL. The absorbance of the samples

at 596 nm was measured after equilibration of the plate at 25 ºC, then measured again at 37 ºC using a Molecular Devices OPTImax™ tuneable microplate reader. The CMC was determined as the lowest concentration of LTA at which the increase in absorbance becomes non-linear.

2.2.8 Measurement of Immune Response

The immune stimulating ability of each sample was determined by measuring the induction of an immune response in peripheral blood mononuclear cells (PBMCs) by the sample. This response was detected and quantified using a cytometric bead array (CBA) technique to measure cytokines secreted by the blood cells.

2.2.8.1 Limulus Amoebocyte Lysate (LAL) Assay

The Limulus Amoebocyte Lysate (LAL) assay was used to determine the amount of any contaminating lipopolysaccharide (LPS) in samples prior to testing for immune activity, primarily to confirm that samples to be tested did not contain sufficient LPS to induce a detectable immune response. The assay was carried out in a sterile microplate according to the supplier's instructions, except that the volumes of sample, lysate, chromogenic substrate and stop reagent (25 % v/v glacial acetic acid) were reduced to 20, 20, 40 and 40 µL, respectively. The absorbance at 405 nm was read in a Molecular Devices OPTImax™ tuneable microplate reader. Samples were tested in duplicate. The detection range for this assay is 0.1 – 1.0 Endotoxin units per millilitre (EU/mL).

2.2.8.2 PBMC Assay

PBMCs are a subset of blood cells including a number of different types of immune cells. These cells provide a convenient method of measuring immune responses. When examining the immune responses to probiotics, the immune response in the intestinal tract is often considered to be the most relevant. While the immune response in the blood may not necessarily approximate that in the gut exactly, the same major immune cell types are represented.

Cell culture media was prepared by filter-sterilising a freshly made solution of RPMI 1640 + GlutaMAX™ containing 10 % v/v fetal bovine serum (FBS) and a

penicillin/streptomycin mixture (at final concentrations of 100 U and 100 µg per mL, respectively). A 1 mL vial of PBMC stored in liquid nitrogen was quickly thawed, then resuspended gently in 25 mL of culture media prewarmed to 37 ºC. Cells were then centrifuged at 200 ×g for 15 minutes at room temperature before being resuspended in 10 mL of media. At this point a 20 µL aliquot was stained with Trypan blue and counted on a haemocytometer to assess viability. The remaining PBMC suspension was diluted with culture media to give a concentration of 1.1×10^6 viable cells/mL, and 180 µL pipetted into each well of sterile flat-bottomed microplates. Thus after addition of 20 µL of control or sample, each well contained PBMCs at a final concentration of 1.0×10^6 viable cells/mL in all wells, in a final volume of 200 µL.

Samples to be tested in this assay had previously been resuspended or diluted as required into phosphate buffered saline (PBS) and tested for the presence of LPS using the LAL assay (section 2.2.8.1). All samples free from LPS were then mixed thoroughly by vortexing before addition of duplicate or triplicate 20 µL aliquots of each sample to wells containing the PBMCs. Wells testing whole bacterial cells were prepared so that the final concentration of bacteria was 1.0×10^6 cfu/mL. Controls were included with the assay containing final concentrations of each of the following: PBS from the same batch used to prepare the samples (negative control), 1 µg/ml bacterial lipopolysaccharide (positive control), a combination of 25 ng/ml phorbol 12-myristate 13-acetate (PMA) with 1 µg/ml ionomycin (T cell mitogens, positive control for TNF, IFN-γ and other cytokines), and 10 µg/mL phytohemagglutinin (PHA-P) (also a T cell mitogen known to induce cytokine secretion in PBMCs). The plates were incubated in 5 % CO_2 at 37 ºC for 24 hours, after which they were sealed and stored at -80 ºC until analysed.

2.2.8.3 CBA Analysis

The concentrations of cytokines secreted into the cell culture supernatants by the PBMCs were determined using the BD™ CBA Assay Flex Set kits in conjunction with analysis on a BD™ FACSArray™ Bioanalyser. The CBA analysis was performed according to the supplier's instructions for the BD™ CBA Flex Set kits, the BD™ Soluble Protein Master Buffer Kit and the manuals for the BD™ FACSArray™ instrument, with the following modifications. The volumes of sample/standard, capture

beads and PE detection reagent were reduced from 50 µL per well to 25 µL per well. Each sample well was resuspended in 100 µL of wash buffer in the final step before analysis on the FACSArray™. For the acquisition settings in the FACSArray™ software controlling the instrument, the number of mixes was changed to three, and the sample volume was increased to 40 µL.

Where possible, each PBMC plate was analysed for all relevant cytokines simultaneously. One notable exception is the analysis of IL-8, which was expressed in PBMC at levels beyond the range of the IL-8 standards, and had to be analysed separately after dilution of the samples in the CBA sample diluent. The number of freeze-thaw cycles of the PBMC plates was kept to as few as possible to minimise the denaturation of cytokine proteins prior to analysis.

After CBA analysis, each PBMC plate was checked for differences across the plate, particularly at the edges, which may be caused by issues such as uneven temperature gradients across a plate during incubation (for example, by plotting cytokine data for every set of twelve wells across the plate). No such effects were observed in this study.

2.2.9 Structural Analysis Using NMR

Aliquots of LTA from the same preparations used for cytokine analysis were concentrated by freeze-drying then resuspended in deuterium oxide (D_2O) giving slightly cloudy suspensions. These samples were then transferred to 5 mm NMR tubes. Spectra were recorded on a Bruker 700 MHz spectrometer equipped with a cryoprobe. 1D ^{1}H spectra were typically recorded with a spectral width of 20.52 kHz using 65k points and averaged for 4096 scans. Spectra were processed using standard parameters and baseline corrected prior to peak integration. Peak identities were made based on the assignments of Morath *et al.* (2001) and verified using ^{1}H-^{1}H DQF-COSY (Double Quantum Filtered Correlation Spectroscopy), ^{1}H-^{1}H TOCSY (Total Correlation Spectroscopy), ^{1}H-^{1}H NOESY (Nuclear Overhauser Effect Spectroscopy) and ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Correlation) 2D experiments. The COSY, TOCSY and NOESY experiments used a spectral width of 4.90 kHz using 256 rows of 2k points each averaged for 80 scans. The HSQC spectra used a spectral width of 4.77 kHz (with 1k points) and 29.1 kHz (with 256 points) for the ^{1}H and ^{13}C dimensions

respectively. Each FID (Free Induction Decay) was averaged for 128 scans. Average poly(glycerol-phosphate) (PGP) and fatty acid chain lengths, as well as the relative amounts of substituents to the PGP were calculated using the ratios of the peak integrals from the 1D NMR spectra.

Chapter 3 - Results & Discussion

3.1 Phosphate and Glycerol Determination

3.1.1 Phosphate Determination

Phosphate concentration is a commonly used indicator of LTA concentration, due to the presence of phosphate in the repeating units of both glycerol- and ribitol-phosphate LTA. The molybdenum blue assay described in the methods (section 2.2.4) is commonly used to quantitate LTA. It is presumed that the molybdate dye forms complexes only with the accessible phosphate groups on the LTA, such as the terminal glycerol-phosphate group. The LTA must be completely hydrolysed in acid before the actual value of phosphate can be determined using a colourimetric assay. The acid hydrolysis step is, however, time-consuming, so during the purification of LTA in this study, the assay was carried out on non-hydrolysed samples. This underestimates the concentration of LTA-phosphate, but gives a useful indication of LTA concentration, suitable for selecting LTA-phosphate-containing chromatography fractions, for example. Quantitation of LTA without acid-hydrolysis gives consistent results for comparing samples within the strain, for each of the three strains in this study. Once calibrated with concentrations determined for acid-hydrolysed samples, the phosphate data from non-hydrolysed samples allowed the estimation of real phosphate concentrations where necessary. For reliable comparison of amounts of LTA between strains, however, acid hydrolysis was performed prior to analysis using the molybdenum blue assay. The standard curve for this assay allowed quantitation in the range of 2 – 150 μM phosphate, and was highly linear, as indicated by high R^2 values for the best-fit line, typified by the standard curve in Figure 11.

LTA from each of the three strains has a different ratio of acid-hydrolysed phosphate to non-hydrolysed phosphate, which may be due to the varied accessibility of phosphate groups for dye-binding. This suggested that there may be structural differences between these LTAs, such as PGP chain length, that may affect formation of the phosphomolybdate complex, as discussed in section 3.5.

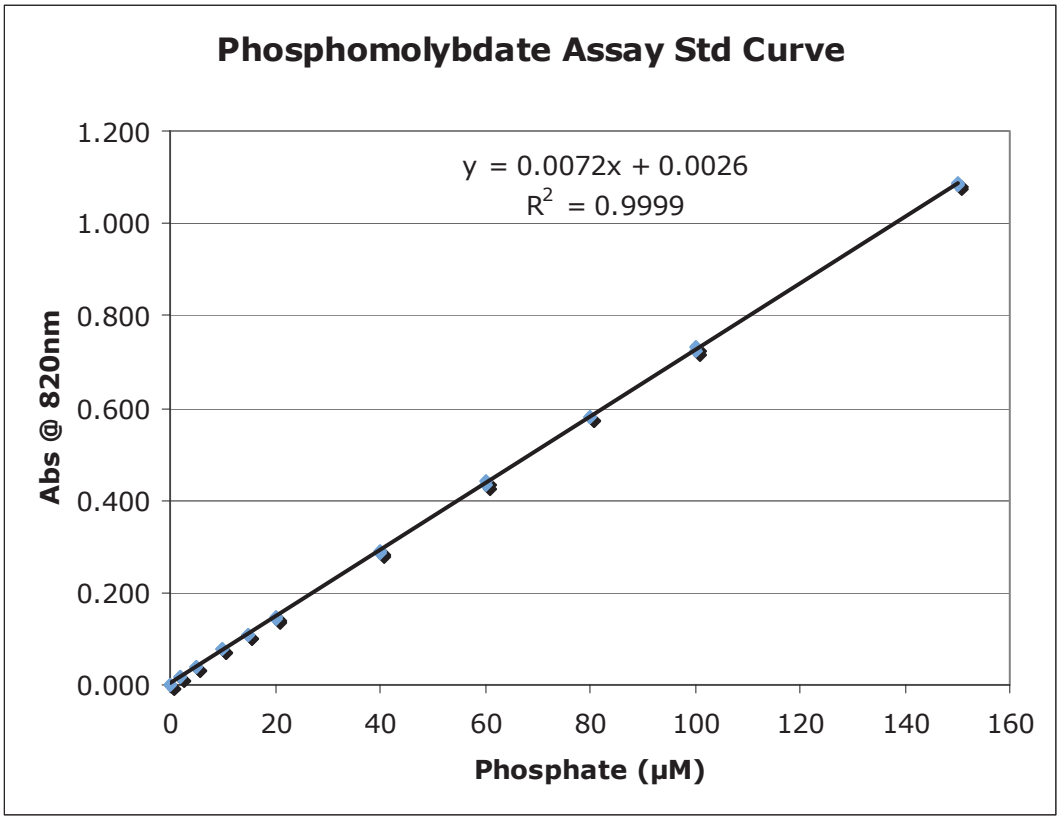

Figure 11: Phosphomolybdate Assay Standard Curve
A typical standard curve for the phosphomolybdate assay using disodium orthophosphate as the standard, used to determine phosphate concentrations of LTA samples. Duplicate standards were measured at each concentration. The formula for the linear trend line and its R^2 value are displayed on the chart.

3.1.2 Glycerol Determination

The concentration of glycerol was measured, as glycerol is a common component of the repeating unit in the hydrophilic polymer of LTA. The commercial kit used to measure glycerol concentrations employs a dye product that can be quantified using both fluorescent and visible spectra, giving an effective concentration range of 2 - 1000 µM. Standard curves were plotted for each of these ranges, examples of which are given in Figure 12. The LTA samples analysed in this thesis were generally in the fluorescent range. The lines of best fit generated were a very close approximation of the standard data, as indicated by the high R^2 values.

3.1.3 Standardisation by Phosphate and Glycerol

To enable comparison of the immune responses to structurally different LTAs, it is critical that identical concentrations of each LTA are used in the immune cell assay. In

order to achieve this, the LTA concentrations of acid-hydrolysed samples from the three strains in this study were determined from the phosphate and glycerol concentrations.

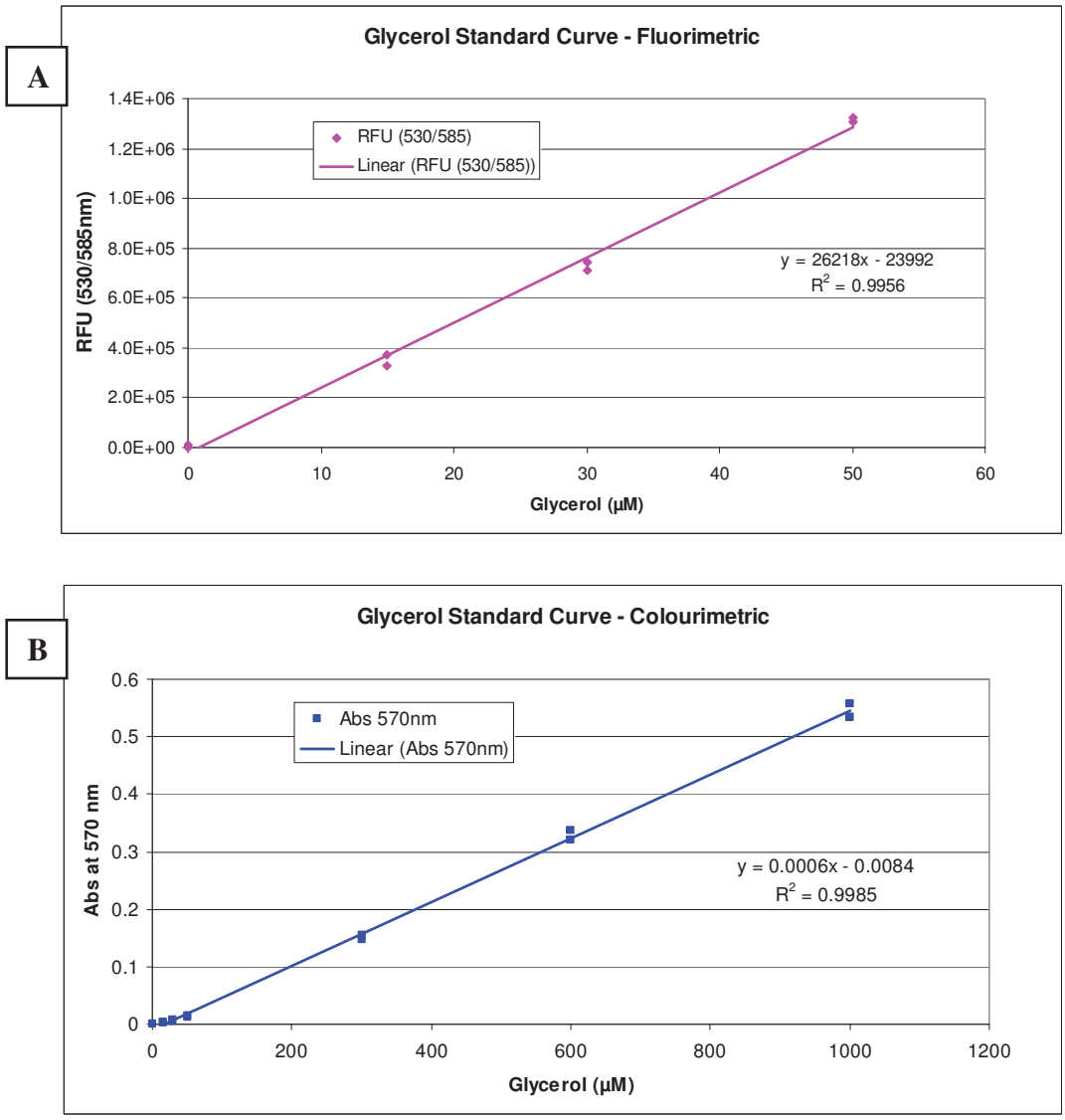

Figure 12: Glycerol Assay Standard Curves
Typical standard curves for the enzymatic glycerol assay used in this study. Duplicate standards were measured at each concentration. The dye end-product from this assay gives a signal in both (A) fluorescent and (B) visible spectra, so standard curves are constructed for each of these. RFU: Relative Fluorescence Units. The formulae for the best-fit lines and their R^2 values are displayed on the chart.

To account for losses or gains occurring during the acid hydrolysis and subsequent neutralisation procedures, both a glycerol/phosphate standard control and a water only control were hydrolysed in HCl, then neutralised alongside the LTA samples. Analysis of the water blank showed negligible phosphate or glycerol, indicating that there was no contamination from exogenous sources, (e.g., from the HCl stock or the water used in the neutralisation procedure). Unfortunately, when the hydrolysed glycerol/phosphate

standard control was compared with the pre-hydrolysis glycerol standard, it was apparent that during the procedure, large losses of both glycerol and phosphate (close to 50 % of each) occurred. To account for this, the phosphate and glycerol concentrations measured after acid-hydrolysis were multiplied by the ratio of non-hydrolysed to acid-hydrolysed phosphate or glycerol standard, respectively.

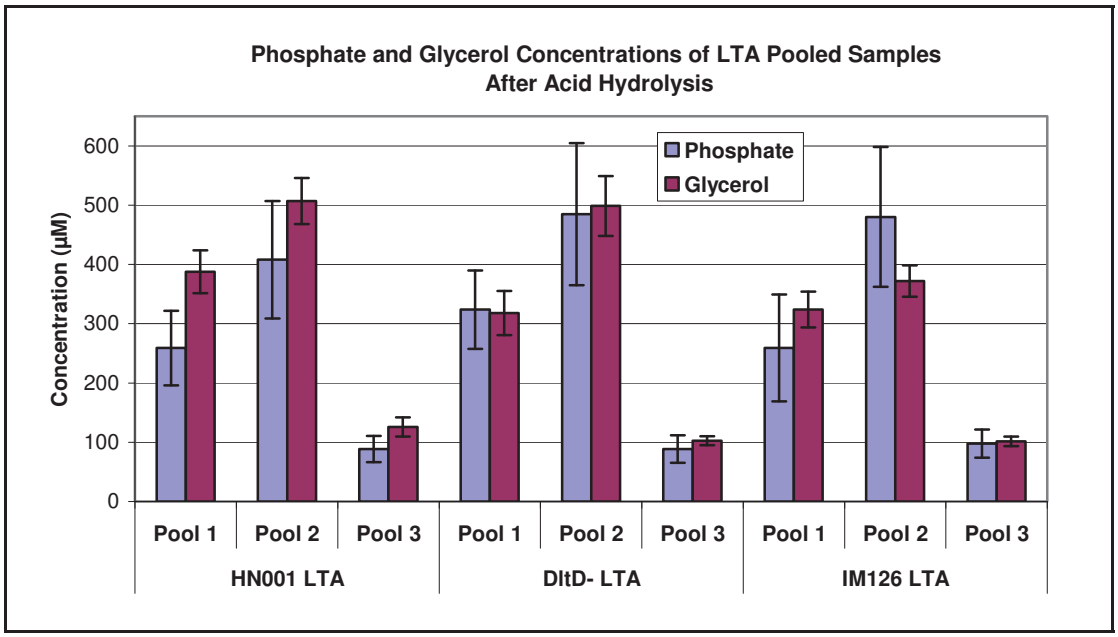

Figure 13: Phosphate and Glycerol Concentrations of LTA after Acid Hydrolysis
Phosphate and glycerol concentrations were determined for each of the three original pools of LTA HIC fractions from each of the three strains HN001, the DltD- mutant and IM126. The chart shows mean values +/- SEM (standard error of the mean) of three replicates for each sample.

There was also considerable variability between the replicates for many of the acid-hydrolysed samples, despite consistent acid-hydrolysis and neutralisation protocols. The large number of steps involved in this process is the most likely cause of this inconsistency. It is also possible that variations in the microwave power or mixing during the automated hydrolysis program are partly responsible. The time taken to remove the acid post-hydrolysis may have also been significant, with the vacuum strength or temperature of the cold-trap apparatus and freeze-dryer possibly playing a role. To partially counter these errors, three aliquots of each control and LTA sample were acid-hydrolysed, and each of these was analysed in triplicate using both the phosphate and glycerol assays. Although it would have been useful to include an internal standard, there was not an obvious or convenient one suitable for this protocol. Figure 13 shows the results of phosphate and glycerol quantitation (after acid hydrolysis) of LTA samples from HN001, the DltD- mutant and IM126.

LTA is expected to contain a ratio of phosphate to glycerol of approximately 1:1, as phosphate and glycerol are the repeating units in the hydrophilic polymer, and other substituents containing phosphate or glycerol are not normally found as substituents to the GroP chain of LTA. There is commonly a glycerol molecule in the lipid anchor moiety of LTA, thus the glycerol concentration may be slightly higher than the phosphate concentration for each fraction. The ratios of glycerol to phosphate for the LTA samples tested in Figure 13 are close to the expected 1:1, suggesting that LTA from these three strains is of the glycerol-phosphate polymer type normally found in lactic acid bacteria (rather than ribitol-phosphate polymer). This also suggests that the samples do not contain large quantities of phosphate or glycerol containing contaminants. Both of these major marker molecules provide a valid reference to normalise the amount of each LTA added into the immune cell assays. For these samples, the glycerol concentrations appear to have slightly smaller errors than the phosphate concentrations; however, phosphate was chosen as the reference, primarily because it is more commonly reported in the literature as the method used to quantitate LTA than is measurement of glycerol. The phosphate assay is also more economical to perform that the glycerol assay.

3.2 Limulus Amoebocyte Lysate (LAL) Assay

As it was essential to use endotoxin-free reagents, the Limulus Amoebocyte Lysate (LAL) assay was used to test for the presence of contaminating lipopolysaccharide (LPS). An example of a typical standard curve generated using the LAL assay, along with the R-squared value is shown in Figure 14. The samples analysed in immune cell experiments that were confirmed to have LPS concentrations below the limit of detection for the Lonza QCL-1000® LAL kit (0.1 EU/mL, ~ 10 pg/mL of LPS) were considered to be endotoxin free.

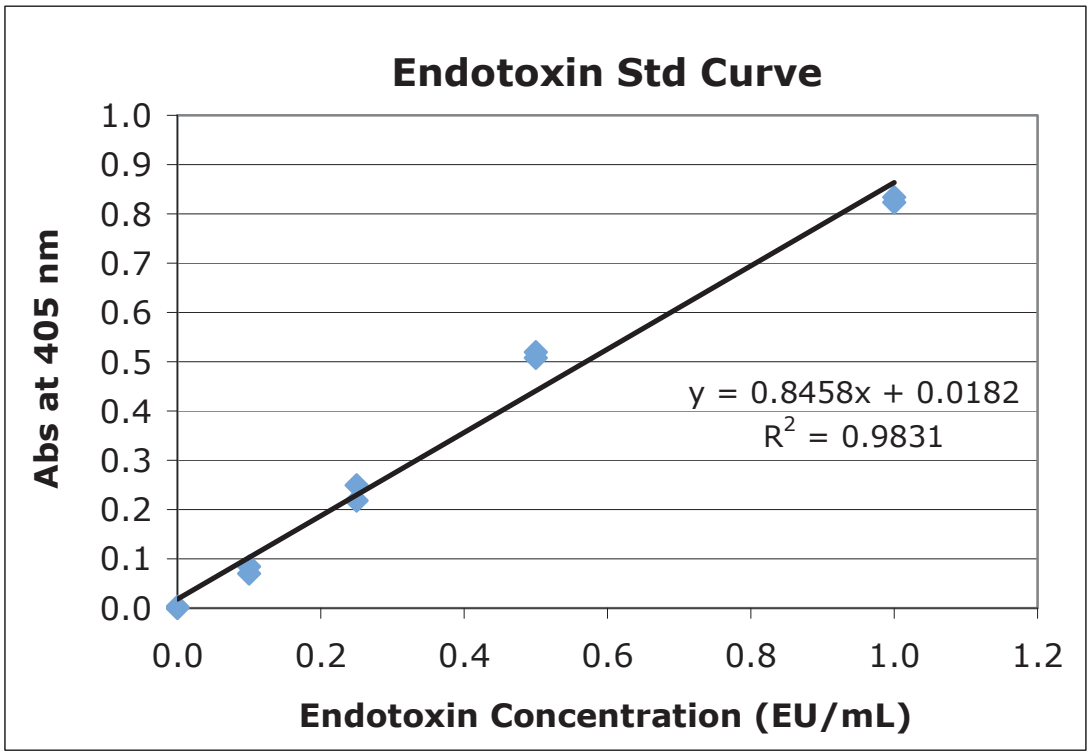

Figure 14: LAL Standard Curve
Typical standard curve for Lonza Limulus Amoebocyte Lysate Assay. Absorbance was measured at 405 nm for duplicate LPS endotoxin standards. The formula for the best-fit line and its R^2 value are displayed on the chart. EU: endotoxin units, 1 EU is approximately equal to 100 pg of an *E. coli* LPS standard.

3.3 Isolation of LTA from *Lactobacillus* Strains

Conditions for isolation of LTA from *Lactobacillus rhamnosus* were developed from established methods, and optimised to produce high purity, intact LTA.

3.3.1 Butanol Extraction

Butanol extraction of mechanically disrupted *Lactobacillus rhamnosus* cells yielded a viscous yellow aqueous solution. The concentration of phosphate in the butanol extracted material was measured for each preparation, and representative values for the total yield for each are shown in Table 2. The crude LTA appeared to contain a large amount of non-LTA phosphate-containing material, as revealed by phosphate analysis after HIC (section 3.3.3.1). Generally, larger cultures were grown for the mutant strain, as the cell density at early stationary phase was lower, and the butanol-extracted crude material was found to have a lower LTA-phosphate to total phosphate ratio than the

other two strains (discussed in section 3.3.4). The phosphate concentration was used as a guide to determine the volume of material to be loaded onto the HIC column.

Strain	Volume of Culture (L)	Optical Density	Cell Density (cfu/mL)	Total Yield of Crude LTA (mg)	Total Amt PO_4 in Crude LTA µmoles(PNH)
HN001	4.4	9.41 @650nm	8.52×10^9	4 140	446
DltD- Mutant	8.8	7.12 @650nm	6.52×10^8	14 290	1 283
IM126	4.4	9.18 @600nm	2.45×10^9	6 730	647

Table 2: Summary of Butanol Extraction for HN001, the DltD- Mutant and IM126
Table containing data from representative preparations of crude LTA from each of the three strains used in this study. The yield values are for the total material extracted from each respective culture. The cell density was calculated from the optical density, as described in the methods.

3.3.2 Hydrophobic Interaction Chromatography (HIC) of LTA

3.3.2.1 Preliminary HIC of LTA from HN001

Butanol extracted crude LTA was further purified by hydrophobic interaction chromatography (HIC) by FPLC on a column of octyl sepharose, using a solvent gradient of 15 to 60 % N-propanol to elute the bound LTA, as described in the methods (section 2.2.3.2).

In the initial HIC purification of LTA from HN001, a volume of HN001 crude LTA containing 60 µmoles (PNH) of total phosphate were loaded onto the column and 5 mL fractions were collected throughout the entire chromatography program. Phosphate concentrations were measured for every fraction and plotted together with the solvent gradient to generate the HIC elution profile shown in Figure 15. The first two fractions within the wash segment of the FPLC program contained a very large amount of phosphate, and these fractions coincided with a very high UV signal (data not shown). It is probable that this represents the elution of nucleic acids that did not bind to the HIC column. The solvent gradient increases from fraction 20 onwards, and the expected LTA-phosphate peak is seen in Figure 15 between fractions 25 - 51. No UV signal was detected for these fractions indicating that the LTA-phosphate peak was free of detectable contaminating proteins and nucleic acids (data not shown).

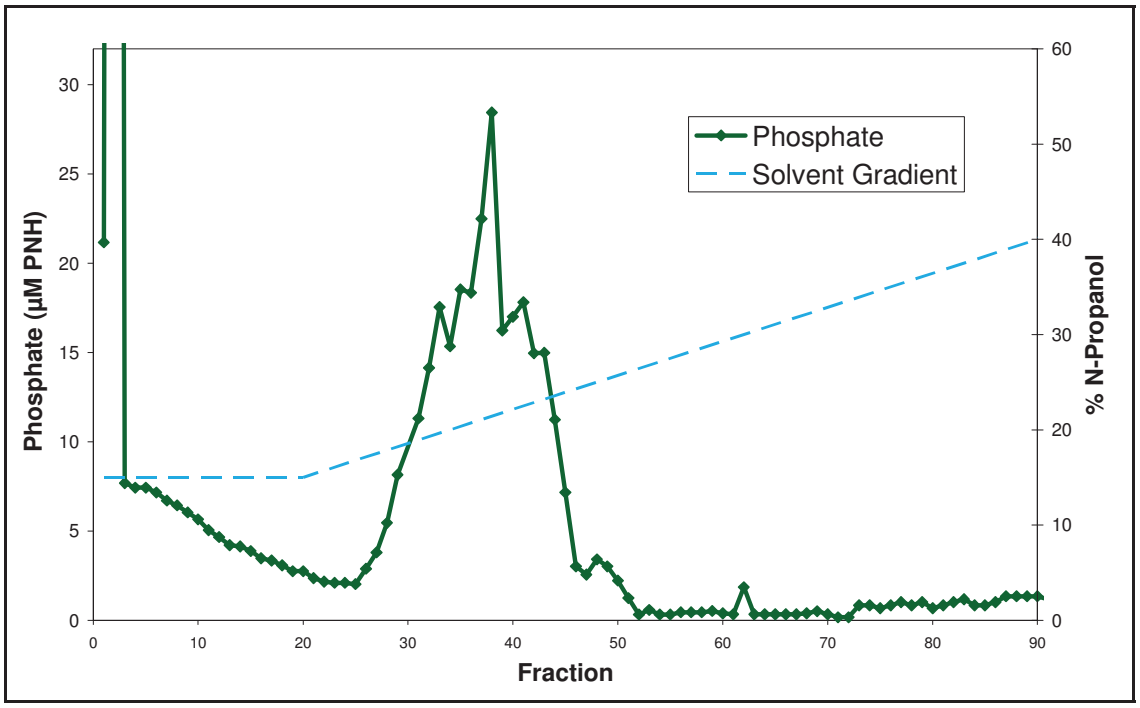

Figure 15: Chromatogram of Preliminary HIC Purification of LTA from HN001
The phosphate concentration of each 5 mL HIC fraction was determined. The phosphate concentrations (without acid hydrolysis) of the first 90 fractions from the preliminary HIC run of HN001 LTA are shown in the chromatogram in green, along with the N-propanol solvent gradient indicated in blue. Phosphate concentrations are shown as mean values of duplicate phosphomolybdate assays for each fraction.

Fractions 3 - 24 in the wash segment of the FPLC program (after the UV absorbing peak and before the expected LTA-phosphate peak) contained levels of phosphate well above the baseline, when compared with fractions 55 - 90. As these fractions contained phosphate but did not absorb at UV wavelengths, this material may have contained LTA that did not bind to the column due to overloading.

3.3.2.2 Immune Response to LTA from Preliminary Purification

Many early studies on immune responses to LTA were carried out using LTA preparations that may have been contaminated with the gram negative endotoxin lipopolysaccharide (LPS), a potent immune stimulator. To eliminate immune responses due to contaminating LPS, LTA samples were tested for the presence of LPS (section 3.2). Only those containing less than 0.1 EU/mL of LPS were used in the immune cell assays.

All fractions from the HIC run of LTA from HN001 shown in Figure 15 were analysed to see if they induced the production of TNF cytokine in an immune cell assay. An

equal volume of each fraction was incubated separately with human peripheral blood mononuclear cells (PBMC) for 24 hours. The cell culture supernatants were then analysed by cytometric bead array (CBA) to determine the concentration of TNF present. The results of the assay are shown in Figure 16. High levels of TNF secretion were observed in a number of fractions, including many from the phosphate peak expected to contain LTA (fractions 25 - 50). The high levels of TNF-inducing activity in the wash fractions indicate that the column was most probably overloaded and that a considerable amount of LTA had not bound to the column. A significant decrease in TNF secretion corresponding to the highest phosphate concentrations in the predicted LTA peak (fractions 30 - 45) was also observed.

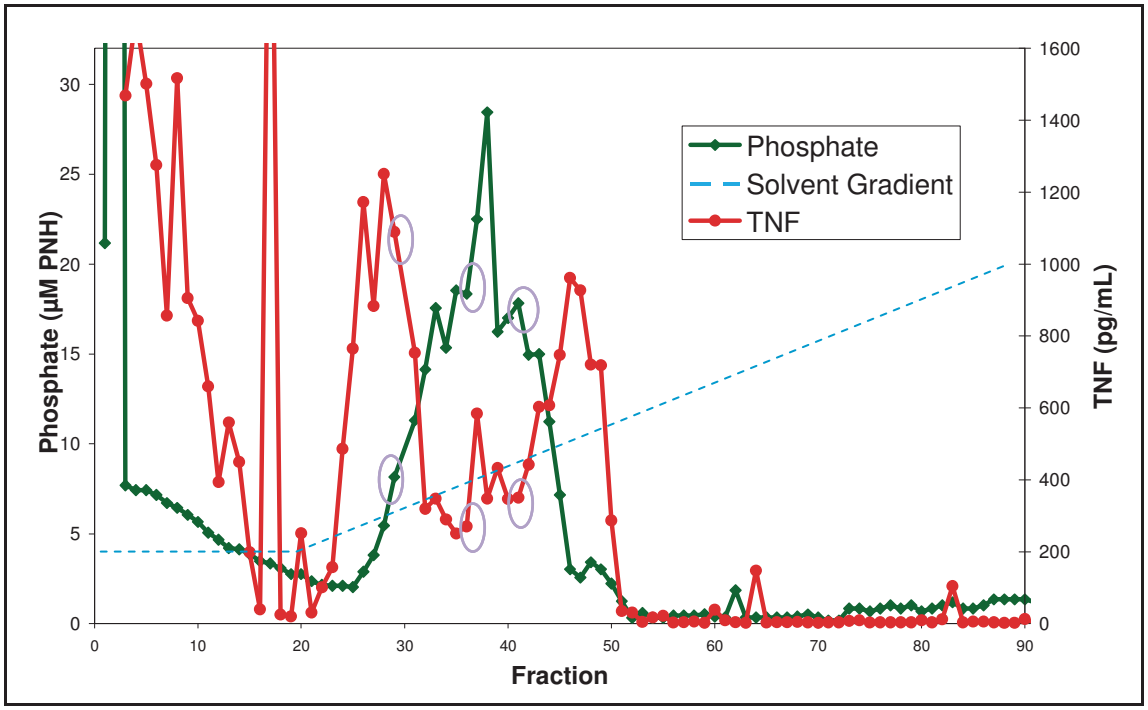

Figure 16: TNF Cytokine Induction by HIC Fractions from HN001
The concentrations of TNF (red line) secreted by PBMCs incubated for 24 hrs with fractions collected during HIC purification of LTA from HN001 are overlaid on the chromatogram from the purification (green line, phosphate; dashed line, N-propanol gradient). TNF values are mean values of duplicate PBMC assays. Fractions 29, 35 and 40 are circled in both the TNF and phosphate traces.

To investigate the affect of high concentrations of LTA on induction of cytokines, a three-fold dilution series was made for fractions 29, 35 and 40 from the LTA-phosphate peak, circled in Figure 16, and the diluted samples analysed by PBMC/CBA assay (Figure 17). Fraction 29 is near the point in the chromatogram where TNF secretion begins to decrease, indicating some reduction of the ability of the sample to induce a TNF response at high concentration. When this fraction is diluted 3-fold an increase in

TNF response was observed, indicating that the high concentrations of LTA might be attenuating its effect on cytokine production. This effect is more pronounced for fraction 35, which induced the secretion of even higher TNF concentrations when diluted three- and nine-fold. A twenty-seven fold dilution did not result in decreased TNF when compared to the nine-fold dilution. The results of dilution of fraction 40 were inconclusive.

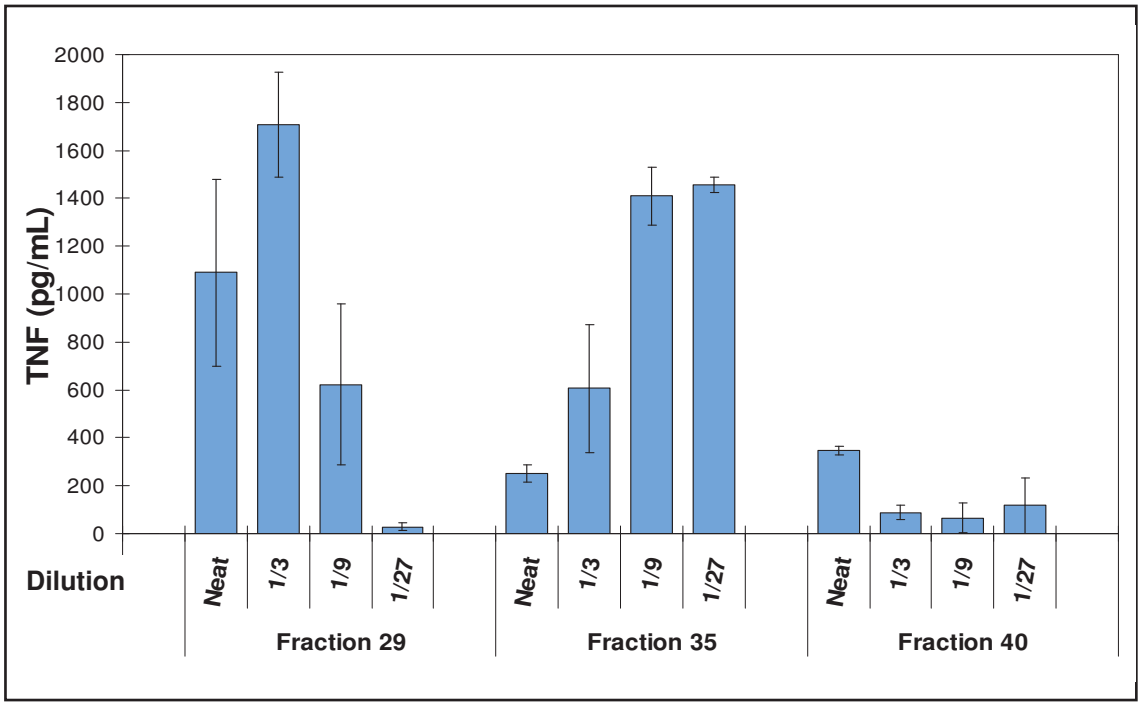

Figure 17: TNF-Induction by Diluted LTA-Phosphate Peak Fractions
A dilution series of three LTA-PO_4 peak fractions from HN001 was analysed by PBMC-CBA assay. The fractions tested: 29, 35 and 40 represent fractions from the HIC purification shown in Figure 16. TNF values are mean values +/- SEM obtained from duplicate PBMC assays.

Although the unexpected TNF response profile could have been due to a difference in cytokine-inducing activity of different populations across the LTA-phosphate peak, it seemed unlikely as such a pattern had not previously been observed in similar studies. The reduction in response could therefore be due to structural or conformational changes in the LTA. These observations, and the known propensity of LTA to form micelles at high concentrations due to its amphipathic structure (Courtney *et al.* 1986; Wicken *et al.* 1986), suggest that the immune responses to micellar LTA and the free molecule are different. The formation of LTA micelles was investigated for its potential role in the immune response.

3.3.3 Determination of Critical Micelle Concentration

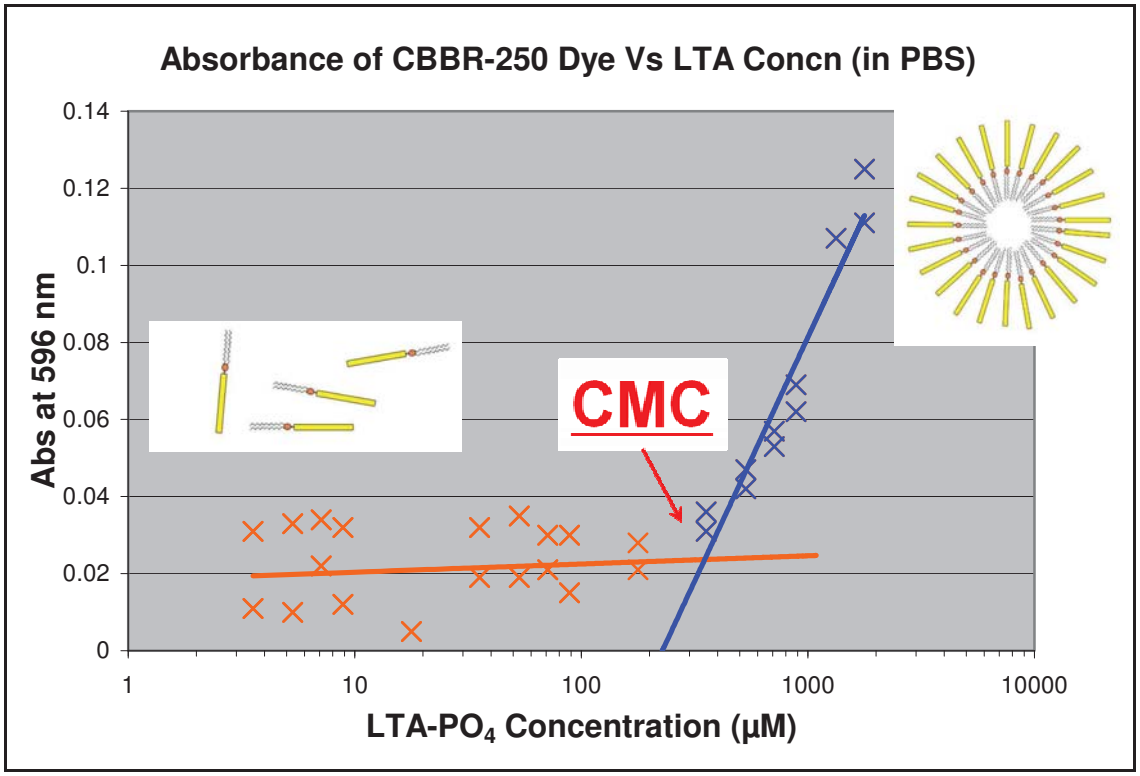

Figure 18: Determination of Critical Micelle Concentration of LTA from HN001 Using CBBR-250 Results of a typical CMC determination experiment, carried out in PBS buffer. The increase in absorbance at 596 nm of CBBR-250 dye in solution with LTA indicates the formation of micelles. The point at which the absorbance begins to rapidly increase is defined as the CMC. At concentrations below the CMC the LTA is monomeric, and above the CMC the LTA is incorporated into micelles, as indicated by the schematic diagrams in the figure.

Lipoteichoic acid is an amphipathic molecule, and has the potential to form micelles at sufficiently high concentrations. The micellar form of LTA may not interact with PRRs on immune cells in the same way as monomeric LTA, due to the differences in size and shape. The clustering of MAMP-PRR complexes on immune cells may also be affected by the presentation of LTA as micelles or monomers. After binding of LTA to a PRR such as TLR2, the interaction of the LTA-PRR complex with other receptors on immune cells is likely to be altered. The potential for recognition of the lipid part of LTA would certainly be diminished by micelle formation, as the location of the hydrophobic moiety would be buried within the micelles, making it unavailable for interaction with PRRs. For these reasons, LTA micelles might induce a different immune response than would monomeric LTA. It is therefore important to know the concentration at which micelles begin to form, i.e. the critical micelle concentration (CMC) for each LTA. The CMCs of the purified LTA from all three strains in this study

were determined using Coomassie Brilliant Blue R-250 dye (CBBR-250). The green form of this dye is incorporated preferentially into micelles, causing an equilibrium driven increase in the concentration of the blue form, which absorbs at 596 nm. The absorbance of a fixed concentration of CBBR-250 was measured in the presence of a range of LTA concentrations, and the CMC was determined from the point at which the colour begins to change, as shown in Figure 18.

The CMC of LTA from HN001 was initially determined in PBS (Figure 18). Note that LTA purified using the optimised chromatography protocol (as shown in Figure 19) was used to determine the CMC, not the LTA of uncertain purity shown in Figure 15. Experiments for all strains with the LTA in RPMI cell-culture medium demonstrated that the CMCs were lower at room temperature than at 37 ºC (the immune cell assay is incubated at 37 ºC, section 2.2.8.2). For this reason, the CMCs were determined at room temperature, the lowest temperature expected to be encountered during the set-up of the immune assay. As each of the samples contained a heterogeneous mixture of LTAs which may have different individual CMCs, the CMC for each sample was determined to be the lowest concentration at which micelles appeared to form. The critical micelle concentrations determined in RPMI media, in terms of phosphate concentration, are listed in Table 3. The values obtained are in a similar range to those for *S. aureus* LTA found in the literature of 28 – 60 µg/mL in PBS at ambient temperature (Courtney *et al.* 1986). It seems therefore unlikely that the specific immune responses induced by these bacteria are due to the CMCs of their respective LTAs.

	CMC in RPMI Media	
	CMC (µM LTA-PO_4)	**CMC (µg/mL LTA)**
(at room temp.)		
HN001	266	64
DltD- Mutant	221	49
IM126	158	31

Table 3: Critical Micelle Concentrations of LTA Purified from *L. rhamnosus* Strains
The CMC of LTA purified from each of the strains HN001, the DltD- mutant and IM126 was determined by measuring the absorbance of Coomassie Brilliant Blue R-250 dye in solutions containing LTA at a range of concentrations. The CMCs shown were determined in RPMI media, the closest approximation to the conditions of the PBMC assay. Each concentration is shown both in units of µM LTA-PO_4, as referred to predominantly in this thesis, and also in µg/mL, for comparison with literature values. The total combined LTA was used for each strain and the CMC represents the lowest concentration where micelle formation was observed.

Using the information in Table 3, the concentrations of the fractions 29 – 45 in Figure 16 were determined to be above the CMC for LTA from HN001. The formation of micelles in these samples is the probable explanation for the lower than expected TNF responses to these fractions.

3.3.3.1 Optimised HIC of LTA

Based on the results of the preliminary HIC purification of LTA, the HIC separation protocol was optimised to provide high purity LTA. To eliminate the problem of column overloading, trials were undertaken to establish the optimum load for HN001 crude LTA, which was determined to be 15 µmoles of total phosphate (PNH). The FPLC elution program was modified to include a longer wash step of seven column volumes, (compared to five), to ensure separation of the LTA-phosphate peak from the unbound material. Fractions of 5 mL were collected and the phosphate concentration of each was measured as described. The results are shown in Figure 19. Monitoring of the UV signal again revealed a large peak in the early unbound fractions that correlated with a large unbound phosphate peak. It also showed that the LTA-phosphate peak was free of detectable contaminating proteins and nucleic acids (data not shown). The LTA-phosphate peak is now well resolved from the unbound phosphate-containing material with no evidence of column overloading, and consists of the main peak (fractions 43 - 52, Figure 19), and a small shoulder (fractions 53 – 57, Figure 19). Immune cell assays of the fractions shown in Figure 19 revealed that TNF inducing activity was now greatest for fractions in the LTA-phosphate peak, and that there was little TNF induced by the flow-through fractions (section 3.4.1). This HIC profile is typical for LTA purified from gram positive bacteria reported in a number of published studies. The apex of the main peak was sometimes observed to be split, as in Figure 19, which may indicate the presence of two closely overlapping peaks. Although this split peak was not always apparent, its shape was taken into consideration later when pooling fractions for parallel NMR and immune response experiments (section 3.3.4.1).

Trials were carried out to determine the optimum loading amount for HIC of LTA from the DltD- mutant and IM126 strains. For IM126, as with HN001, loading was 15 µmoles of phosphate (PNH), but for the DltD- mutant, 90 µmoles (PNH) of DltD-

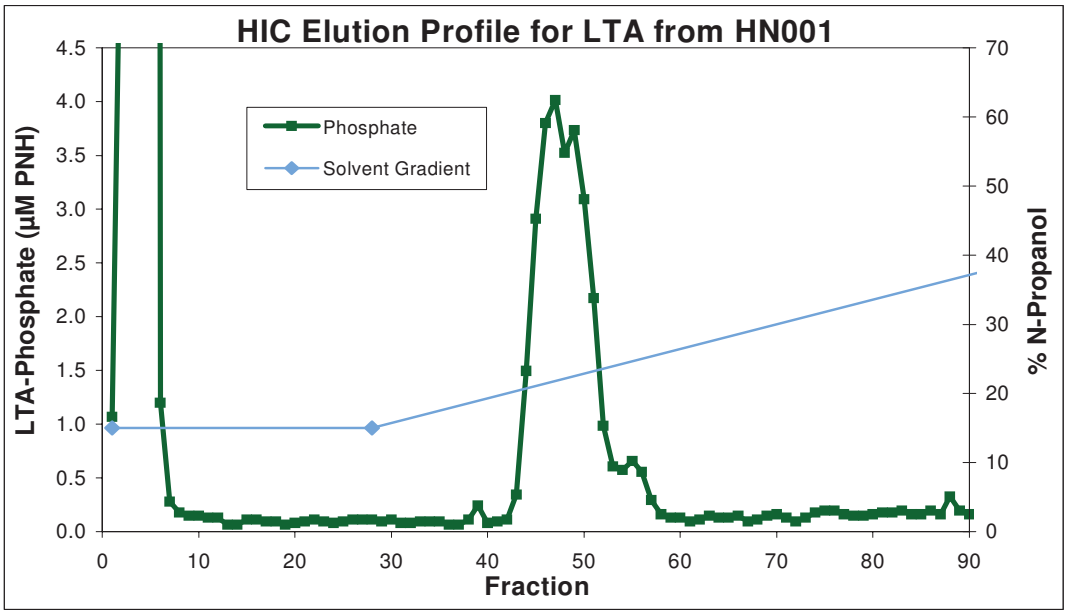

Figure 19: Elution Profile of LTA from HN001 Purified by Optimised HIC
Butanol-extracted material was applied to an octyl sepharose HIC column in 15 % N-propanol in 0.1 M ammonium acetate buffer at pH 4.7. Unbound material was removed by washing with the loading buffer. The LTA peak was eluted with a solvent gradient of N-propanol in buffer, and 5 mL fractions were collected throughout. The phosphate concentration of each fraction is shown in the chromatogram along with the solvent gradient.

crude LTA in terms of phosphate was loaded, as the starting material from this mutant strain appeared to contain much less LTA-phosphate per total phosphate loaded. The elution profiles of LTA from IM126 and the DltD- mutant were similar to that of HN001 seen in Figure 19, when the same FPLC protocol was applied.

3.3.4 Purification of LTA for Parallel NMR and Immune Cell Assays

Once the correlation between the LTA-phosphate peak and cytokine induction was established, the HIC protocol was modified slightly to shorten the run time (Table 1). Initial NMR trials revealed that high concentrations of LTA were required in order to achieve an acceptable signal to noise ratio (S/N). Several HIC runs were performed for each strain to generate high purity LTA in sufficient amounts to be analysed by NMR. The amounts of crude LTA (the aqueous phase after butanol extraction of disrupted cells) processed using HIC and the yield of LTA are summarised in Table 4. The LTA constituted a considerably smaller proportion (0.4 %) of the crude butanol-extracted material from the DltD- mutant strain than it did for HN001 (3.1 %) and IM126 (1.9 %). Therefore more starting material was processed from the DltD- mutant to generate sufficient purified LTA for NMR experiments, compared to the other two strains.

	HN001	DltD-Mutant	IM126
LTA Yield (mg)			
Pool 1	24	21	17
Pool 2	28	22	26
Pool 3	9	11	7
Total LTA Yield (mg)	61	54	50
Crude LTA Processed (mg)	1 950	13 530	2 650
% LTA/Crude Material	3.1 %	0.4 %	1.9 %

Table 4: Yield of LTA Purified for Parallel PBMC/NMR Experiments
This table lists the dry weight of purified LTA in each of the pooled fractions for each strain, as well as the total amount of LTA purified from each strain, from several HIC runs. The "Crude LTA" dry weights describe the total amount of butanol-extracted starting material processed by HIC for each strain.

3.3.4.1 Fractions Pooled for NMR and PBMC-CBA Analysis

The retention of LTA during HIC purification is dependent on hydrophobicity, therefore fractions of LTA eluting at different concentrations of propanol are likely to be structurally different. With structural difference comes potential for differences in immune response, so the collected fractions containing LTA-phosphate were pooled to give three fractions. The main LTA peak sometimes appeared to be a split peak (section 3.3.3.1) and so was divided into two halves at the apex, and the two pools labelled as Pool 1 and Pool 2. The distinct smaller secondary peak of LTA-phosphate was kept separate as Pool 3. This pooling scheme is illustrated in Figure 20.

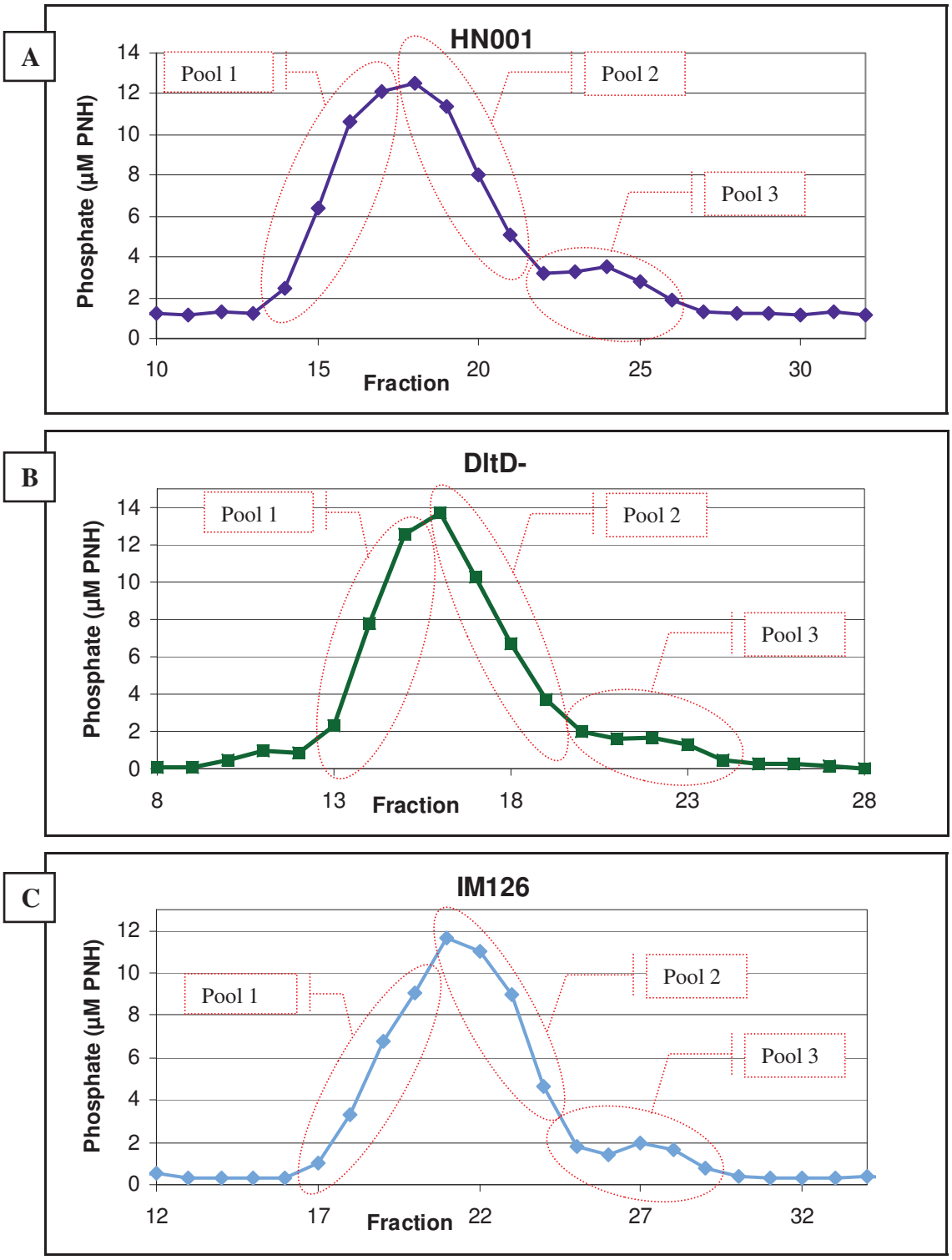

Figure 20: LTA Peak Fractions Selected for Pooling
The chromatograms show LTA-phosphate peak fractions from example HIC runs for the three strains in this study. The fractions that were pooled to create Pool 1, Pool 2 and Pool 3 are indicated by the ellipses. Fractions making up Pool 1, Pool 2 and Pool 3, respectively: (A) HN001 LTA fractions 14 – 17, 18 – 21 and 22 – 26; (B) DltD- LTA fractions 13 – 15, 16 – 19 and 20 – 24; (C) IM126 LTA fractions 17 – 20, 21 – 24 and 25 – 29. The other HIC runs to be analysed by NMR and PBMC-CBA were pooled according to this pattern for each strain.

3.4 Immune Response to LTA

3.4.1 TNF Response to Fractions from Optimised HIC

After optimisation of the LTA purification (section 3.3.3.1), equal volumes of each HIC fraction were analysed by PBMC assay as before, and the concentrations of secreted TNF were measured. To ensure fair comparison between fractions, the amount of sample added to each of the PBMC assays was calculated to ensure that the concentrations of all LTA-phosphate peak fractions were below their CMC. Figure 21 shows the TNF concentrations for each fraction overlaid onto the chromatogram. The peak of TNF concentration corresponds to the fractions containing LTA-phosphate (fractions 43 - 55), suggesting that LTA from HN001 induces a cytokine response in

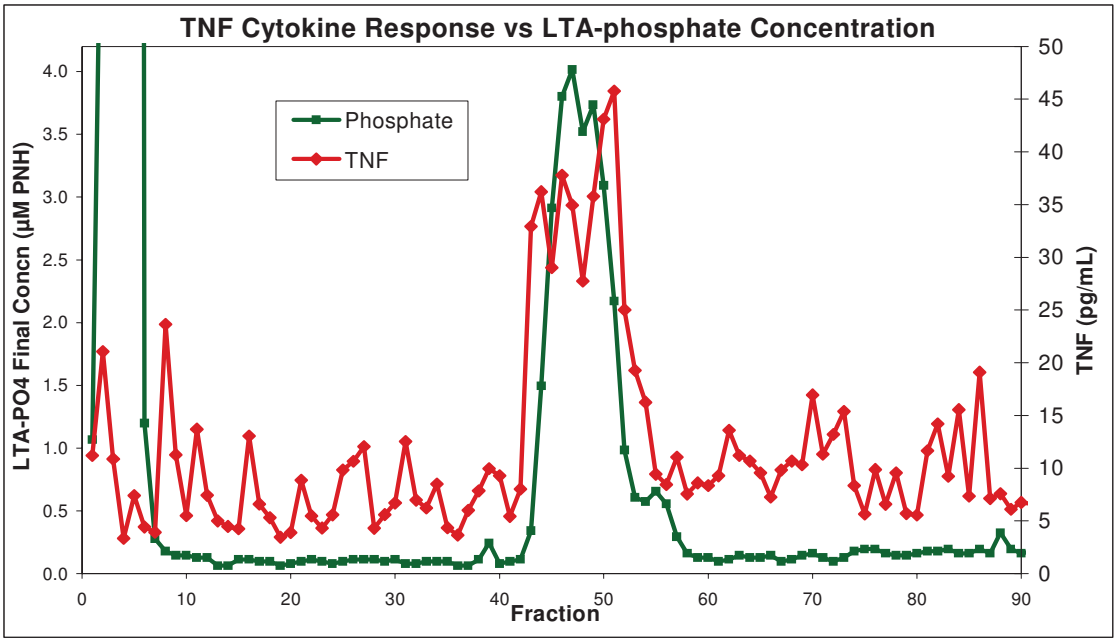

Figure 21: TNF Cytokine Response of Human PBMCs to LTA from HN001
Each fraction from the LTA purification was incubated with human PBMCs for 24 hrs, and the amount of TNF cytokine in the cell culture supernatant was measured. The level of TNF induced is overlaid on the chromatogram, and the peak of TNF is co-located with the LTA-phosphate peak. TNF values are means of duplicate PBMC assays. Phosphate values are the means of duplicate assays, and represent phosphate values from non-hydrolysed LTA.

PBMCs. It appears that LTA has not formed micelles at the concentrations used in this experiment, as the greatest amount of TNF induced coincides with the highest concentrations of LTA-phosphate. There is much less TNF-inducing activity in the wash fractions, which suggests that in contrast to earlier experiments, the column was not overloaded. Unfortunately, the amounts of TNF secreted were relatively low when

compared with the experiment in Figure 16. It has been reported that PBMC from different donors vary in their ability to respond to particular stimuli such as LTA, which may explain the low TNF values in Figure 21.

3.4.2 Comparison of Immune Responses to Three *Lactobacillus rhamnosus* Strains

In order to directly compare purified LTAs from three different strains, preparations of purified LTA and of intact bacteria from HN001, the DltD- mutant and IM126 (confirmed to be free of detectable LPS contamination) were tested using the PBMC assay simultaneously, using identical conditions for all strains. Equivalent amounts of LTA in terms of phosphate (after acid hydrolysis) from the three strains HN001, the DltD- mutant and IM126 were incubated separately with PBMCs from a single donor. LTA from each strain was pooled into three samples: the main LTA-PO_4 peak was divided into two separate pools, Pool 1 and Pool 2, while the second smaller LTA-PO_4 peak was kept separate as Pool 3. These three pools were also combined in the same ratio as they were recovered from HIC. An experiment where aliquots of the three pools were combined in a 1:1:1 equimolar mixture in terms of phosphate gave similar cytokine responses to the aforementioned combined LTA, so the results are not shown here. A series of two-fold dilutions of each LTA sample were made to cover a concentration range from 400 to 12.5 μM phosphate. As the CMC of each LTA is between 150 and 300 μM phosphate, the highest concentrations of LTA in the PBMC assays may contain micellar LTA, while the 100, 50, 25 and 12.5 μM phosphate samples are expected to contain monomeric LTA. Levels of secretion of several cytokines were measured by CBA assay. TNF, IL-1β, IL-8 and IL-10 were all induced in PBMC by both LTA and intact bacteria from HN001, the DltD- mutant and IM126. Cytokines IL-4, IL-17, IL-12 and IFN-γ were also analysed, but were not secreted in detectable quantities from the PBMC from this donor by either intact bacteria or by LTA from the three target strains. All of the cytokines that were tested for except IL-12 were secreted in detectable amounts by PBMC exposed to the positive control, a PMA/ionomycin mixture (results not shown). IL-4 and IL-17 are produced by Th2 and Th17 cells, respectively, and these responses are not typically induced by LAB. It was somewhat surprising that IL-12 and IFN-γ were not detected in the PBMCs exposed to the whole cells, as HN001 has been shown to induce production of these cytokines in

PBMC (M. Collett, personal communication). Expression of some cytokines is known to vary considerably between PBMC donors, which may explain why IL-12 and IFN-γ were not detected for the PBMC exposed to bacteria or LTA. The samples must be tested using several different donors to investigate this further.

Cytokine responses to the intact bacteria for each strain are shown in Figure 22. All three strains induced measurable amounts of TNF, IL-1β, IL-10 (Figure 22A) and IL-8 (Figure 22B). The DltD- mutant induced substantially more of all of these cytokines, but particularly TNF and IL-10, compared to WT HN001. These results clearly show that the inactivation of the *dlt*D gene in HN001 has had a considerable impact on the magnitude and the relative cytokine profile of the immune response by PBMCs to the intact bacteria. This alone does not prove that altered LTA is responsible for the changed response, making it necessary to investigate immune responses to the purified LTA from these strains. IM126 bacteria induce slightly higher levels of TNF, IL-1β and IL-8 compared to HN001 bacteria, and approximately three-fold higher amounts of the anti-inflammatory cytokine IL-10.

The TNF responses to all LTA samples in this experiment are shown in Figure 23. There was some TNF response to every sample for at least one concentration of LTA for all three strains. Generally, LTA from the DltD- mutant strain induced high levels of TNF secretion at much lower concentrations of LTA-PO_4 than HN001, suggesting that LTA from the mutant is a more potent stimulator of cytokine release than HN001 LTA. The TNF response to LTA from IM126 was lower than the response for the other two strains. The maximum TNF response was similar for both HN001 and DltD- mutant LTAs, even though DltD- LTA was much more potent at lower LTA-PO_4 concentrations. This effect may be due to limiting cellular factor(s) involved in the LTA-specific TNF response by PBMCs, and the results suggest that at higher concentrations of LTA, the assay is saturated. It must also be remembered that it is not sensible to compare the different LTAs above their CMC, where the proportion of micellar LTA may vary between strains. The exception to this is the combined LTA from the DltD- mutant, which at the 50 and 100 μM concentration induced a slightly higher TNF concentration than any of the separate pools for this strain (Figure 24). This was less apparent for IL-1β secretion (IL-8 and IL-10 not shown). The increase in TNF activity for the combined LTA is small, and was not observed for HN001 or IM126, but

indicates that there may be some synergy between the fractions for this strain for the pathway responsible for TNF secretion.

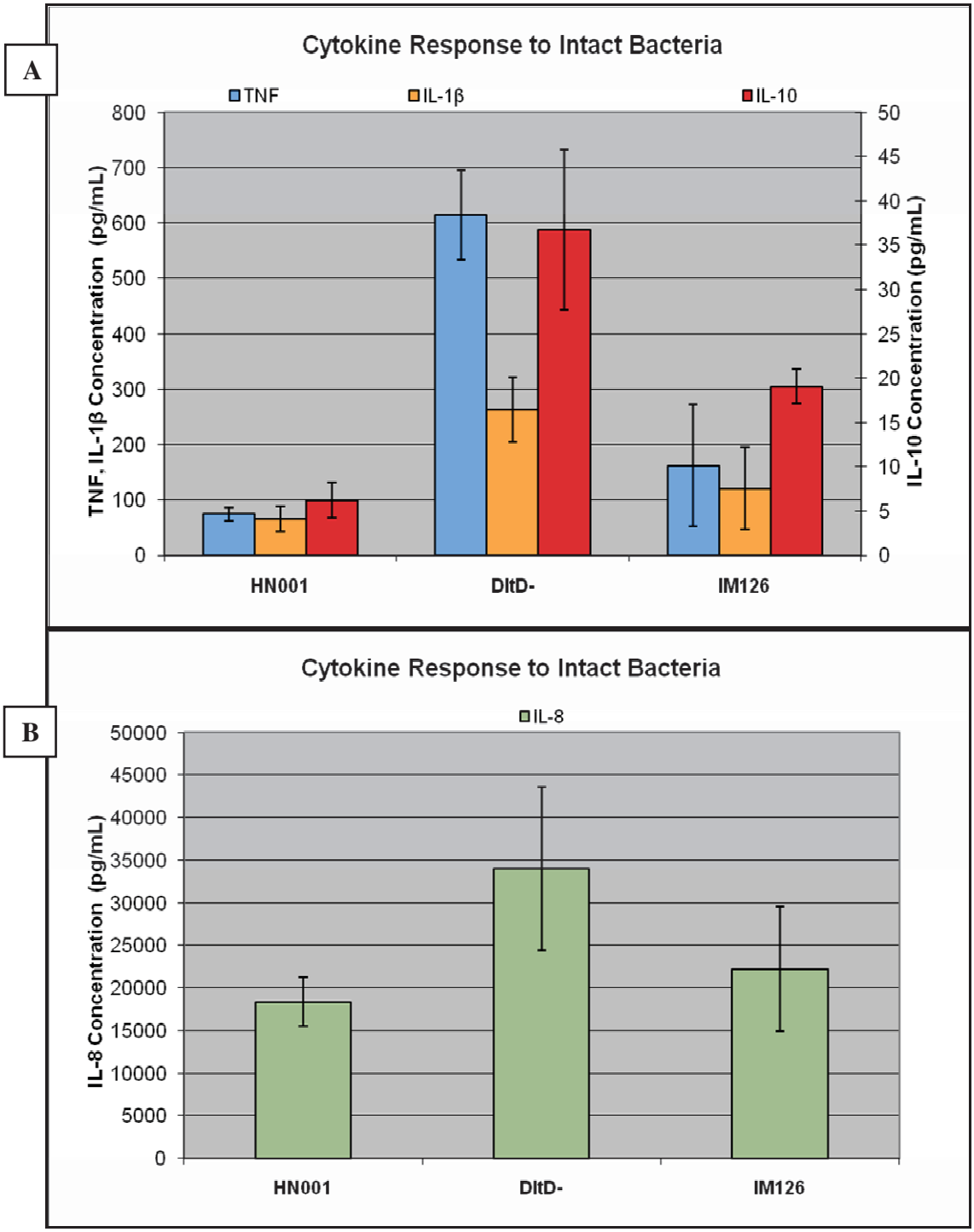

Figure 22: Immune Response to Intact Bacteria
PBMCs were exposed to preparations of each of the three strains HN001, the DltD- mutant and IM126, and secreted cytokines were measured. (A) TNF, IL-1β (left hand y-axis) and IL-10 (right-hand y-axis). (B) IL-8. Bacteria were at a final concentration of 10^6 cfu/mL. Cytokine concentrations are mean values +/- SEM from triplicate PBMC assays.

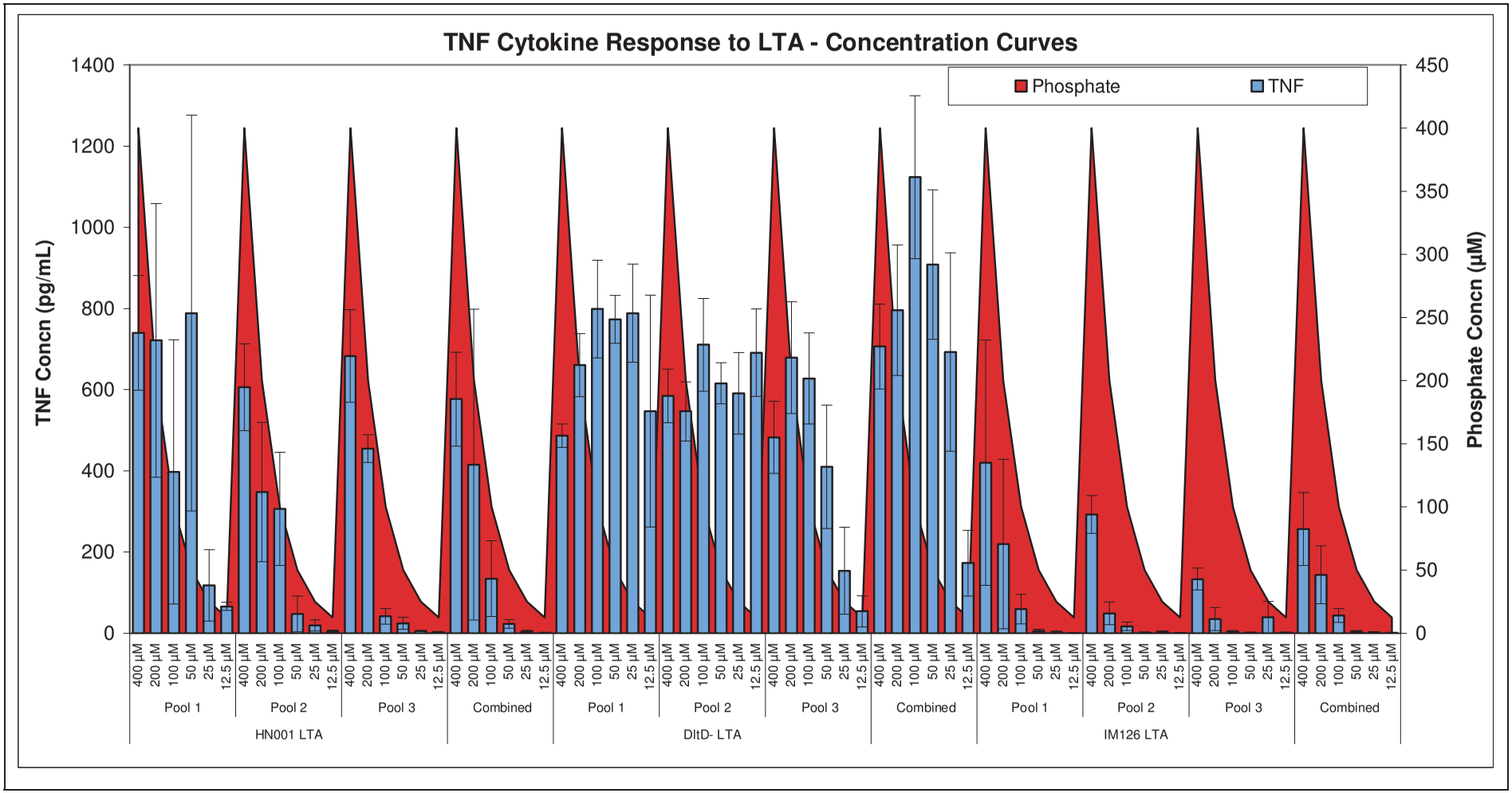

Figure 23: TNF Cytokine Response to LTA from HN001, the DltD- Mutant and IM126
Each of three pooled samples and a combined sample for each strain were added at final concentrations of 400 to 12.5 µM PO_4 to PBMCs and incubated for 24hrs. Pools 1 - 3 are separate pools from HIC purification for each strain. Combined is total LTA combined in the original proportions of the three pools from HIC. The LTA-PO_4 concentration is indicated by the red shaded area. TNF concentrations are mean values +/- SEM from triplicate PBMC assays.

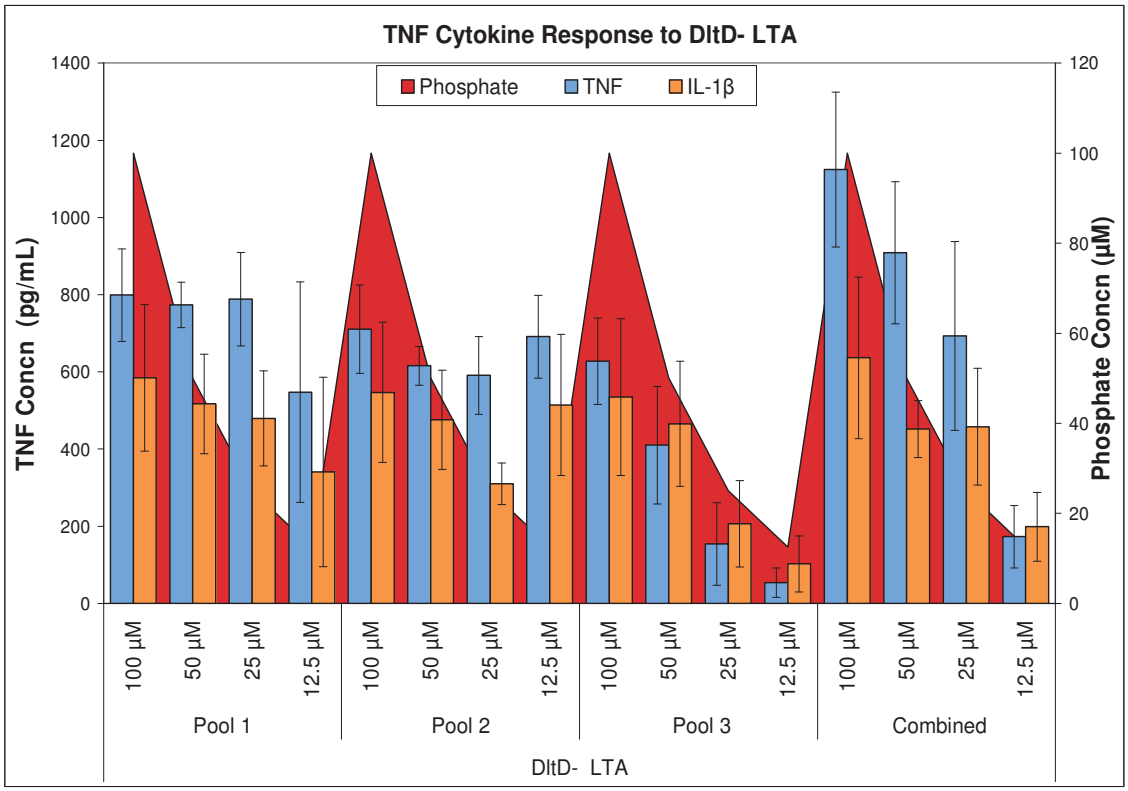

Figure 24: TNF and IL-1β Cytokine Response to DltD- LTA
TNF and IL-1β secretion by PBMCs in response to each of three pooled samples and a combined sample for DltD- LTA at final concentrations of 100 to 12.5 µM PO_4. The LTA-PO_4 concentration is indicated by the red shaded area. Cytokine concentrations are mean values +/- SEM from triplicate PBMC assays

There was measurable TNF-induction by all of the LTA samples at 400 µM PO_4, even though this concentration was determined to be above the CMC of LTA for all three strains. It may be that at this concentration some, but not all of the LTA forms micelles. Testing at higher concentrations of LTA may have revealed a more noticeable impact on cytokine induction, but the effect of micelles had previously been shown to attenuate this induction. The micelle affect can be observed in the DltD- LTA samples in Figure 23, where the 400 µM sample for Pool 1 induced less TNF than the 200 and the 100 µM samples. To simplify the comparison, only the monomeric LTA for each strain was examined further; that is, only samples containing LTA at concentrations of 100 µM or lower (and not the 200 and 400 µM samples), were compared.

3.4.3 Cytokine Response from Purified LTA versus Whole Bacteria

Of the cytokines analysed in this experiment, TNF, IL-1β, IL-8 and IL-10 were secreted in measurable amounts. The responses to these cytokines are each shown in Figure 25 for the 25, 50 and 100 µM combined total LTA samples (the three pools combined in a

ratio related to the LTA-PO_4 peak from HIC), as well as the responses to whole HN001, DltD- mutant and IM126 bacterial cells, and as a positive control lipopolysaccharide from *E. coli* (LPS) at 1 µg/mL. IL-8 was secreted at a rate approximately forty times that for either TNF or IL-1β, while IL-10 levels were an order of magnitude lower than those for either TNF or IL-1β. While LTA from all three strains exhibited cytokine inducing activity, none were as potent as LPS in this assay, as it required many times more LTA material to produce a similar or lesser response in PBMCs compared to LPS.

Figure 25 clearly shows that LTA from the DltD- mutant is a highly potent stimulator of TNF, IL-8, IL-1β and IL-10, (Figures 25 A, B, C and D, respectively), at concentrations of 25, 50 and 100 µM LTA-PO_4, when compared to LTA from HN001 or IM126 at the same concentrations. This suggests that at least part of the increased immune response induced by the DltD- mutant bacteria in comparison to that induced by HN001 is due to the altered LTA. The LTA from IM126 induced lower concentrations of the cytokines than LTA from HN001 (Figure 25). This is in contrast to the slightly greater amounts of TNF, IL-1β, IL-8 and in particular IL-10 induced by IM126 bacteria in comparison to HN001 bacteria. This indicates that for the cytokines measured, the LTA in HN001 has a greater influence on the immune response to the whole bacteria than does LTA from IM126. In the case of IM126, it is likely that factors other than LTA contribute more to the cytokine response than they do in HN001.

The pattern of cytokine secretion exhibited, i.e., the relative amounts of cytokines secreted by PBMCs after incubation with purified HN001 LTA was similar to the cytokine profile for HN001 entire cells. The pro-inflammatory cytokines TNF, IL-1β and IL-8 were induced in a similar ratio in PBMC by both LTA from HN001, and the parent cells, as shown in Figure 26A. This similarity supports the hypothesis that the LTA is responsible for some part of the immune response of PBMCs to HN001. However, the amount of IL-10 induced relative to that of TNF and IL-1β is higher for the intact cells than for the LTA from this strain (Figure 27A), indicating that LTA is not solely responsible for inducing this anti-inflammatory cytokine in HN001.

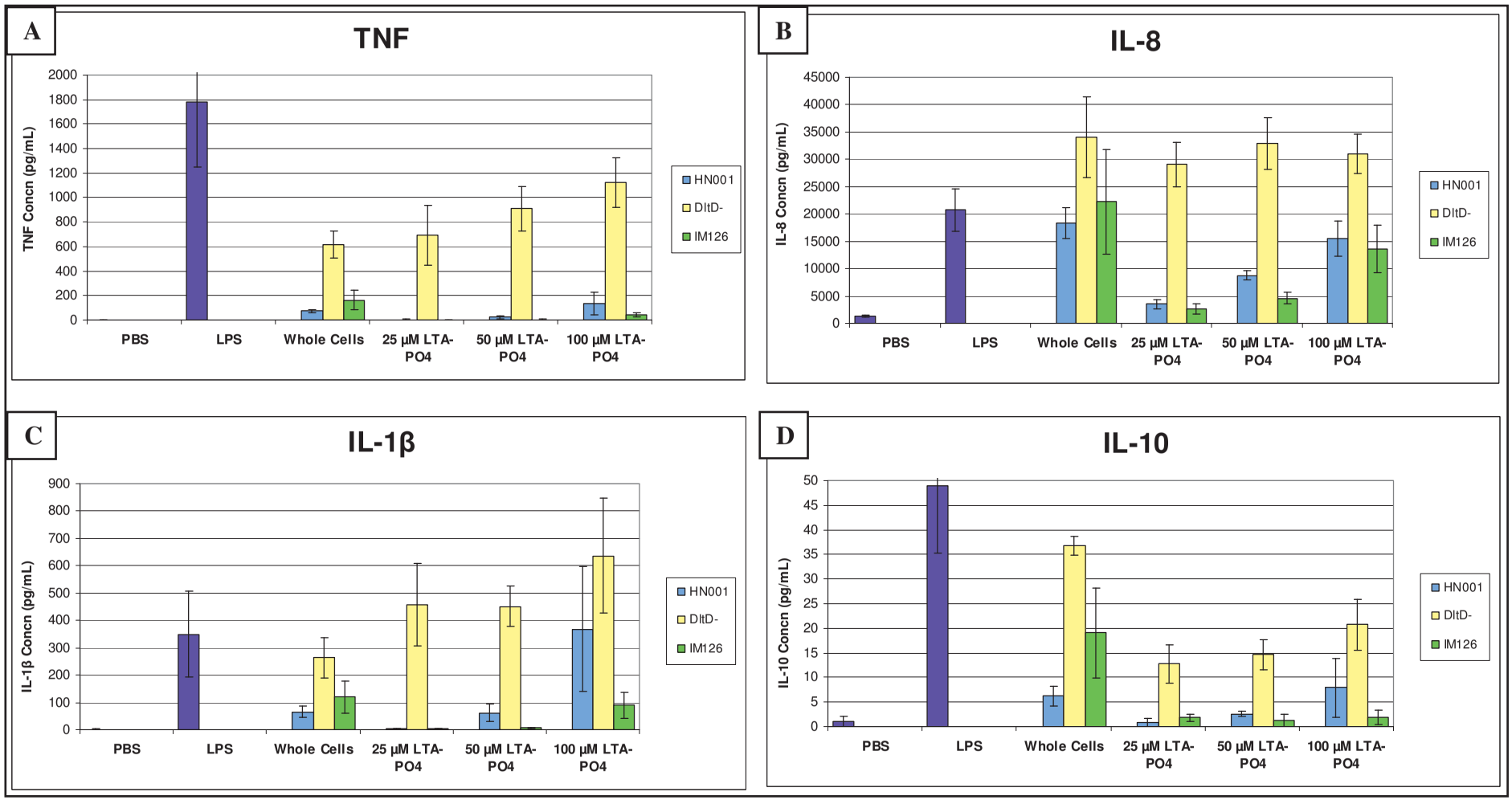

Figure 25: TNF, IL-1β, IL-8 and IL-10 Induction by LTA

PBS as a control, LPS at 1 µg/mL, whole bacteria at 10^6 cfu/mL and LTA at final concentrations of 25, 50 and 100 µM LTA-PO_4 from the strains HN001 (~ 6, 12, 24 µg/mL), the DltD- mutant (~ 5.5, 11, 22 µg/mL) or IM126 (~ 5, 10, 20 µg/mL) were incubated with PBMCs for 24 hours. Legend: (A) TNF, (B) IL-8, (C) IL-1β and (D) IL-10. Mean values +/- SEM of three PBMC replicates for each sample are shown for each cytokine.

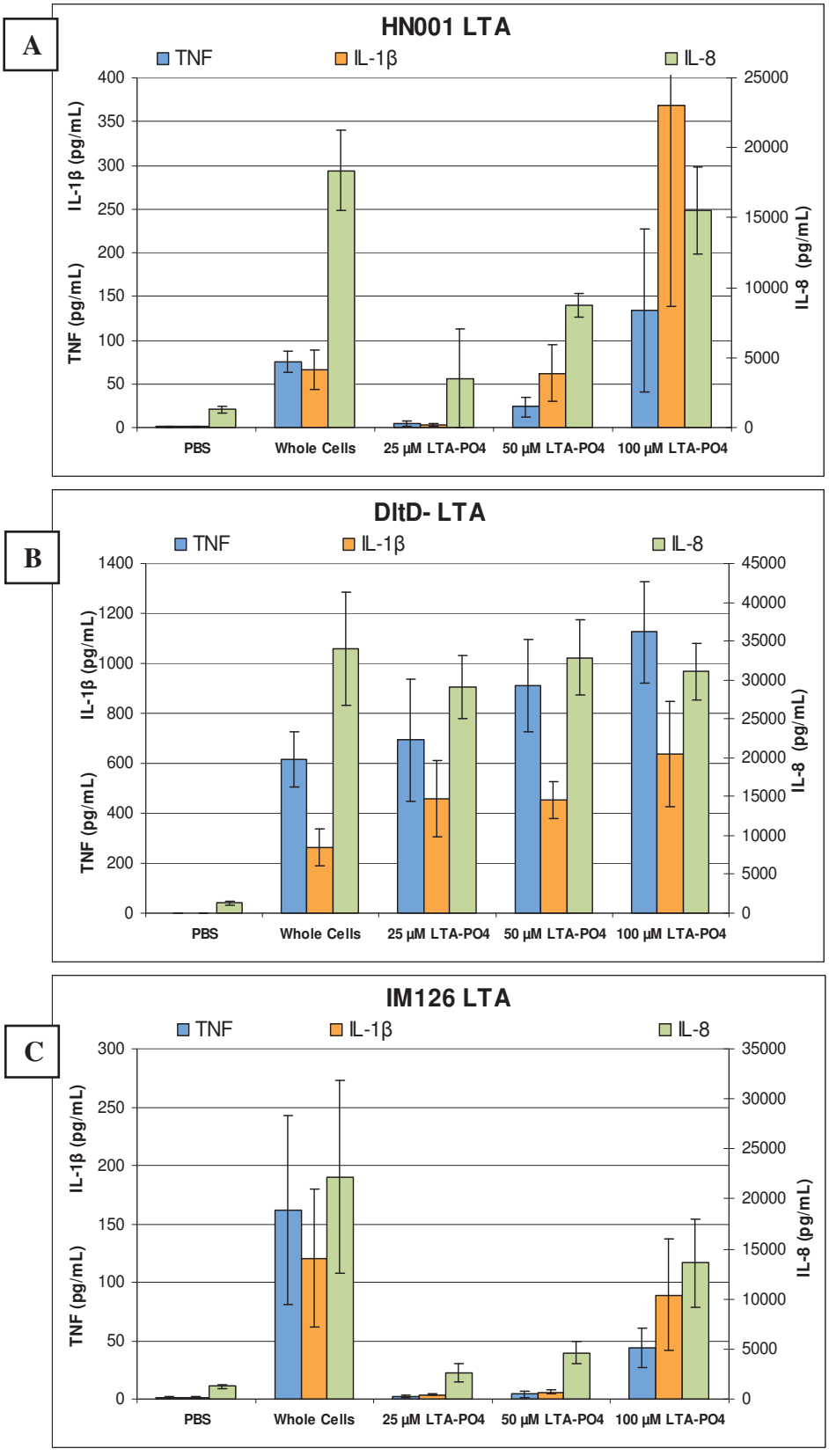

Figure 26: Pro-inflammatory Cytokine Induction by LTA versus Entire Bacterial Cells
PBS as a control, whole bacteria at 10^6 cfu/mL and LTA at final concentrations of 25, 50 and 100 µM LTA-PO_4 from the *L. rhamnosus* strains (A) HN001, (B) the DltD- mutant or (C) IM126 were incubated with PBMCs for 24 hours. The mean values +/- SEM of three PBMC replicates for each sample are shown for the cytokines secreted TNF, IL-1β (left-hand scale) and IL-8 (right-hand scale).

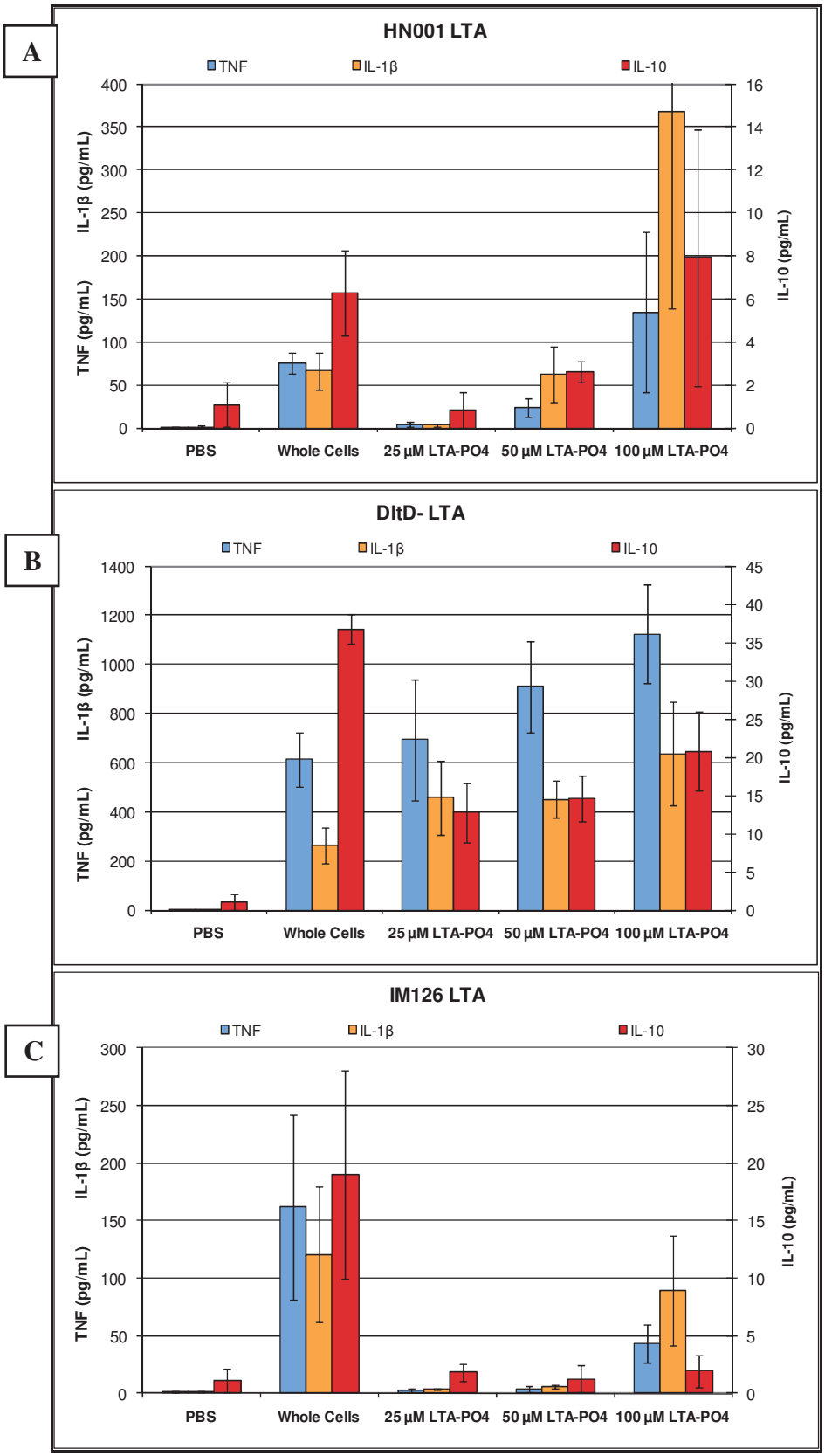

Figure 27: Anti-inflammatory Compared with Pro-inflammatory Cytokine Induction by LTA versus Entire Bacterial Cells

PBS as a control, whole bacteria at 10^6 cfu/mL and LTA at final concentrations of 25, 50 and 100 µM LTA-PO_4 from the *L. rhamnosus* strains (A) HN001, (B) the DltD- mutant or (C) IM126 were incubated with PBMCs for 24 hours. The mean values +/- SEM of three PBMC replicates for each sample are shown for the cytokines secreted TNF, IL-1β (left-hand scale) and IL-10 (right-hand scale).

The immune response to the altered LTA from the DltD- mutant corresponds to the immune response for DltD- mutant bacteria, both in relative magnitude and the ratios of the pro-inflammatory cytokines induced. Very similar relative amounts of TNF, IL-1β and IL-8 were induced by both DltD- mutant cells and the LTA purified from this strain (Figure 26B). The increase in the amount of TNF induction relative to IL-1β by the DltD- mutant bacteria compared to that induced by the HN001 bacteria is also reflected in the relative amounts of TNF and IL-1β induced by the purified LTA from both strains, indicating that the LTA is probably responsible for this. The ratio of secreted TNF to IL-10 for PBMCs exposed to purified DltD- LTA is different to that of PBMCs incubated with whole DltD- mutant cells (Figure 27B). As was the case with HN001, the DltD- mutant bacterial cells induced higher levels of the anti-inflammatory IL-10 relative to TNF and IL-1β than can be explained by the LTA alone, again, suggesting that part of this anti-inflammatory ability may be due to molecules other than LTA. It may be that LTAs from HN001 and the DltD- mutant are able to potentiate the IL-10 response in PBMCs, but play a lesser role in the *triggering* of the IL-10 response than they do for TNF, IL-1β and IL-8. A similar observation has been made for *Streptococcus gordonii* PM14, which induces the production of both IL-12 and IL-10 from mouse DCs; however, LTA isolated from this strain induced neither cytokine. After inactivation of the *dlt*A gene, which has the effect of eliminating the D-ala substituents from the LTA, less IL-12 and IL-10 were produced by mouse DCs (Chan *et al.* 2007). This suggested that although the LTA alone could not trigger production of these cytokines, it was involved in the response to whole cells. These observations suggest that the changes in the LTA brought about by the *dlt*D- mutation are responsible for the dramatically different immune responses to the DltD- mutant bacteria observed when compared to those elicited by HN001, but that LTA may not be the sole factor responsible for the cytokine induction by these bacteria.

LTA from IM126 was a weak inducer of TNF, IL-1β and IL-10 cytokines relative to intact IM126 bacteria (Figure 27C). Because of these low concentrations, it is difficult to compare the ratios of cytokines induced by the LTA with those induced by IM126 bacteria. However, the results suggest that the LTA may not be the main contributing factor in the ability of this strain to induce secretion of these cytokines, and that LTA is less involved in the cytokine response for these cells than it is for HN001 cells. The exception is IL-8, which IM126 LTA was able to induce to levels only slightly lower

than LTA from HN001 (Figure 25B). This is further evidence that immune responses to LTA are highly strain specific, and demonstrates that it is possible for some cytokine responses to be more dependent on LTA than others. For IM126, as for all three strains of bacteria used in this study, the amount of IL-10 induced relative to that of TNF and IL-1β was higher for the intact bacteria than for purified LTA (Figure 27C). It is therefore likely that there is some non-LTA factor in the strains of *L. rhamnosus* used in this study that contributes to the IL-10 response in PBMC that is possibly potentiated by LTA on cells. As anti-inflammatory effects such as IL-10 secretion are often desired in probiotics, it would be interesting to know if this trend extended to other strains of *L. rhamnosus*, as this knowledge may help to identify and characterise these other IL-10 inducing factors. If other MAMPs involved in triggering IL-10 responses can be identified and isolated from these strains in the future, then experiments can be carried out to determine the potential for LTA to enhance these responses.

3.4.4 Estimated Amount of LTA per Cell

The amount of LTA per cell was estimated for each of the three strains in this assay. Several HIC purification runs were pooled from a single initial culture, and the dry weight and phosphate concentration of the purified LTA were measured. The concentration of LTA was calculated by dividing the phosphate concentration by the number of GroP units in the molecule, as determined by NMR (Table 11). An estimate for the total yield from the culture was calculated from the proportion of the culture processed in these selected runs. The estimated total yield was divided by the total cfu in the culture to give the yield of LTA per cell (Figure 28). Assumptions were made that the LTA was purified from each strain with 100 % yield, and that the HIC runs pooled were representative for that culture.

The estimated values suggest that the *dlt*D- mutant of HN001 has ~ 2.5 – 4 times the amount of LTA per bacteria than the wild type, and that IM126 has ~ 3 – 3.5 times as much LTA as HN001. The morphology and growth habits of the DltD- mutant cells are very different to those of the HN001 cells. The DltD- mutant cells were found to be on average ~ 2.6 × longer, many with incomplete septae, suggestive of defective cell division. The mutant exhibits slower growth in MRS broth, plateaus at a much lower cell density, and loses viability in spent media faster than, the WT parent (M. Collett,

personal communication). Therefore, the increased quantity of LTA in the mutant may be due either to the altered structure of the LTA, or perhaps as a consequence of the changed characteristics of the cell, or both. In any case, this highlights the importance of analysing the purified LTA from an organism with such a pleiotropic mutation. Literature values for the amount of LTA per cell have been reported for: *L. rhamnosus* GG, 1.25 mg/g of lyophilised cells and 0.8 mg/g for a *dlt*D- mutant (Perea Velez *et al.* 2007); *Streptococcus mutans,* 1.68 mg/g (Josephson *et al.* 1986); and *Staphylococcus aureus,* 89 µmoles of phosphorus per gram of lyophilised cells (Fischer *et al.* 1983). The average dry weight of a single cell of HN001 is ~ 1.1 pg, and that of the DltD- mutant is ~ 1.8 pg (M. Collett, personal communication). Therefore, the values shown in Figure 28 are estimated to correspond in terms of LTA per dry weight of cells to be: for HN001, ~ 3.6 mg/g (~ 15 µmol phosphorous/g); and for the DltD- mutant, ~ 5.5 mg/g (~ 25 µmol phosphorous/g). The amounts of LTA per cell calculated for the strains in this study are higher than those reported for the GG and *S. mutans* strains, but lower than the value for *S. aureus*. However, to make an accurate comparison, a side-by-side purification of LTA from these bacteria using identical protocols would be required. Also, the molar amount of LTA per colony forming unit is perhaps a better measure for comparison than the dry weights.

3.4.5 Amount of LTA per Cell and the Cytokine Response

It is possible that the amount of LTA present in each cell is another factor in the immunomodulating ability of bacteria. Purified LTA from IM126 was shown to be a poor inducer of cytokine secretion from PBMCs when compared to LTA from HN001; however, IM126 cells were estimated to contain more than three times the amount of LTA per cell as HN001 (section 3.4.4). A number of bacteria have been shown to vary the total cellular LTA content in response to environmental changes (section 1.2.2). It may be that the greater amount of LTA in IM126 cells compensates for the weaker cytokine inducing activity of the LTA to give the significant cytokine response induced by IM126 bacteria in PBMC. The DltD- mutant strain was estimated to have ~ 4 times the molar amount of LTA per cell as the wild type HN001, and slightly more than IM126 cells. If the amount of LTA per cell alone were responsible for the LTA-induced immune response of bacteria then it would be logical to expect IM126 to induce the same levels of cytokine secretion from PBMCs seen for the DltD- mutant. This is not

the case, so it appears that the differences in cytokine inducing activity between these three strains is not due solely to the amount of LTA in the cell.

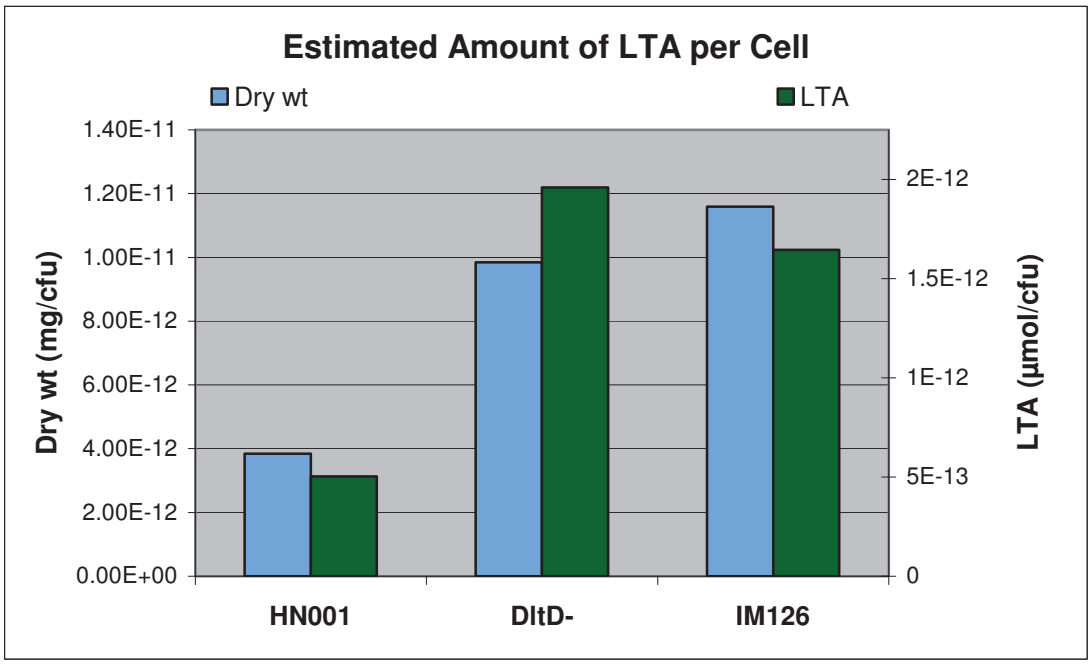

Figure 28: Estimated Amount of LTA per Cell
The amount of LTA per cell for HN001, the DltD- mutant and IM126, as estimated from the yield of LTA from purification, and the number of colony forming units in the bacterial culture used in the preparation. Blue bars: the dry weight of LTA in mg, left-hand axis. Dark green bars: the amount of LTA in µmoles (calculated by dividing the amt of phosphate by the number of GroP per chain, see Table 11), right-hand axis. The concentrations of phosphate per cfu for HN001, the DltD- mutant and IM126 were 3.6×10^{-13}, 9.2×10^{-13} and 9.5×10^{-13} µmol/cfu, respectively. The concentrations of LTA in the graph are calculated by dividing the concentrations of LTA-PO_4 by the estimated PGP chain lengths

Using the data for the amount of LTA per bacterial cell, the estimated final concentrations of cell-associated LTA for each of the whole cell samples: HN001, the DltD- mutant and IM126, were calculated (Table 11). Each of these samples contained intact cells at 1×10^6 cfu/mL, which is equivalent to 1×10^9 cfu/L. The amount of LTA-phosphate per cfu calculated for each strain in section 3.4.4 was divided by 1×10^9 cfu/L to give approximate concentrations of cell-associated LTA in the whole cell samples of 0.016, 0.045 and 0.060 µM phosphate for HN001, the DltD- mutant and IM126, respectively. Using HN001 as an example, the amount of LTA present in the whole cell sample in Figures 26A and 27A containing 0.016 µM PO_4 is approximately 3000 times less than that present in the 50 µM LTA-PO_4 purified LTA sample. Interestingly, the whole cells induced roughly similar levels of secreted TNF, IL-1β, IL-8 and IL-10

cytokines as the 50 μM LTA-PO_4 sample (Figures 26A and 27A), suggesting that the LTA alone is a much less potent inducer of these cytokines than when it is on cells. It is possible that localised LTA concentrations on the bacterial cell surface may be significantly higher, increasing the effective concentration per cell and leading to a greater immune response. LTA on the bacterial cell surface is anchored to a scaffold, and thus may be presented to immune receptors in a very different way than is purified LTA, potentially increasing the efficiency of the immune response. Immobilisation of purified *S. aureus* LTA on a polystyrene surface has been shown to increase the induction of the pro-inflammatory cytokines IL-1β, TNF and IL-6 and the chemokines IL-8 and G-CSF by LTA in whole blood, when compared to soluble LTA (Deininger *et al.* 2008). Interestingly, the induction of the anti-inflammatory cytokine IL-10 was unaffected by immobilisation. It may be that the interaction of host cell receptors with LTA on the surface of bacterial cells leads to localised increases in signalling and/or cross-linking between receptors, resulting in a more efficient immune response. There are MAMPs other than LTA on *L. rhamnosus*, such as peptidoglycan, that are also able to interact with PRRs on PBMCs, and the presence of these other molecules may have a synergistic effect on the cytokine response that would not be possible for purified LTA.

3.5 Structural Analysis of LTA

Purified lipoteichoic acids from HN001, the DltD- mutant and IM126 were prepared in three pools per strain, as described in section 3.3.4.1, that is, for each strain the main LTA-PO_4 peak was divided in half to create Pools 1 and 2, and the smaller, later eluting LTA-PO_4 peak was collected as Pool 3. Each of these nine fractions was assayed separately in the immune assay, and also individually analysed by NMR spectroscopy. A representative 1D ^{1}H NMR spectra with the structural components labelled is shown in Figure 29. The 1D ^{1}H spectra for all three fractions of LTA from each of HN001, the DltD- mutant and IM126 are shown in Figures 30, 31 and 32, respectively. The NMR profile in each case revealed that the samples contained Type I lipoteichoic acid, consisting of a polymer of glycerol-phosphate linked to a glycolipid anchor, based on comparison with published NMR studies of LTA (Morath *et al.* 2001; Neuhaus and Baddiley 2003). Many of the peaks in the ^{1}H NMR spectra are broader than those seen in published spectra for *S. aureus* (Morath *et al.* 2001), which makes the assignment of structural elements from the spectra more difficult. This is indicative of a more complex sample, and suggests that the samples of LTA examined in this thesis may be more heterogeneous than those from *S. aureus*. As a result, identification of the specific LTA structure(s) that are responsible for immune activity may prove to be difficult. It must be remembered that the NMR data for each sample represent an average structure for a heterogeneous mixture of different LTA molecules. The structural heterogeneity of LTA may play a role in the immune response to bacteria. If, for instance, a particular structural motif was responsible for the immunomodulation, then it could be possible for a strain with more homogeneous LTA to contain a greater proportion of LTA with this motif, compared to a strain with greater structural variation in its LTA.

3.5.1 Purity

In general, the LTA samples were found to be free of major contaminants, as the peaks in the NMR spectra could be accounted for by the LTA. Taken together with the lack of UV absorbance, and the absence of detectable levels of LPS in the samples, it would seem that the samples were highly pure, although this is difficult to quantitate. Some small, unexpected peaks, however, were observed in the spectra, which may represent minor contaminants, and these are discussed in sections 3.5.1 - 3.5.3. The Pool 3 LTA

from the DltD- mutant contained signals of relatively low intensity in the aromatic region of the spectrum (~ 7 parts per million (ppm)) and some others around the Gro sugar region (~ 5 - 4 ppm) that cannot be accounted for by the LTA structure (Figure 31c). This should be taken into account when examining the immune responses to this sample. One of the possible contaminants potentially responsible for this is lipoprotein, which one group asserts is co-purified with LTA and is responsible for the observed immune stimulating activity of LTA (Hashimoto *et al.* 2006; 2007), although this has been disputed (von Aulock *et al.* 2007; Bunk *et al.* 2010).

Evidence of low concentrations of free acetate ions, most likely carried through from the ammonium acetate buffer used in the chromatographic purification of the LTAs, were seen in some of the spectra at ~ 1.9 ppm (Figures 30, 31 and 32), but did not obscure any of the peaks used for structural analysis, and are not expected to be at levels that would interfere with the PBMC assay. The possibility that these free acetate ions might represent acetyl groups cleaved from the GlcNAc substituents of the LTA cannot be excluded; however, the relative area of the free acetate peak is small compared to that of the GlcNAc substituents. Sanchez Carballo *et al.* (2010) reported a similar free acetate peak in the ^{1}H NMR spectra of LTA from *Lactobacillus brevis*, which was found to have glucose but not GlcNAc substituents; the authors suggest that the source of this acetate was the chromatography buffer, as do Fabretti *et al.* (2006).

3.5.2 Unexplained Peak at ~ 0 ppm in the ^{1}H NMR Spectra

A small unexpected peak appeared in all the 1D ^{1}H spectra at ~ 0 ppm (Figures 30, 31 and 32). However, this showed no correlation in the 2D ^{1}H-^{1}H COSY or ^{1}H-^{1}H TOCSY spectra (Figure 34a) with any structural components of the LTA, which indicates that it was not related to the protons in the LTA. Interestingly though, 2D ^{1}H-^{1}H NOESY experiments carried out on two of the samples showed some correlation of the ~ 0 ppm peak with peaks from the FA chains of the LTA. As NOESY identifies through-space rather than through-bond correlation, this may be indicative of a hydrophobic contaminant having this unusual chemical shift associating with the FA moiety of the LTA. Very few molecules have a chemical shift at this position. One possibility is that it arises from a methyl group attached to either silicon or a metal ion; another is that it is due to a cyclopropane derivative (as indicated in Figure 29). Although a silylated

reference compound is often used in NMR experiments, it was not used in this study, and the presence of this compound in the solvent and in the NMR tubes prior to use was excluded by control experiments.

One possible explanation for this could be contamination of the samples with silicone grease, which may have been transferred from glassware either during the cell disruption or butanol extraction processes prior to chromatography, or from glass containers used for freeze-drying post-chromatography, despite rigorous cleaning procedures. If small amounts of silicone grease were associated with the hydrophobic moiety of the LTA, it is possible that it could have remained soluble in the aqueous solvent and passed through the HIC purification. Another potential explanation may be that silica compounds released from the glass beads used to disrupt the bacteria have also been carried through the purification, associated with the acyl chains of the LTA, although this seems unlikely.

Another possibility that may account for this ~ 0 ppm peak is the presence of a cyclopropane derivative. Many lactobacilli have lipids that contain cyclopropane rings in the acyl chain (Grogan and Cronan 1997), including *L. rhamnosus* (Shaw *et al.* 1968). *Lactococcus lactis* G121 has been shown to protect against allergy, and possesses LTA (Figure 35d) containing some lactobacillic acid (a 19 carbon FA with a single cyclopropyl group) (Fischer *et al.* 2011). Cyclopropane fatty acids (CFA) are formed when the Cfa synthase enzyme adds a methyl group to a *cis*-unsaturated bond in the acyl chain to form a *cis*-substituted cyclopropyl ring (Grogan and Cronan 1997; Zhang and Rock 2008); the genome of *L. rhamnosus* HN001 has a predicted gene for this enzyme (M. Collett, personal communication). Probiotic strains of *Lactobacillus reuteri* have been shown to inhibit the secretion of TNF; however, mutant strains that lack active Cfa synthase genes did not exhibit TNF-inhibitory activity (Jones *et al.* 2011). The authors found that purified lactobacillic acid from TNF-inhibitory strains did not have this activity. One scenario that the authors did not mention is the incorporation of lactobacillic acid into the LTA of *L. reuteri*, which might explain the increased TNF-inhibitory potential of the WT bacteria. The presence of lactobacillic acid or other CFAs in the LTA of HN001 may help to explain its immunomodulatory activity, such as its ability to protect against eczema (section 1.1.3).

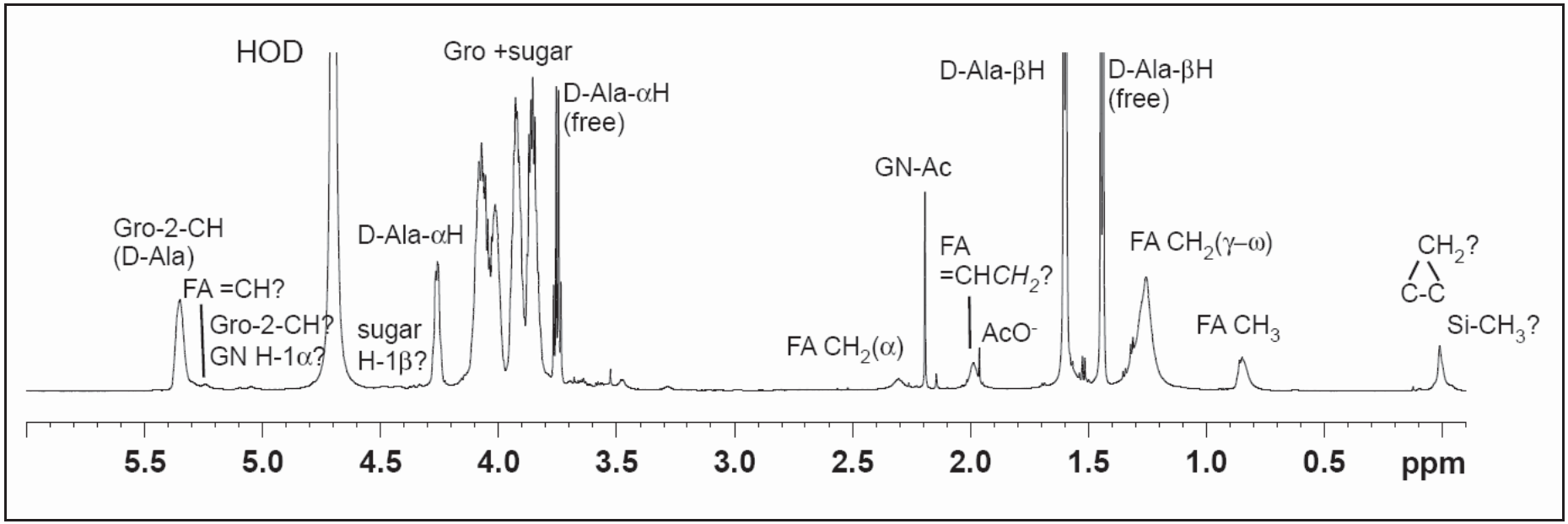

Figure 29: 1D ^{1}H NMR Spectrum of LTA from HN001 Pool 1

LTA from HN001 was analysed by ^{1}H NMR at 700 MHz. The ^{1}H spectrum for HN001 LTA Pool 1 is shown with the chemical shift peaks representing structural elements labelled. The area marked Gro+sugar represents a combination of signals from the glycerol (Gro) units substituted with sugars and other structural elements. The peaks labelled D-ala-αH(free) and D-ala-βH(free) represent free D-alanine that was not attached to the LTA but was present in the samples. The AcO^{-} peak at ~ 1.9 ppm is a free acetate remnant from the chromatography buffer. The unusual peak at ~ 0 ppm could not be assigned as a component of the LTA. It is possible that this chemical shift represents either silicon attached to a methyl group, or the CH_2 group of a cyclopropane ring. The peak labelled HOD represents residual water after partial exchange of hydrogen/deuterium with the D_2O solvent.

If the peak at ~ 0 ppm was a cyclopropane group of a CFA, the ^{1}H NMR spectra should also contain peaks at chemical shifts characteristic of a *cis*-substituted ring, and these were not detected. However, if a cyclopropyl group was *trans*-substituted, those peaks would not be present. *Trans*-substituted cyclopropyl groups are, however, rare in bacteria, and cannot be formed by Cfa synthase. Together with the absence of any correlation of the ~ 0 ppm peak with any other proton signals in the 2D COSY and TOCSY spectra, this strongly suggests that this peak is not part of the LTA (or of free CFA contaminants). A peak at the same position was reported in the ^{1}H spectra for LTA from *Lactobacillus brevis* (Sanchez Carballo *et al.* 2010); however, the authors saw no evidence of lactobacillic acid using GC-MS analysis of the free FAs (Otto Holst, personal communication). This does not, however, exclude the possibility that the LTAs from the strains used in this study contain CFAs, and this should be investigated in future studies.

The relative integral of this peak at ~ 0 ppm seemed to be much greater for the LTA samples from the DltD- mutant (Figure 31). Much more (~ 7-fold) butanol-extracted crude material from the DltD- mutant was processed by HIC to generate a similar amount of purified LTA than from either HN001 or IM126 (Table 4). For this reason, it seems likely that this compound was present in the butanol-extracted starting material, and has been carried through the HIC purification. Interestingly, the relative integral of the ~ 0 ppm peak (Table 5) was very small for the Pool 2 fractions for all three strains, including the DltD- mutant (Figures 30b, 31b and 32b). After the initial pooling and freeze-drying of the respective HIC fractions, Pools 1 and 3 of the DltD- LTA material had a slight yellow tinge, whereas Pool 2 LTA from the mutant appeared white; the dried LTA from IM126 and HN001 was also white. The compound represented by the ~ 0 ppm peak may be responsible for this colouring, perhaps due to higher concentrations of it in Pools 1 and 3 from the DltD- mutant. Silicone grease would not be expected to add colour to the samples, so perhaps the contaminant is a product of CFA breakdown, or some novel cyclopropyl-containing compound produced by the bacteria. It would seem that this compound co-eluted with the early and late LTA-PO_4 peak fractions in Pools 1 and 3, but less so with the Pool 2 fraction. Such an elution pattern seems difficult to explain. All three fractions of LTA from the mutant induced concentrations of cytokines that were consistently higher than those induced by LTA from HN001 or IM126 (Figure 23), while only pools 1 and 3 from the mutant appear to

have significant quantities of the peak at ~ 0 ppm. This suggests that the higher concentration of this contaminant is not likely to be solely responsible for the increased activity. It cannot be ruled out, however, that this putative contaminant is a potent cytokine inducer at very low concentrations, and that the response was saturated at the high concentrations in Pools 1 and 3. It is also a possibility that the LTAs in Pools 1 and 3 have little to no activity, and that the contaminant in these fractions induces a cytokine response similar to that induced by the LTA in Pool 2, which has a very low intensity peak at ~ 0 ppm, although this seems unlikely. The NMR studies of LTA to date that have used the modern purification protocols have not, for the most part, shown the ~ 0 ppm regions of their spectra, so it is difficult to determine whether this may be a common contaminant in preparations of LTA. Further investigation is necessary to identify this compound and to explore its relationship to the LTA.

3.5.3 Unexpected Hydrolysis of D-Alanine in NMR samples

Peaks in the ^{1}H NMR spectra corresponding to free D-alanine (that was not chemically bound to the LTA) were seen in all the spectra for HN001 and IM126, at ~ 1.45 and ~ 3.75 ppm (marked in Figure 29). Interestingly, they were not reported in a number of previous studies of D-alanylated LTA (Morath *et al.* 2001; Grangette *et al.* 2005; Perea Velez *et al.* 2007). However, these peaks representing unbound D-alanine have been observed in some NMR spectra for D-alanylated LTA, without mention of the source (Theilacker *et al.* 2006; Fischer *et al.* 2011). Hydrolysis of D-ala from LTA is known to occur in mild alkaline conditions, a reaction that has been used by researchers to de-D-alanylate LTA for analysis (Morath *et al.* 2001). The improved preparation procedure developed by Morath *et al.* (2001) was designed to prevent this, and employs buffers at pH 4.7 throughout the butanol extraction and chromatography of LTA, to prevent alkaline hydrolysis of D-alanyl esters during the purification (section 2.1.1.3). As free alanine is unlikely to co-elute with LTA during HIC, the source of the unbound D-alanine in the NMR spectra is most likely to be hydrolysis of D-ala substituents from the LTA either during or after chromatography. The signals for this free D-alanine were not present in the ^{1}H NMR spectra of the LTA from the DltD- mutant (Figure 31a - c), which was shown to possess no D-ala substituents on its PGP chain. The inactivation of the *dlt*D gene in HN001 prevents the transfer of D-ala residues to the LTA; however, it can be assumed that the DltD- mutant cells still contain free D-alanine. For these

reasons, it is improbable that the free D-alanine in the LTA from HN001 and IM126 is from a non-LTA endogenous source.

Prior to NMR analysis, fractions from HIC were dried and resuspended into MQ water and then D_2O, as in Morath *et al.* (2001). The MQ water used is slightly acidic, and thus unlikely to cause alkaline hydrolysis of LTA. The absence of buffering may, however, have contributed to gradual loss of D-alanine. The portion of each LTA fraction reserved for the immune assays was resuspended in and stored in PBS buffer, pH 7.2, so it is unlikely that the D-alanyl esters were hydrolysed in these samples during storage, although this cannot be confirmed. Unequal rates of removal of ammonium and acetate ions from the solvent during freeze-drying of the chromatography fractions may also have an effect. Such a phenomenon may have led to fluctuations in pH during drying, resulting in partial alkaline hydrolysis of D-alanyl esters. This has not been reported by other researchers using similar methods; however, if this were the case then the free D-ala would have been hydrolysed prior to the immune cell assays. Consequently the cytokine inducing activity observed for each LTA would have to be attributed to the partially hydrolysed structures. The cytokine-induction in PBMCs by the samples is discussed in relation to this possibility in section 3.5.7. There does not appear to be any direct correlation between the integral of the unidentified peak at ~ 0 ppm and the loss of D-ala from LTA (Table 5), although if this peak does represent a contaminant, it is possible that this is responsible for the hydrolysis.

The presence of this free alanine is problematic for calculating the level of D-alanine substitution, as although it is most probable that this free alanine was originally bound to the LTA, it is by no means certain. The point at which the hydrolysis occurred is also unknown. As similar loss of D-alanine is very rarely reported in studies using comparable methods (Morath *et al.* 2001; Grangette *et al.* 2005; Perea Velez *et al.* 2007), it may be that LTAs from the strains used in this thesis are more susceptible to D-ala hydrolysis compared to those of other strains; although this seems unlikely. However, in a recent study of the LTA from *Lactococcus lactis*, a peak attributed to free D-alanine appears in the ^{1}H NMR spectrum; unfortunately there is no mention of this in the text (Fischer *et al.* 2011). Free D-ala was also seen in the ^{1}H spectrum for LTA from *Enterococcus faecalis* strain 12030 (Theilacker *et al.* 2006). Further experiments to determine the stability of LTA in different solvents and storage conditions are necessary

to determine the cause of the partial D-alanine hydrolysis. As freeze-drying was the only method used to remove solvents post-chromatography in this study, any D-alanine hydrolysed from the LTA remained in the samples, so the total amount of D-alanine should be consistent with the original degree of D-ala substitution of the LTA. The structure-function relationships of LTAs in the following sections are generally discussed with the assumption that the sum of the GroP-bound D-ala plus the free D-ala for each sample represents the D-ala substitution of intact samples in the immune cell assays. The consequences for the structure-function relationships assuming that the immune responses are due to only the D-ala that was shown to be chemically bound to the LTA by NMR are discussed in section 3.5.7.

3.5.4 Structural Elements of LTA from HN001, the DltD- Mutant and IM126

3.5.4.1 Estimation of PGP Chain Length

The average number of glycerol-phosphate (GroP) units in the hydrophilic chain of each LTA can be calculated using the integrals (Table 5) of the glycerol (Gro) peaks (Gro-D-ala at 5.35 ppm and Gro at 3.7 – 4.1 ppm) and the FA terminal CH3 peak (at 0.85 ppm). This requires an assumption about the number of FA terminal CH3 protons per molecule, for instance, if two acyl chains were present, then there would be six protons contributing to this CH3 peak, but if three acyl chains are present, this would be nine protons. As the *numbers* of acyl chains in the LTAs from the strains in this thesis have not yet been determined, the calculation was made based on the diacyl LTA structure, as this is the most commonly found in the literature. If there were some triacyl-anchored LTAs in the samples then this would underestimate the average poly(glycerol-phosphate) (PGP) lengths slightly. Five protons in total contribute to the glycerol signals for each sample, thus the PGP chain lengths were determined from the integrals using the calculation:

$$n = 6/5 \times ((\text{GroDAla} + \text{Gro}) / \text{FA\_CH3})$$

These estimated chain lengths for LTA from HN001, the DltD- mutant and IM126 are given in Tables 6, 7 and 8, respectively, and are summarised in Table 9.

3.5.4.2 D-Alanine Substituents on the PGP Chain

As it seems likely that the free D-alanine detected in the NMR spectra for LTA from HN001 and IM126 was originally bound to the LTA (section 3.5.3), the average percentage of the GroP units that were substituted with D-ala was calculated for each sample using the sum of the integrals of the GroP-bound D-ala Hα peak (at 4.25 ppm) and the free D-ala Hα peak (at 3.75 ppm). There are five protons which contribute to the glycerol signals (GroDAla at 5.35 ppm and Gro at 3.7 – 4.1 ppm) and a single proton on D-ala Hα, therefore the proportion of D-ala substitution was calculated from the integrals (Table 5) as follows:

$$\% \text{ D-ala} = 100 \times (5/1) \times ((\text{Ala}\alpha + \text{Ala}\alpha \text{ free}) / (\text{GroDAla} + \text{Gro}))$$

The % D-ala values for LTA from HN001, the DltD- mutant and IM126 are listed in Tables 6, 7, and 8, respectively, and are summarised in Table 9. The amounts of D-ala substitution calculated using the total D-ala were very similar for all of the LTA fractions from both HN001 and IM126, at ~ 80 %. In fact, this total amount of D-ala seems to be related to the amount of GroP in each sample; the percentage of total D-ala remains approximately constant even though the amount of GroP (i.e., the length of the PGP) varies between samples (Table 9). This is consistent with the assumption that all of the D-ala originated from the LTA. Interestingly, the HN001 LTA Pool 2 sample shows a decrease in D-ala % compared to that observed for pools 1 and 3. This decrease is accompanied by an increase in *free* D-ala relative to the PGP (Table 12), possibly implying slightly more hydrolysis of a similarly D-alanylated LTA.

The alternative percentages of D-ala substitution considering only the integral for the chemically bound D-ala peak at 4.25 ppm were calculated using the following:

$$\% \text{ D-ala} = 100 \times (5/1) \times (\text{Ala}\alpha / (\text{GroDAla} + \text{Gro}))$$

The % D-ala values calculated this way are listed in Table 12, and discussed in section 3.5.7.

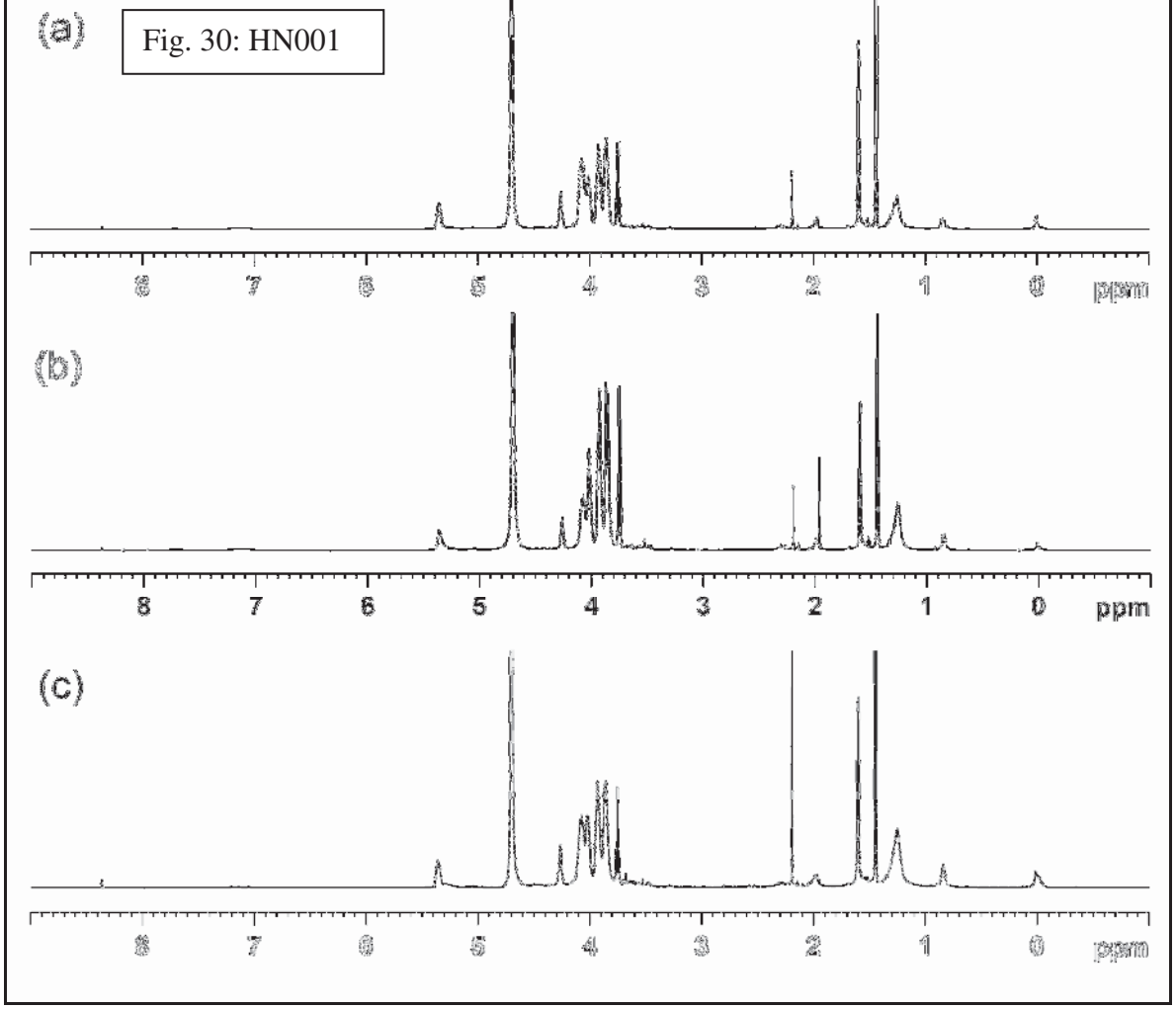

Fig. 30: HN001

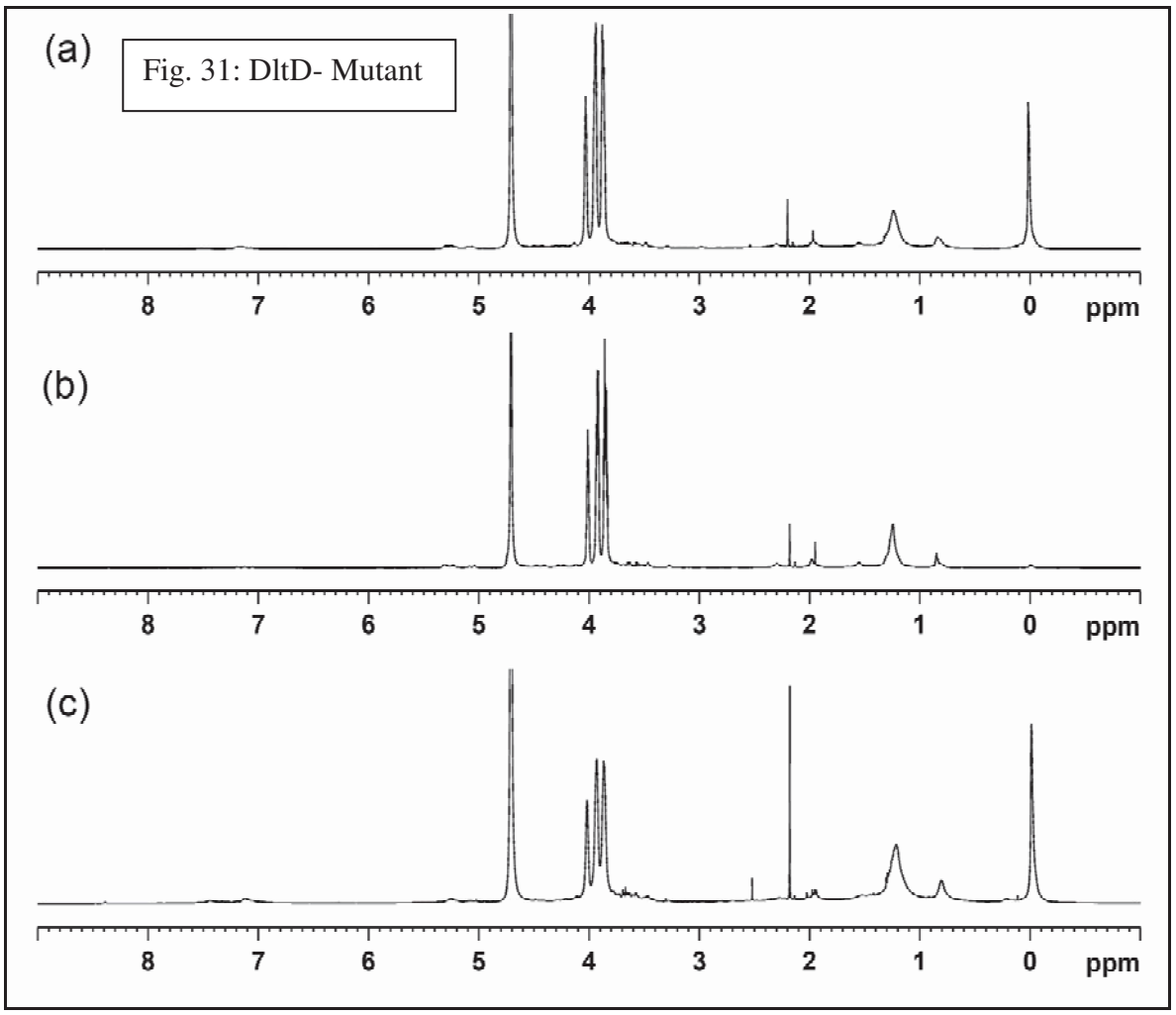

Fig. 31: DltD- Mutant

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Top:

Figure 30: ^{1}H NMR Spectra of LTA from HN001

Each of the three pools of LTA from HN001 were analysed by ^{1}H NMR at 700 MHz. (a) Pool 1, (b) Pool 2, (c) Pool 3. The pooling of the three fractions is described in section 3.3.4.1. The assignments of each peak are described in the representative ^{1}H spectrum in Figure 29.

Bottom:

Figure 31: ^{1}H NMR Spectra of LTA from the DltD- Mutant

Each of the three pools of LTA from the DltD- mutant were analysed by ^{1}H NMR at 700 MHz. (a) Pool 1, (b) Pool 2, (c) Pool 3. The pooling of the three fractions is described in section 3.3.4.1. The assignments of each peak are described in the representative ^{1}H spectrum in Figure 29. Peaks relating to D-alanine substituents to the LTA, as well as free D-ala, were completely absent in the DltD- LTA samples. The peak at ~ 0 ppm is more prominent in Pool 1 (a) and Pool 3 (b) from the mutant than it is for either HN001 or IM126.

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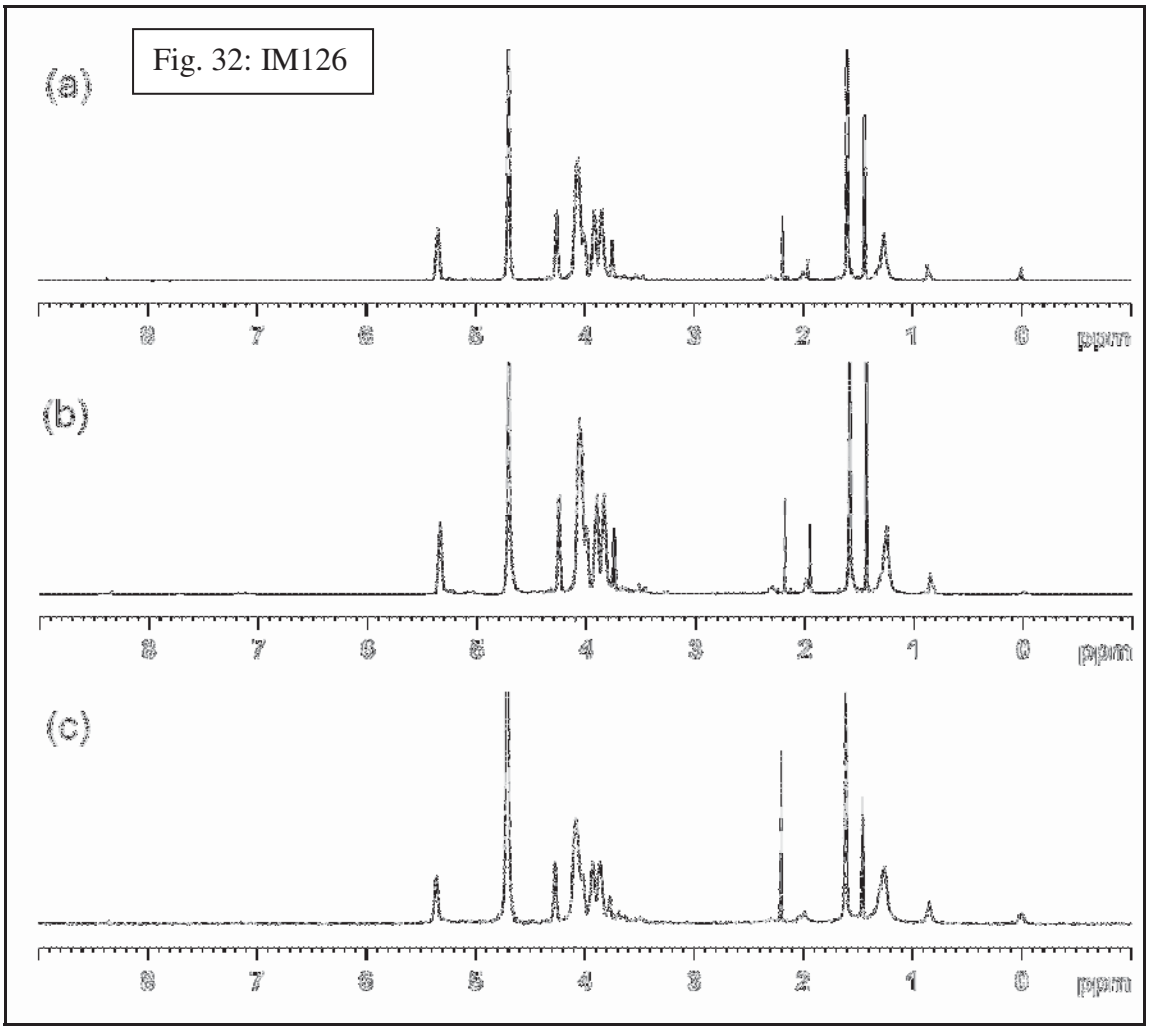
Figure 32: ^{1}H NMR Spectra of LTA from IM126

Each of the three pools of LTA from IM126 were analysed by ^{1}H NMR at 700 MHz. (a) Pool 1, (b) Pool 2, (c) Pool 3. The pooling of the three fractions is described in section 3.3.4.1. The assignments of each peak are described in the representative ^{1}H spectrum in Figure 29.



^{1}H NMR Peak	Chemical Shift (ppm)	Peak Integral									
		HN001			DltD- Mutant			IM126			
		Pool 1	Pool 2	Pool 3	Pool 1	Pool 2	Pool 3	Pool 1	Pool 2	Pool 3	MEAN
FA_CH2α	**2.30**	1.6	1.6	1.4	1.4	1.5	1.1	2.0	1.9	1.5	**1.6**
FA_CH2CH=	**2.00**	2.9	4.1	2.5	2.4	2.9	1.9	3.5	3.7	2.8	**3.0**
FA_CH2β	**1.55**	n.d.	n.d.	n.d.	1.8	2.0	1.5	n.d.	n.d.	n.d.	**1.8**
FA_CH2γ-ω	**1.30**	16.3	17.5	14.9	13.0	16.9	11.6	18.3	19.2	16.0	**16.0**
FA_CH3	**0.85**	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	**3.0**
0ppm	**~ 0.0**	2.8	1.2	2.5	13.5	0.7	9.0	1.9	0.3	1.7	
GroDAla	**5.35**	6.5	4.3	3.2	0	0	0	11.3	11.0	5.1	
Gro	**~ 3.7 - 4.1**	75.0	82.0	43.0	55.0	67.8	21.7	89.5	87.0	41.9	
Ala α	**4.25**	6.0	4.2	3.6	0	0	0	11.1	10.3	5.1	
Ala α free	**3.75**	6.9	9.1	4.2	0	0	0	4.2	4.0	2.2	
Ala α total		12.9	13.3	7.8	0	0	0	15.3	14.3	7.3	
Ala β	**1.60**	16.8	10.0	7.7	0	0	0	32.0	30.0	14.7	
Ala β free	**1.45**	18.4	23.4	9.1	0	0	0	10.8	10.1	4.9	
GlcNAc	**2.20**	1.6	1.3	2.6	0.8	0.8	1.0	2.0	1.4	3.6	
Acetate (free)	**1.96**	0.3	1.8	0	0.3	0.4	0.2	0.6	1.1	0	

Table 5: Integrals of Peaks in the ^{1}H NMR Spectra

The area under each peak in the ^{1}H NMR spectra for LTA from HN001 (Figure 30a-c), the DltD- mutant (Figure 31a-c) and IM126 (Figure 32a-c) were integrated, and are given in the table. To allow comparison between samples, the integrals for each sample are normalised so that the integral of the terminal methyl group of the FA (FA_CH3) has a value of 3. The FA CH2β peaks for the HN001 and IM126 samples could not be accurately integrated as they were partially overlapping with the D-ala-β proton peak. The mean integrals of the FA peaks are given on the far right of the table.

3.5.4.3 Saccharide Substituents on the PGP Chain

It is important to note that the sugar components of the glycolipid moiety of the LTAs (usually a di- or tri-saccharide) could not be determined using the NMR methods employed in this study (section 3.5.4.5). The current section relates only to the sugar substituents on the PGP chain. Further analyses of the saccharide substituents of these samples using a method specific for sugar analysis, such as HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection), are required.

The peak at 2.2 ppm in the ^{1}H spectra (Figure 29) is characteristic of the N-acetyl group of N-acetyl glucosamine (GlcNAc), and appears in all of the fractions for all three strains (Figures 30a-c, 31a-c and 32a-c). The presence of GlcNAc in all of the samples is confirmed by correlation with peaks in the 2D ^{1}H-^{13}C HSQC spectra. Unfortunately however, the signal for the H-1 protons of the sugar substituents is poorly defined (~ 4.9 – 5.3 ppm) and the other sugar peaks overlap with the glycerol phosphate signals (~ 3 – 4 ppm) (Figure 29). Also, the ^{1}H-^{13}C HSQC spectra for several of the samples showed significant signal dispersion in the sugar CHα-1 region. This dispersion may be caused either by different chemical environments around each GlcNAc, or by the presence of sugars other than GlcNAc. Morath *et al.* (2001) showed that selective removal of the D-ala substituents from *S. aureus* LTA by mild alkaline hydrolysis significantly improved the resolution of the saccharide signals in the ^{1}H NMR spectrum, as the GlcNAc substituents were now in identical chemical shift environments. The NMR spectra after this hydrolysis indicated that GlcNAc was the only sugar substituent on *S. aureus* LTA. However, the LTA from the DltD- mutant in the current study was shown to have no D-ala substituents at all (Table 5), yet the H-1 sugar peaks were still highly dispersed. It therefore seems unlikely that this approach will have the same effect on these samples. The dispersion of these sugar peaks despite the absence of D-ala substituents provides tentative evidence that there may be sugars other than GlcNAc present. Although this has not been excluded, the spectra suggest that GlcNAc is the most abundant saccharide substituent in all of these samples. As the peak at 2.2 ppm is the only one that could be reliably integrated, this integral, along with those for the glycerol peaks (Table 5) was used to calculate the average amount of GlcNAc

substitution on the PGP chain for each sample. As there are five protons which contribute to the glycerol signals (GroDAla at 5.35 ppm and Gro at 3.7 – 4.1 ppm) and three protons responsible for the GlcNAc peak (2.20 ppm), the degree of GlcNAc substitution was calculated from the integrals as follows:

$$\% \text{ GlcNAc} = 100 \times (5/3) \times (\text{GlcNAc} / (\text{GroDAla} + \text{Gro}))$$

The % GlcNAc values for LTA from HN001, the DltD- mutant and IM126 are listed in Tables 6, 7 and 8, respectively, and are summarised in Table 9. Note that this calculation pertains to the sugar substituents on the glycerol-phosphate units in the polymer, not to the di-/tri-saccharide linker in the anchor of LTA. For all strains, the LTA in Pool 1 had a similar proportion of GlcNAc substitution to Pool 2, while Pool 3 contained a higher percentage of GlcNAc on the repeating units.

The LAB that have so far had the structures of their LTAs characterised (three of which are shown in Figure 35b, c and d) have been reported to contain only glucose and/or galactose substituents, or no sugar substituents (Grangette *et al.* 2005; Henneke *et al.* 2005; Theilacker *et al.* 2006; Perea Velez *et al.* 2007; Kramer *et al.* 2008; Sanchez Carballo *et al.* 2010; Fischer *et al.* 2011; Jang *et al.* 2011), so it is interesting that the strains of *L. rhamnosus* in the present study are substituted with GlcNAc.

3.5.4.4 Non-substituted GroP Repeating Units

GroP units of LTA that are not substituted do not give a unique signal in the ^{1}H NMR spectrum (unlike those decorated with an amino acid or sugar), as the proton of the glycerol 2' hydroxyl group exchanges with the deuterium in the solvent. The proportion of non-substituted GroP units in each LTA sample was simply calculated by subtracting the percentage of GroP units that were found to have D-ala and GlcNAc substitutions from 100 % GroP. Therefore this does not take into account the presence of undetected substituents to the GroP, such as sugars other than GlcNAc. Additional experiments must be performed to find whether D-ala and GlcNAc are the only substituents to these LTAs, however, based on the spectra, the amounts of any as-yet undetected substituents are expected to be low. If there are substituents to the LTA other than D-ala or GlcNAc, then this value will be slightly overestimated.

3.5.4.5 The Glycolipid Anchor

The glycolipid moiety of the LTAs could not be completely characterised using the methods described in this thesis. The identities of the sugar components of the glycolipids were also not determined in this study. Further analyses using methods such as NMR, GC-MS or HPAEC-PAD following hydrolysis of the LTA are required to identify these sugars.

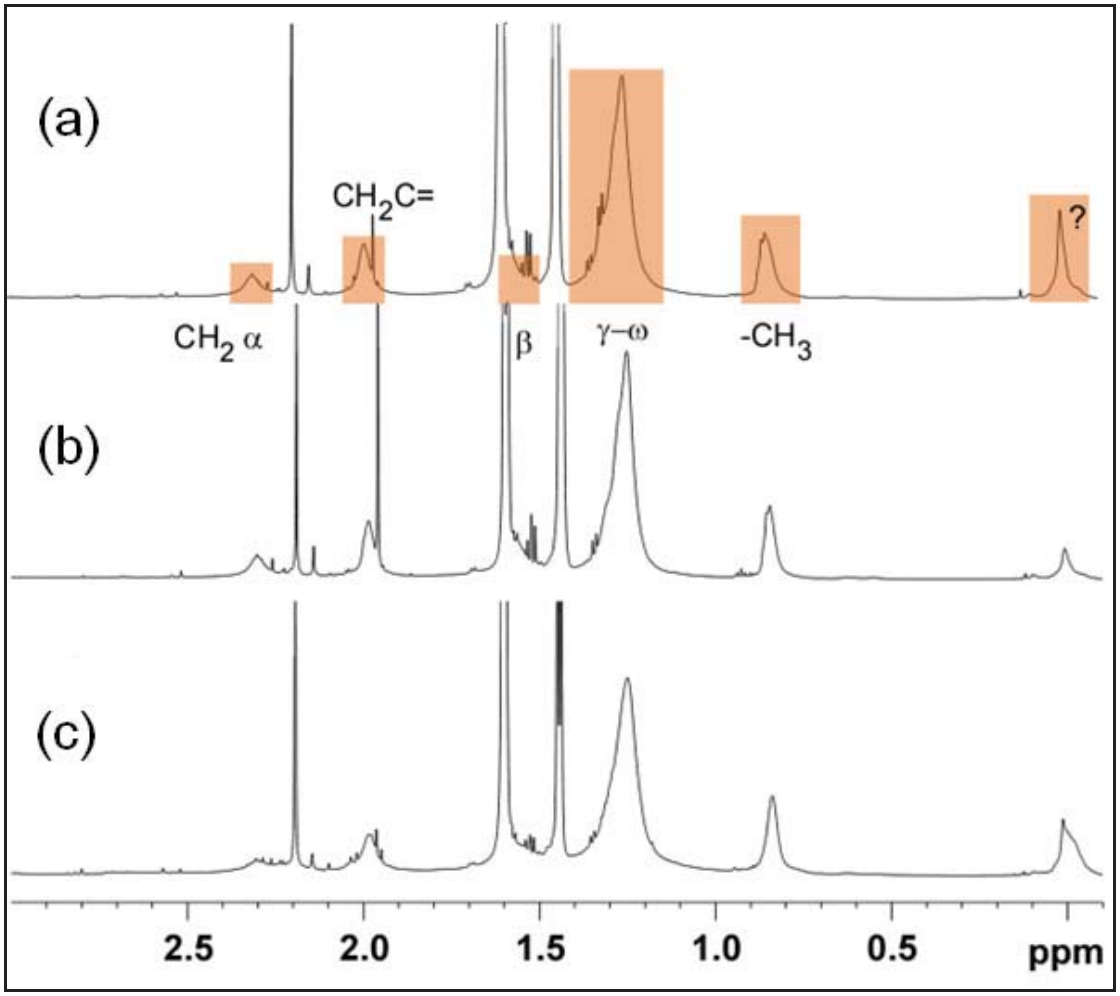


Figure 33: The Fatty Acid Region of the ^{1}H NMR Spectra of LTA from HN001
A selected region of the ^{1}H NMR spectra for the three fractions of LTA from HN001 (Figure 30). The peaks relating to the protons of the fatty acid components in the LTA are shaded. (a) Pool 1, (b) Pool 2, (c) Pool 3. With the exception of the unidentified peak at ~ 0 ppm, the profiles of these peaks are similar to those of the LTA from the DltD- mutant (Figure 31) and IM126 (Figure 32).

NMR analysis of the LTA was able to give average values for the fatty acid (FA) components of the glycolipid anchor; however, the specific types and distribution of the FAs in the membrane anchors could not be determined. Analysis of the glycolipids using GC-MS methods are required to provide these details.

With the exception of the unexplained peak at ~ 0 ppm (section 3.5.2), the peaks in the FA region of the ^{1}H NMR spectra were similar for all of the samples, indicating similar average structures. These peaks (highlighted by shading in Figure 33) were quite broad, indicating heterogeneity of the FAs within each sample, as expected based on the literature (Morath *et al.* 2002b; Fischer *et al.* 2011; Jang *et al.* 2011). The broadness of these peaks and the fact that they were often overlapping with other peaks, limited the accuracy to which their integrals could be calculated (Table 5). Also, the integrals for the FA peaks within a sample are not always consistent, for instance, the ratios of the integrals of the FA terminal CH3 peak to the CH2α peak should be 3:2 if these protons were in the same environment for each sample. The inconsistency of this ratio (Table 5) is indicative of heterogeneity of the FA in each sample.

The ^{1}H NMR peak at ~ 2.0 ppm indicates the presence of a double bond in the acyl chain of the FA, and was recently used as evidence of unsaturated bonds in the fatty acids of LTA from *Lactobacillus plantarum*, confirmed using mass spectrometry (Jang *et al.* 2011). This peak does not appear in the ^{1}H spectra for LTA from *S. aureus*, which does not have unsaturated fatty acids (UFAs) in its LTA (Morath *et al.* 2001). A peak with the same chemical shift also appears in the ^{1}H spectra of LTA from *Lactococcus lactis* G121 (Fischer *et al.* 2011) and *Lactobacillus brevis* (Sanchez Carballo *et al.* 2010), which have both been shown to possess LTA containing some unsaturated fatty acids. Schematic diagrams of some of these LTAs are shown in Figure 35. The ^{1}H spectra for all nine of the samples in this study contained the peak at ~ 2.0 ppm (Figures 30a-c, 31a-c and 32a-c), and the assignment of this to the unsaturated bond in the FAs was confirmed by correlations in the ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC spectra (Figures 34 a and b, respectively). In all nine samples tested, these correlations provided evidence of at least one FA chain with a double bond on average per LTA molecule, in agreement with results reported for *L. plantarum* and *L. brevis* LTAs (Sanchez Carballo *et al.* 2010; Jang *et al.* 2011). It is not possible to accurately calculate the number of unsaturated bonds per molecule (and hence the total average length of the acyl chains) from the current data, because of the inability to precisely integrate some of the peaks, and because of the heterogeneity within the FAs. Rough estimations of the relative lengths of the acyl chains can be made by comparing the integrals of the FA CH2γ-ω peaks, and a comparison of the relative proportions of UFAs in the different samples

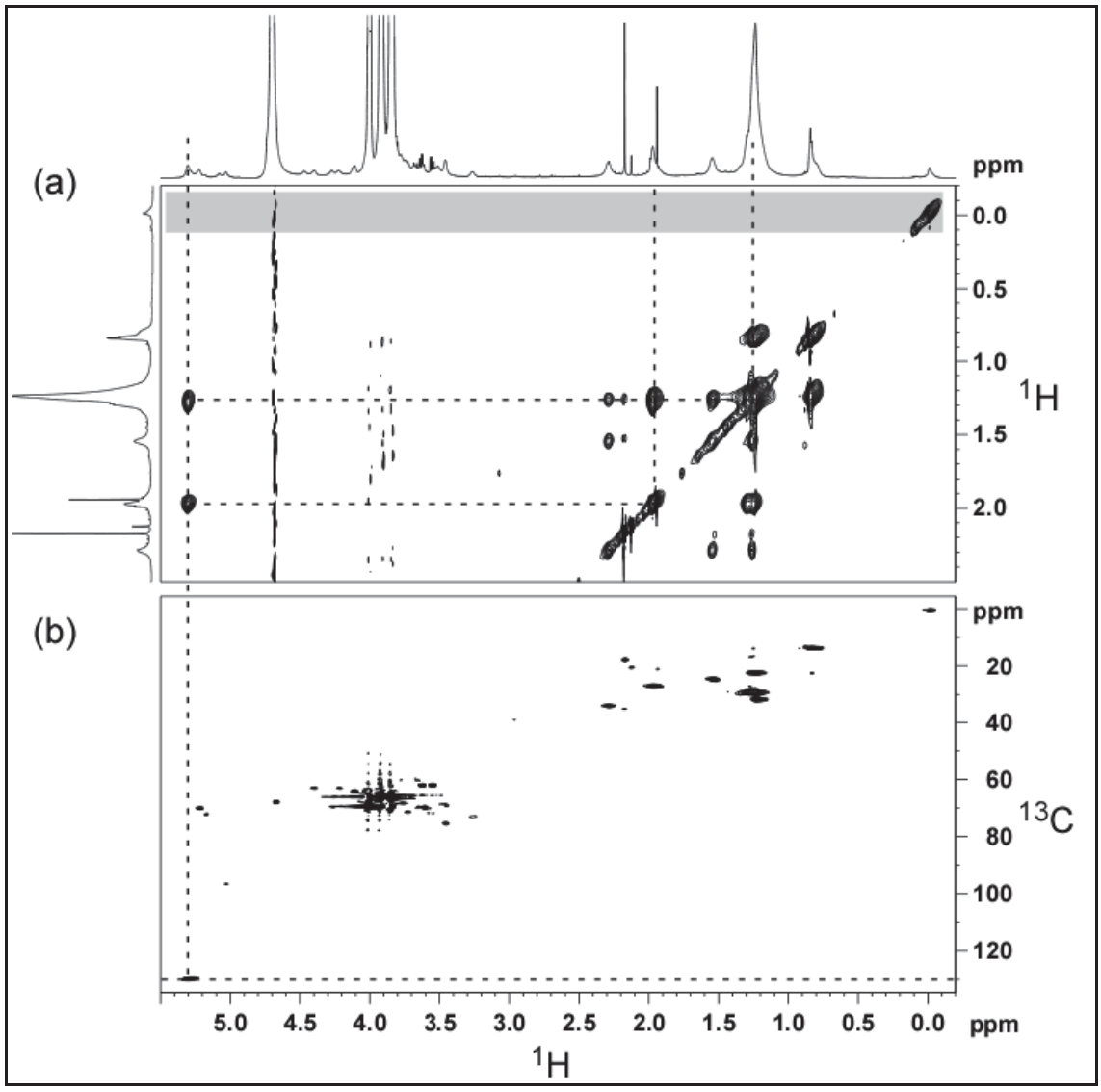


Figure 34: Partial 2D NMR ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC Spectra
Representative partial 2D spectra: (a) ^{1}H-^{1}H TOCSY and (b) ^{1}H-^{13}C HSQC (DltD- Pool 2 LTA). Correlations between the alkenic protons at 2.0 ppm and 5.3 ppm in the TOCSY spectrum and between those at 5.3 ppm and 129 ppm in the HSQC spectrum, are consistent with the presence of unsaturated bonds in the FA chains, and are shown by the dotted lines. The shaded band at the top of the TOCSY spectrum indicates the region where correlations with the unexplained peak at ~ 0 ppm would be expected if those protons were covalently attached to the LTA.

can be made from the FA CH2CH= peak integrals (Table 5). The Pool 3 LTA samples from all three strains appear to contain on average shorter FA chains than either Pool 1 or 2 LTA samples, based on the CH2γ-ω integrals.

As the intensities of the FA signals in the ^{1}H NMR spectra were similar for all three strains, an estimate of the average FA chain length was calculated using the mean values of the integrals of these peaks from all samples (Table 5, right hand column) assuming a single double bond per acyl side-chain. An assumption is made that there

will be one CH3, one CH2α and one CH2β carbon per chain, and four carbons around each double bond (two *CH2*CH= and two CH= carbons that do not contribute to the CH2γ-ω signal). The number of remaining carbons was calculated from the integral of the CH2γ-ω peak (at 1.3 ppm) relative to the integral of the terminal CH3 peak (at 0.85 ppm):

$$n = 3/2 \times (\text{mean FA\_CH2}\gamma\text{-}\omega / \text{mean FA\_CH3})$$

Substituting the mean integral values:

$$n = 3/2 \times (16.0 / 3.0) = 8 \text{ carbons}$$

Therefore the approximate total length is:

$$1 + 1 + 1 + 4 + 8 = 15 \text{ carbons.}$$

This average chain length is similar to that reported in the literature for many strains with differing immune activity (Morath *et al.* 2001; Perea Velez *et al.* 2007; Ryu *et al.* 2009; Dehus *et al.* 2010; Fischer *et al.* 2011). This suggests that although the presence of the lipid anchor may be important for the recognition of LTA, the average length of the lipid anchor does not play a major role in distinguishing between different LTAs. The potential impact of the distribution of different chain lengths within each heterogeneous sample, however, requires further investigation.

The number of acyl chains on each LTA was not determined in this study. LTA is most commonly reported as having only two acyl chains in the membrane anchor, as in *S. aureus* (Figure 35a) (Morath *et al.* 2001). As strains of *L. plantarum* and *L. rhamnosus* have both been shown to contain a combination of diacyl- and triacyl-anchored LTAs (Figure 35c) (Fischer *et al.* 1980; Jang *et al.* 2011), determination of the number of acyl chains on each LTA should be included in further analysis of the glycolipids.

3.5.4.6 HN001 LTA

Three separate fractions of purified LTA from HN001 (as described in section 3.3.4.1) were analysed by ^{1}H NMR (Figure 30a, b, c). The average lengths of the PGP polymer and the average proportions of substituents on the PGP chain were calculated from the relative integrals of the peaks in the proton NMR spectrum for HN001 LTA, and are

summarised in Table 6. It should be noted that each population of LTA is expected to have some microheterogeneity, thus the average values describe the average structure of, but not the variation within, each population.

HN001 Sample	PGP Length	D-Ala %	GlcNAc %	Non-substituted %
Pool 1	33	79	3	18
Pool 2	35	77	3	20
Pool 3	18	84	9	7

Table 6: Structural Elements of LTA from HN001, as Determined by ^{1}H NMR
The average chain lengths of the PGP head group of each LTA, as well as the average proportion of substituents to the PGP (either D-ala or GlcNAc) were calculated from the integrals of peaks in the ^{1}H NMR spectra. The values for the D-ala % represent the total substitution assuming that the free D-ala is derived from the LTA.

Using the values in Table 6 (calculated including the free D-ala, see section 3.5.3), HN001 Pools 1 and 2 (representing the main LTA-phosphate peak from HIC) were both found to have average PGP chain lengths of 33 and 35 GroP residues, 79 % and 77 % of which were substituted with D-alanine, respectively. Approximately 3 % of the GroP units of both Pools 1 and 2 were substituted with GlcNAc. This indicates the presence of an average of approximately one GlcNAc residue and 26 - 27 D-ala residues per LTA molecule for Pool 1 and Pool 2, suggesting that the hydrophilic head-groups of these fractions are similar. Pool 3 LTA (from the less abundant, later-eluting LTA-PO_4 peak) had 84 % D-alanine substitution, similar to that for the main fraction (Pools 1 and 2) from HN001; however, at 18 GroP units in length, its PGP chain was much shorter. Also, 11 % of the GroP units in Pool 3 were substituted with GlcNAc on average (implying ~ two GlcNAc and 16 D-ala residues per PGP chain). It is interesting that the LTA population in the later eluting peak represented by Pool 3 has a PGP chain approximately half the length of the population in the main peak, and is substituted with approximately twice as many GlcNAc residues on average. This may suggest that the substitution with GlcNAc is not merely a function of the PGP chain length, but that this shorter LTA is synthesised by the bacteria for a specific purpose, such as modulating cell surface charge under certain conditions. Only limited structural information could be gathered on the FA chains; however, the integrals of the FA peaks in the ^{1}H NMR spectra suggest that all three were similar (Table 5), and that all three contained some unsaturated fatty acids (section 3.5.4.5). The integrals of the FA_CH2γ-ω peaks

suggested that Pool 2 may have slightly longer average FA chains than Pool 1 (integrals of 17.5 vs. 16.3), while the Pool 3 FA are slightly shorter (integral of 14.9).

These observations of LTA from *L. rhamnosus* HN001 are in contrast to those of LTA from *L. rhamnosus* GG. NMR experiments determined that GG LTA has a hydrophilic chain length of 50 GroP residues, 74 % of which are substituted with D-alanine, and has no sugar substituents, as shown in Figure 35b (Perea Velez *et al.* 2007). Both strains were found to contain unsaturated FAs. It is perhaps surprising that these two strains of the same species of bacteria seem to have LTA that is structurally quite different.

3.5.4.7 DltD- Mutant LTA

The three separate fractions of purified LTA from the DltD- mutant (as described in section 3.3.4.1) were analysed by ^{1}H NMR spectroscopy (Figure 31 a, b, c). Table 7 summarises the average proportions of structural elements of the LTA that were determined from the peak integrals in the ^{1}H NMR spectra. HN001 contains a single *dlt*D gene (complete genome sequence available in the NCBI database, http://www.ncbi.nlm.nih.gov/genome). The D-alanylation of LTA in this mutant was completely abolished by inactivation of this single *dlt*D gene, suggesting that there is no redundancy for this gene function in this strain. Some mutants with inactivated *dlt* genes have been reported to show only a reduction in the degree of D-alanylation of LTA, rather than the total elimination of D-ala (Table 10) (Grangette *et al.* 2005; Steen *et al.* 2005; Hasty *et al.* 2006). It may be that in some strains of bacteria there are redundant genes, which is not the case for *dlt*D in HN001. Alternatively, redundant pathways of D-alanyl ester substitution may exist that are active only under certain culture conditions. Inactivation of the *dlt*D gene in HN001 resulted in dramatic changes in morphology, growth rates and susceptibility to lysis (M. Collett, personal communication). This is similar to observations that have been reported to occur after disruption of *dlt* genes from a number of bacteria (Steen *et al.* 2005; Palumbo *et al.* 2006; Chan *et al.* 2007; Perea Velez *et al.* 2007; Walter *et al.* 2007).

The PGP head group of LTA from the DltD- mutant is shorter on average than that of the isogenic parent strain HN001 (Tables 6 and 7), but similar to LTA from a *dlt*D- mutant of *L. rhamnosus* GG relative to its WT (Perea Velez *et al.* 2007). Conversely,

the LTA of a *dlt*B- mutant of *L. plantarum* was determined to have a PGP chain that was approximately three-fold longer than that of the WT (Grangette *et al.* 2005). This indicates that the degree of D-alanylation of LTA may have some influence on other aspects of LTA biosynthesis, although as the outcome varies significantly between strains it is difficult to suggest how.

DltD- Mutant Sample	PGP Length	D-Ala %	GlcNAc %	Non-substituted %
Pool 1	22	nil	2	98
Pool 2	27	nil	2	98
Pool 3	9	nil	8	92

Table 7: Structural Elements of LTA from the DltD- Mutant, as Determined by ^{1}H NMR
The average chain lengths of the PGP head group of each LTA, as well as the average proportion of substituents to the PGP (either D-ala or GlcNAc) were calculated from the integrals of peaks in the ^{1}H NMR spectra. The signals for D-ala were completely absent in the spectra for the mutant LTAs.

The percentages of GlcNAc attached to the PGP of DltD- LTA for Pools 1, 2 and 3 are 2, 2 and 8 %, respectively. These are similar to those for the three fractions from HN001, and follow the same trend; although, due to the shorter PGP chains, this only corresponds to ~ 0.5 GlcNAc residues per chain for the mutant Pool 1 and 2 fractions, and ~ 0.7 residues for Pool 3. This suggests that a greater proportion of the LTA molecules from the DltD- mutant would have no GlcNAc substituents, when compared to those from HN001, which were shown to have an average substitution of between one and two GlcNAc per chain. The differences between the proportions of PGP-GlcNAc from HN001 and the DltD- mutant are not large; however, if the impact of D-ala elimination on cytokine induction is dependent on the presence of PGP-GlcNAc, it can be deduced that the increased immune response of human cells seen for DltD- LTA must be due to only a subset of the total LTA population. The purification procedures used in this study do not separate LTA populations on the basis of the degree of PGP-saccharide substituents. Such a separation would be very useful to examine how the putative interdependence between D-alanine and saccharide substitution might influence the immune response to LTA.

The NMR analysis of the fatty acids (section 3.5.4.5) showed that the fatty acid region of LTA from the DltD- mutant was structurally similar to that of the WT HN001;

however, comparison of the integrals of the FA_CH2γ-ω peaks (Table 5) indicated that the FA chains were slightly shorter in the mutant than in the WT, particularly for Pools 1 and 3 (Table 5). That is, the integrals for this peak for Pools 1, 2 and 3 were 16.3, 17.5 and 14.9 for HN001 LTA, but 13.0, 16.9 and 11.6 for the mutant LTA. In contrast, the *dlt*D- mutant of *L. rhamnosus* GG actually has LTA with FAs that are two carbons longer than those of the WT GG, even though the PGP chains of the mutant were shorter (Figure 35b)(Perea Velez *et al.* 2007), as in the corresponding mutant of HN001. Once again this demonstrates the influence of D-alanylation of LTA on other structural features of LTA, even though the mechanism behind this is unknown. As the lipid anchor has been shown to be required for the immune activity of some LTAs, these potential differences in FA length may contribute to the various immune responses seen to LTA from different strains in this study.

The LTAs in Pool 1 and Pool 2 from HN001 and IM126 seem to be structurally quite similar on the basis of the features analysed in Tables 6 and 8; however, Pools 1 and 2 from the DltD- mutant have averages of 22 and 27 GroP units, respectively (Table 7). As Pool 1 and Pool 2 are two halves of the main LTA-phosphate peak (Figure 20A, B and C), it is not surprising that they are similar in each of HN001 and IM126. The shape of the LTA-phosphate peak in the HIC chromatogram for the DltD- mutant appeared to be similar to those for HN001 and IM126. In comparison, for the mutant, the LTA species under the main peak appears to be more diverse. For HN001 and IM126, the fractions of LTA with the longest hydrophilic PGP chain eluted first on HIC, which was also observed for LTA from *L. rhamnosus* GG (Perea Velez *et al.* 2007). The situation is different for the DltD- mutant in the current study, where the fraction of LTA that eluted first did not have the longest PGP chain. It is conceivable that the absence of alanine in the structure is affecting the retention time of the mutant LTA on the octyl-sepharose column. The difference in the elution order for the DltD- LTA indicates that the glycolipid moiety of LTA from Pools 1 and 2 may be different; sufficiently so to alter the elution from the HIC column. This is supported by the integrals for the FA_CH2γ-ω peaks (Table 5), which suggest that, for the DltD- mutant, Pool 1 has much shorter FA than Pool 2 with integrals of 13.0 and 16.9, respectively, compared with the integrals of 16.3 and 17.5, respectively, for HN001 LTA. The lipid portion of LTA is expected to contribute to the hydrophobic interactions with the octyl-sepharose column;

however, the effect will not be obvious when comparing LTA samples with very similar lipids, which may be the case with HN001, IM126 and GG.

Fractions of LTA from the DltD- mutant collected during chromatographic purification were pooled according to a similar scheme as the other two strains, yet this yielded what appear to be three different subpopulations of LTA, with average PGP chain lengths of 22, 27 and 9. HN001 and IM126 fractions pooled in the same way were shown to have two subpopulations according to PGP length, of ~ 35 and 18 GroP units for HN001 and ~ 40 and 19 units for IM126. The values for the PGP lengths determined in this study are averages for heterogeneous populations and the distribution of different LTAs within these is not known. It may, however, indicate that there is more diversity of PGP chain length in the DltD- mutant than in either HN001 or IM126. Could D-alanylation of LTA perhaps be involved in the control of PGP elongation? Both processes occur on the outer surface of the membrane, but the order and relationship between them remains undetermined. Perhaps the removal of the D-alanine residues alters the overall shape of the LTA molecules, resulting in different stereochemistry or secondary structure; this may in turn affect the recognition of the nascent PGP chain by the LTA biosynthesis proteins. It is also possible that the many changes to the cell morphology in the DltD- mutant result in the LTA biosynthesis machinery performing less efficiently, and that those LTAs with incompletely elongated PGP chains are responsible for the increased variation.

3.5.4.8 IM126 LTA

The three separate fractions of purified LTA from IM126 (section 3.3.4.1) were analysed individually by ^{1}H NMR spectroscopy (Figure 32), as for the LTAs from HN001 and the DltD- mutant. The structural features analysed by ^{1}H NMR are summarised in Table 8. The LTA samples from IM126 were found to contain free D-alanine, as in the HN001 NMR spectra (discussed in section 3.5.3). It has been assumed that this free D-ala comes from the LTA, and the values in the table are based on the total amount of D-ala in the samples. The alternative values not including this free D-ala are discussed in section 3.5.7. In each case, the values are averages for fractions containing heterogeneous subpopulations of LTA.

IM126 Sample	PGP Length	D-Ala %	GlcNAc %	Non-substituted %
Pool 1	40	76	3	21
Pool 2	39	73	2	25
Pool 3	19	78	13	9

Table 8: Structural Elements of LTA from IM126, as Determined by ^{1}H NMR
The average chain lengths of the PGP head group of each LTA, as well as the average proportion of substituents to the PGP (either D-ala or GlcNAc) were calculated from the integrals of peaks in the ^{1}H NMR spectra. The values for the D-ala % represent the total substitution assuming that the free D-ala is derived from the LTA.

Generally, the LTAs from IM126 (Table 8) are similar to those from HN001 (Table 6). They both have average PGP chain lengths between 18 – 40, are highly substituted with D-alanine and contain comparable numbers of GlcNAc residues. They also seem to have similar anchors, including some unsaturated FAs in all fractions. There appear to be two main populations of LTA for HN001 and IM126, where Pools 1 and 2 make up the first, with small variations between them, and Pool 3 as the second main population, with a much shorter PGP chain. Another strain of *L. rhamnosus*, GG, has been found to have LTA with a PGP length of ~ 50 units, 74 % of which are substituted with D-ala, and none of which are adorned with saccharides (Figure 35b) (Perea Velez *et al.* 2007). All of these *L. rhamnosus* strains contain Type I glycerol-phosphate LTA which is highly D-alanylated. It is interesting that the LTAs from HN001 and IM126 seem to be more similar to each other than they are to LTA from *L. rhamnosus* GG, as HN001 was isolated from cheese, but both IM126 and GG were isolated from human faecal samples. The NMR analysis in the current study only provided limited information on the glycolipid anchor region of LTA (section 3.5.4.5). Therefore, at this point, the complete structures of LTA from HN001 or IM126 cannot be compared with the structure of LTA from *L. rhamnosus* GG.

The NMR results show that the shorter LTA in Pool 3 from IM126 has more GlcNAc per chain than Pools 1 and 2, which was also observed in Pool 3 from HN001, and to a lesser extent for Pool 3 from the DltD- mutant (Table 9). IM126 Pool 3 has GlcNAc attached to 13 % of its GroP, which equates to approximately 2.5 GlcNAc residues per chain. LTA in IM126 Pools 1 and 2 have ~ 0.8 - 1.2 GlcNAc residues per chain. While the increase in GlcNAc substitution may not be large, the fact that it is seen in all three strains suggests that it might be significant. The shorter PGP chains in the Pool 3

samples may simply be partially synthesised LTAs still undergoing GroP chain elongation; however, the Pool 3 LTA samples from all three strains also appear to contain shorter average FA chains than either Pool 1 or 2, based on the CH2γ-ω integrals (Table 5). The elongation of the GroP chain of LTA is thought to begin after translocation of an intact glycolipid from the inner to the outer leaflet of the cell membrane (section 1.2.2), and there is no evidence that the FA chains of LTA are further processed after this point. This suggests that glycolipids with shorter FA chains may be processed differently by the LTA biosynthesis machinery, yielding a unique population of LTA represented by the Pool 3 fractions in this study. A mechanism for the control of the length of the PGP chain in LTA has not yet been identified. It is possible that these different LTAs with shorter PGP polymers and greater sugar substitution are synthesised by the bacteria for some specific purpose, such as regulation of cell wall enzymes, or for modulation of charge in localised parts of the cell wall. Shorter-chain LTAs such as those found in Pool 3 may be less likely to extend beyond the cell wall into the environment. Their different sugar substitution may reflect that they have a specific function inside the cell wall, in contrast to long chain LTAs. The Pool 3 LTAs from both HN001 and IM126 are also more highly substituted overall than the LTA in Pools 1 and 2. The Pool 3 LTAs from HN001 and IM126 were shown to have 93 % and 91 % combined substitution (with D-ala and GlcNAc) of the GroP units, respectively, in contrast to those of Pool 1 (80 – 82 %) and Pool 2 (75 – 80 %) LTAs.

3.5.5 Structure-Function Relationships of LTAs

All of the structural data in Tables 6, 7 and 8 are compared in Table 9. It is important to note that the structural data reported here represent averages for all of the molecules in each sample. This means that while the average structure may reflect the more abundant species in the sample, the immune activity observed could be due to a less abundant molecule, and therefore cannot be absolutely linked to the single reported average structure. Some researchers have constructed synthetic LTAs to obtain homogeneous samples in attempts to address this issue (Morath *et al.* 2002b; Deininger *et al.* 2007; Pedersen *et al.* 2010; Schmidt *et al.* 2011). It is also possible that a combination of the heterogeneous native LTA population is required for the immune response to bacteria.

Sample	PGP Length	D-Ala %	GlcNAc %	Non-substituted %
HN001 LTA				
Pool 1	33	79	3	18
Pool 2	35	77	3	20
Pool 3	18	84	9	7
DltD- Mutant LTA				
Pool 1	22	nil	2	98
Pool 2	27	nil	2	98
Pool 3	9	nil	8	92
IM126 LTA				
Pool 1	40	76	3	21
Pool 2	39	73	2	25
Pool 3	19	78	13	9

Table 9: Structural Elements of LTA from HN001, the DltD- Mutant and IM126, as Determined by ^{1}H NMR

This table summarises all of the data in Tables 6, 7 and 8. The average chain lengths of the PGP head group of each LTA, as well as the average proportion of substituents to the PGP (either D-ala or GlcNAc) were calculated from the integrals of peaks in the ^{1}H NMR spectra. The values for the D-ala % represent the total substitution assuming that the free D-ala is derived from the LTA.

3.5.5.1 The Effect of D-Ala Substitution

The reported effects of D-ala substitution on the immune responses to a number of strains are summarised in Table 10. When the *dlt*D gene was knocked out in *L. rhamnosus* GG, the D-ala substitution of the LTA of this mutant was completely abolished; nevertheless, the cytokine responses from PBMCs incubated with either the WT or the *dlt*D- mutant were similar (Perea Velez *et al.* 2007). When the same *dlt*D gene was inactivated in *L. rhamnosus* HN001, D-alanylation of LTA was eliminated, as in GG; however, the levels of TNF, IL-1β, IL-8 and IL-10 secreted by PBMCs in response to the mutant bacteria were substantially increased compared to that of the WT, in contrast to GG (Table 10). One explanation for this dramatic difference in the impact of D-alanine depletion could be the presence of GlcNAc substituents in the LTAs of HN001 and its DltD- mutant (Table 9), and the absence of sugar substituents on *L. rhamnosus* GG LTA and its *dlt*D- mutant (Table 10, Figure 35b). A *dlt*A knockout of *Streptococcus pyogenes* resulted in mutant bacteria with LTA that were substituted at less than 7 % with D-ala, versus 65 % for the WT; however, both the purified LTA and whole cells of this mutant induced similar pro-inflammatory cytokine responses in human whole blood to the WT LTA and cells (Hasty *et al.* 2006). The LTAs from both *dlt*A mutant and WT strains of *S. pyogenes* have no saccharide substituents on the PGP chain, only D-ala esters (Table 10). The authors propose that the reason for the apparent

lack of influence of D-alanylation of LTA on the cytokine response for *S. pyogenes* is because it is not glycosylated. Similarly, a *dlt*A- mutant of Group B *Streptococcus* (GBS) that contained LTA lacking D-ala esters induced similar cytokine concentrations as the WT (Table 10); the LTA of GBS is also not glycosylated (Henneke *et al.* 2005). *Staphylococcus aureus* contains LTA that is substituted with GlcNAc (Figure 35a), and the cytokine inducing activity of this LTA is reduced after elimination of D-ala (Table 10) (Morath *et al.* 2001; Rockel *et al.* 2010). D-alanylation of LTA has a significant impact on the cytokine induction profile of *Lactobacillus plantarum*, which has glucose substituents on its LTA (Figure 35c) (Grangette *et al.* 2005; Jang *et al.* 2011), adding further weight to the hypothesis that the influence of D-alanylation of LTA is only seen when the PGP chain of LTA is also substituted with saccharide molecules.

While D-alanylation of LTA was shown to play an important role in the cytokine response induced by LTA from both the pathogen *S. aureus* and the probiotic HN001, reduction of D-ala substitution on LTA from each of these strains had quite different outcomes (Table 10). Elimination of D-ala on the LTA from HN001 brought about a large increase in TNF induction in PBMC by both the LTA and the bacteria, whereas removal of D-ala substituents from *S. aureus* LTA by alkaline hydrolysis resulted in a reduction of TNF response to purified LTA in whole blood (Morath *et al.* 2001). The alkaline hydrolysis may have also had other effects on the structure, such as removal of sugar substituents; however, the immune results were consistent with recent findings for LTA that lacked D-ala, isolated from a *dlt*A- mutant of *S. aureus* (Rockel *et al.* 2010). The same was true for a non-pathogenic *Bacillus subtilis*, alkaline treated LTA from which induced less TNF in PBMCs than did intact LTA (Ryu *et al.* 2009); and an oral commensal *Streptococcus gordonii* LTA, which when de-D-alanylated (by creating a *dlt*A knockout), reduced TNF induction in DCs (Table 10) (Chan *et al.* 2007). HN001 is unusual in this sense, as it appears to be the only bacteria studied so far where removal of the D-alanyl esters from its LTA actually *increases* the induction of pro-inflammatory cytokines. Conversely, when a *dlt*B- mutant of *L. plantarum* was created, reduction of the D-ala content of the LTA *decreased* the induction of the pro-inflammatory cytokines TNF, IL-1β, IL-6 and IL-8 (Table 10), similar to what was observed for *S. aureus* and *S. gordonii* (Morath *et al.* 2001; Chan *et al.* 2007); however, the induction of anti-inflammatory IL-10 was *increased* for the *dlt*B- mutant (Grangette

et al. 2005). NMR analysis indicates that the LTA from HN001 contains unsaturated fatty acids; whereas LTAs from *S. aureus* and *B. subtilis* have only SFAs (section 3.5.4.5 and Figure 35a). Structural differences such as this may be partly responsible for the different effects after the elimination of D-ala. The fact that GlcNAc substituents are found on the LTA from HN001, which is unusual among LTA from LAB (section 3.5.4.3 and Figure 35b-d), may also explain the contrasting effects of D-alanine depletion of LTA in HN001 and *L. plantarum*.

Strain	Substituents		Effect on Immune Response to:	
	% D-Ala	Sugar	LTA	Bacteria
HN001	* 79	GlcNAc		
HN001 *dlt*D-	nil	GlcNAc	↑	↑
L. rhamnosus GG	74	nil		
L. rhamnosus GG *dlt*D-	nil	nil	n.d.	** ≈
L. plantarum	42	nil		
*L. plantarum dlt*B-	1	Glc	↓ pro-infl. IL-10 n.d.	↓pro-infl. ↑IL-10
L. lactis	29	Gal	n.d.	
*L. lactis dlt*D-	6	Gal	n.d.	≈
S. aureus	70	GlcNAc		
*S. aureus dlt*A-	nil	n.d.	↓	≈
S. pyogenes	65	nil		
*S. pyogenes dlt*A-	7	nil	≈	≈
GBS	20	nil		
GBS *dlt*A-	nil	nil	n.d.	≈
S. gordonii	†3.2µg/mg	n.d.		
*S. gordonii dlt*A-	nil	n.d.	↓	↓

Table 10: Effects of *dlt* Mutations on Immune Responses
KEY: ↑, increased; ↓, decreased; ≈, similar to WT; n.d., not determined.
Comparison of the effects after inactivation of genes of the *dlt* operon, which leads to decreased D-ala substitution of LTA. The effect of these mutations on the immune response induced by isolated LTA and/or whole bacteria are indicated relative to the WT LTA and/or bacteria. * Weighted average of D-ala content, calculated using the data for the three pools and the proportions of total LTA for each pool. ** Recently shown to exhibit improved probiotic effects on colitis in mice compared to the WT (Claes *et al.* 2010). † Dry wt of D-ala per dry wt of LTA. Strains: *Lactobacillus rhamnosus* GG (Perea Velez *et al.* 2007), *Lactobacillus plantarum* NCIMB8826 (Grangette *et al.* 2005; Palumbo *et al.* 2006), *Lactococcus lactis* MG1363 (Perea Velez *et al.* 2007; Kramer *et al.* 2008), *Staphylococcus aureus* S113 (Peschel *et al.* 1999; Rockel *et al.* 2010), *Streptococcus pyogenes* M type 1 strain 8004 (Hasty *et al.* 2006), Group B *Streptococcus* NEM316 (Henneke *et al.* 2005) and *Streptococcus gordonii* PM14 (Chan *et al.* 2007).

Interestingly, both the *L. plantarum* mutant bacteria, and the DltD- mutant of HN001 in this study induced considerably more of the anti-inflammatory cytokine IL-10 than their respective wild type bacteria (Grangette *et al.* 2005). While the authors did not measure the IL-10 induced by the purified LTAs, it seems likely that the altered LTA is

responsible for at least part of this response. The LTA from the *L. plantarum* mutant, while greatly reduced in D-alanine, was found to be substituted with glucose on 24 % of the GroP units, while the WT LTA had barely detectable amounts of glucose (Table 10). The LTA of both the WT HN001 and the DltD- mutant in this study were substituted with sugars on the PGP chain, but no increase in the amount of these sugar substituents were observed in the mutant LTA, in contrast to *L. plantarum*. Similarly, a *dlt*D- mutant of *Lactococcus lactis* was shown to have LTA with its D-ala content reduced to 6 % (from 29 % for the WT, Table 10), while substitution of the PGP chain with galactose was considerably increased from 12 % to 46 % (Kramer *et al.* 2008). This helps to demonstrate the complexity of the various factors involved in the immune response due to LTA, and it seems unlikely that any one structural component of the LTA is solely responsible for all of the differences seen between different strains of gram positive bacteria.

It has been shown that the D-alanine esters of LTA are able to migrate along the PGP chain, or between chains, after the initial esterification, independent of the D-alanylation enzymes (Childs *et al.* 1985); thus the positions of the D-alanines on the native LTA structure are not known. Nor is there knowledge of the distribution of the sugars along the PGP chain, although there is no evidence that they are redistributed between GroP units. The relative positions of the D-ala and saccharides on the PGP chain may be important for immune recognition, as receptors on immune cells may recognise a specific architecture made up from these molecules. If an LTA molecule was highly substituted with D-ala, the D-ala residues may be quite close together along the chain. In an LTA with low levels of D-ala substitution, however, the D-ala residues may be further apart. Therefore, altering the degree of D-ala substitution may alter this architecture, resulting in changes to the recognition of LTA.

3.5.5.2 The Effect of PGP Chain Length

There is some correlation of PGP chain length with cytokine induction for LTA from these strains, although as this was not the only structural difference identified, it may not necessarily be the sole cause. LTA from IM126 was found to have longer average PGP chains than HN001, and caused lower concentrations of secreted cytokines, while LTA from the DltD- mutant had generally shorter PGP chains than HN001, and

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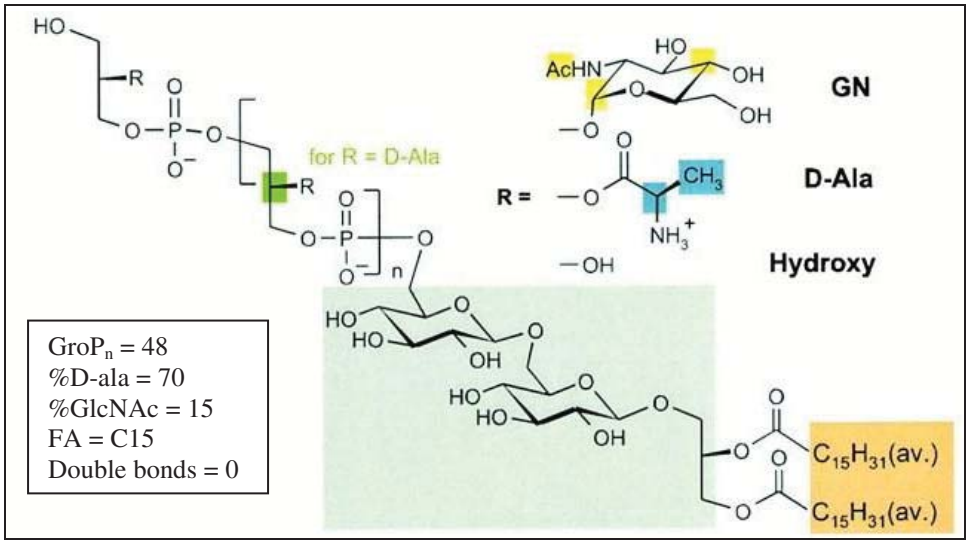
Figure 35: Structures of LTA from *S. aureus*, *L. rhamnosus* GG, *L. plantarum and L. lactis*
Schematic diagrams of four Type I LTA structures as determined by NMR and MS analysis, reproduced with permission. Structural elements and their relative *average* proportions are shown where appropriate. (a) LTA from *Staphylococcus aureus* DSM20233 (Morath *et al.* 2001). (b) LTA from *Lactobacillus rhamnosus* GG, which contains no sugar substitution of GroP (Perea Velez *et al.* 2007). (c) LTA from *Lactobacillus plantarum* KCTC 10887BP (Jang *et al.* 2011). NB: *L. plantarum* NCIMB8826 (not pictured) contained LTA with 22 GroP units, 42 % D-ala substituted, whereas a *dlt*B-mutant of this strain had LTA with 63 GroP units substituted with 1 % D-ala and 24 % glucose (Grangette *et al.* 2005). (d) LTA from *Lactococcus lactis* G121, which contains lactobacillic acid (a cyclopropyl group-containing FA) amongst the FA chains on its LTA (Fischer *et al.* 2011)

exhibited greatly increased cytokine induction in comparison to WT HN001. The PGP chain length does not seem to be a major factor in the immune activity of LTAs reported in the literature. A synthetic LTA based on that of *S. aureus*, with only six GroP repeating units, but with similar percentage substitution of D-ala and GlcNAc, was shown to have comparable cytokine inducing activity to native *S. aureus* LTA containing 48 GroP units (Morath *et al.* 2002b). The patterns of cytokines induced were also similar and the responses to both LTAs were shown to be TLR2 dependent. Thus the PGP length may influence the immune response to LTA, although its contribution to the immune activity is difficult to define in this study.

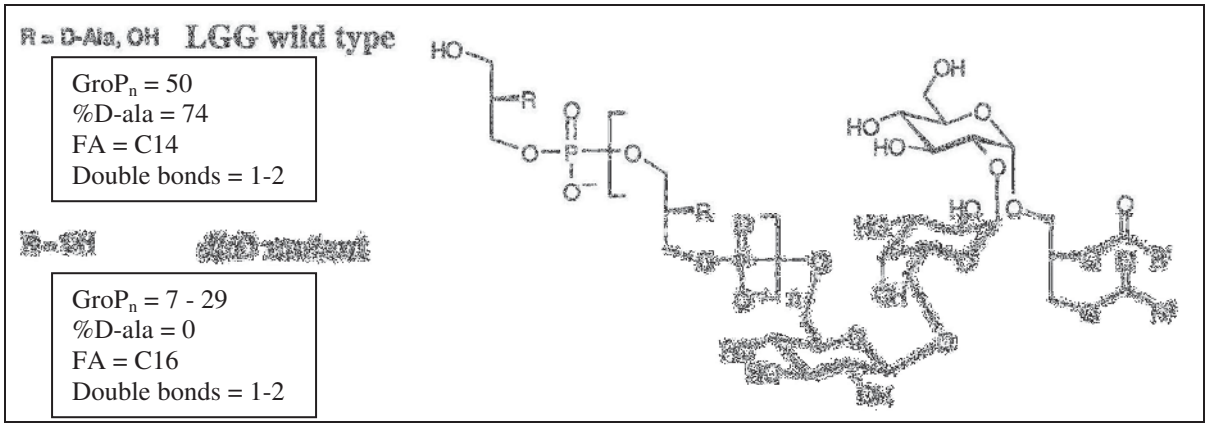
3.5.5.3 The Effect of the Glycolipid Anchor

The average length of the FA chain of LTA from the strains in this thesis was estimated to be approximately 15 carbons (section 3.5.4.5), which is in agreement with the 14 - 16 carbon length typically found in the membrane anchor of LTA (Morath *et al.* 2001; Perea Velez *et al.* 2007). It seems that the average FA chain length is not a major determinant of the immune response to LTA, although other differences in the composition of the FA chain may be important. Structural changes in the lipid anchor may alter the recognition and binding of LTA by PRRs, including co-receptors such as CD36, which is known to interact with ligands containing diacyl chains (section 1.2.5). Further analyses of the lipid moieties of the LTAs in this study are required before the role of the lipid part in cytokine induction can be described in detail.

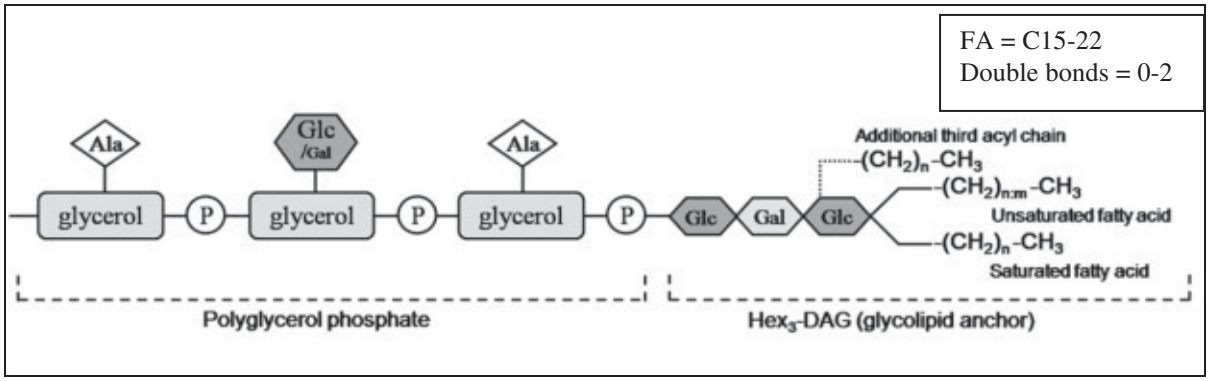
(a) *Staphylococcus aureus* LTA



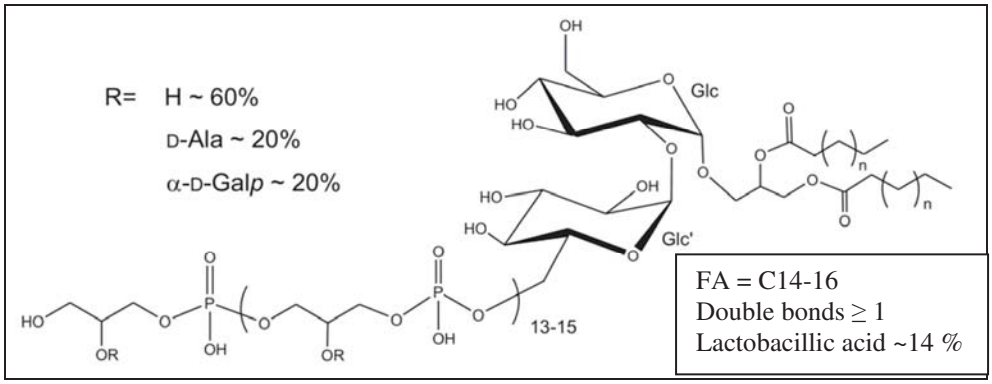
(b) *Lactobacillus rhamnosus* GG LTA



(c) *Lactobacillus plantarum* LTA



(d) *Lactococcus lactis* LTA



NMR analysis indicated the presence of unsaturated fatty acids (UFAs) in LTAs of all three strains in this thesis (section 3.5.4.5). The presence of olefinic bonds in fatty acids can alter the structure of membrane lipids. In particular, *cis*-unsaturated carbon-carbon bonds are known to introduce kinks in the acyl chain, changing the structure dramatically. They also increase membrane fluidity and decrease the tightness of packing in the cell membrane and UFAs have also been shown to be less likely to be incorporated into lipid rafts than SFAs (Zhang and Rock 2008); all of these may change the accessibility of LTA on cells to PRRs. The presence of UFAs in the glycolipid anchor of LTA may alter the recognition by PRRs of both isolated LTA and LTA on cells, and/or the binding affinity between ligand and receptor(s). The activation of TLR2 by lipopeptides has been shown to be potentiated by SFAs, but suppressed by UFAs (Lee *et al.* 2003). Similar modulatory effects were also demonstrated for activation of TLR4 by LPS. It may be expected then, that TLRs may interact differently with LTA containing UFAs, compared with those containing only SFAs. It has been suggested that the degree of saturation of the FA in LTA is a major structural difference between highly pro-inflammatory LTAs, such as those from *S. aureus* and *B. subtilis* and LTAs that are less pro-inflammatory or that have anti-inflammatory properties, for example, those from commensal or probiotic bacteria such as *L. plantarum* and *L. rhamnosus* (Jang *et al.* 2011). LTA from both *S. aureus* and *B. subtilis* have LTA that contain only SFAs, while both *L. rhamnosus* and *L. plantarum* have LTA containing UFAs and SFAs (Figure 35). The identification of UFAs in all fractions of the LTA from all three strains in this thesis may help to explain some of the immunomodulatory properties of HN001 and IM126.

The glycolipid anchor of LTA most commonly has two acyl chains, as found in *S. aureus* and *B. subtilis* (Morath *et al.* 2001; Neuhaus and Baddiley 2003); however, this is not always the case (Figure 35). LTA from *L. rhamnosus* has been previously shown to contain both diacyl and triacyl glycolipids, at a ratio of 60 % to 40 % of the LTA, respectively (Fischer *et al.* 1980). However, this has not yet been established for the *L. rhamnosus* strains studied here. Recently, *L. plantarum* was shown to synthesise both diacyl- and triacyl-anchored LTAs (Jang *et al.* 2011). *Listeria monocytogenes* has been reported to produce two different LTA structures, one with a DAG anchor, and another simultaneously possessing two DAG chains, a total of four acyl chains attached to the glycolipid (Dehus *et al.* 2010). These and other variations in acylation of the

glycolipid may continue to be found in the LTA of other strains, and may have a significant impact on the immune recognition of LTA. The PRR, TLR2 is known to be important for the immune recognition of lipopeptides. It has been shown that diacylated lipopeptides require the formation of a TLR2/TLR6 heterodimer to be recognised by TLR2, whereas recognition of triacylated lipopeptides occurred only after formation of a TLR2/TLR1 heterodimer (Farhat *et al.* 2008; Kang *et al.* 2009). As TLR2 has been implicated in the recognition of LTA, it is quite possible that the formation of TLR heterodimers could also be impacted by the number of acyl chains in the glycolipid moiety of LTA, with potentially different immune outcomes. CD36 is known to recognise diacylated lipid compounds, and is thought to be a co-receptor in the recognition of LTA by TLR2 (Hoebe *et al.* 2005; Jimenez-Dalmaroni *et al.* 2009). It is possible that the roles of co-receptors such as CD36 and CD14 are not the same in the case of the triacylated ligand, which may lead to differences in the recognition and response to diacylated and triacylated LTAs. The presence of triacyl membrane anchors in the LTA of probiotic strains of *L. plantarum* and *L. rhamnosus* may therefore be partly responsible for the unique immune responses to these bacteria, when compared to highly pro-inflammatory strains such as *S. aureus,* which contain only diacylated LTA. This requires further investigation.

Changes to the structure of LTA such as the saturation of the fatty acids or the number of acyl chains anchoring the molecule in the membrane are also likely to impact on the fluidity of LTA in the membrane. As a consequence, the formation of lipid rafts and other complexes involving LTA may be affected. Presentation of ligands such as LTA on a scaffold, where multiple ligands are clustered on the surface, is known to increase the specificity and stability of interactions with receptors, compared with single ligand-receptor binding. Thus, the efficiency of recognition by immune cells of LTA on the bacterial surface may be influenced by the ability of LTA to migrate across the membrane. The interaction of multiple receptors and ligands is common in immune signalling. Thus, a structural change in one component of an immune recognition complex may alter the overall specificity or stability of the complex, and result in different signalling.

LTAs with different lipid anchor structures may be released into the environment at different rates, which may have an impact on immune responses to bacterial cells. This

study found that a considerably higher concentration of purified LTA was required than that naturally occurring on HN001 cells to induce a similar level of cytokine response from PBMCs (section 3.4.5). Therefore, released LTA is unlikely to have much influence. It is not clear however, what impact any increased release of free LTA along with other bacterial components, such as peptidoglycan or nucleic acids, might have on this response.

For IM126, the approximate lengths of the PGP and FA chains, and the degree of substitution with D-ala and GlcNAc were found to be similar to HN001 (Table 9). Although the LTA from IM126 generally demonstrated a weaker cytokine inducing activity than LTA from HN001, the concentrations of cytokines induced in response to LTAs from HN001 and IM126 were more similar than those measured for LTAs from the DltD- mutant (Figure 25). The *pattern* of cytokines induced in PBMCs was also quite similar for HN001 and IM126 LTAs (Figure 26 A and C; Figure 27 A and C). The biggest structural difference between these two LTAs revealed by NMR spectroscopy was an extra 5 – 7 repeating units in the PGP chain of IM126 LTA. It is possible that there may be differences between the glycolipid moieties in the two LTAs that were not identified in this study, or there may be sugars other than GlcNAc attached to some of the GroP units. The small differences between the structures of the LTA from HN001 and IM126 seem, however, to correlate with the small differences between the cytokine responses to these two LTAs. This generally supports the idea that differences in the structure of LTA are responsible for the differences in immune response, as the more obvious differences between the structures of LTAs from the DltD- mutant and WT HN001, compared to the minor differences between IM126 and HN001 LTAs, were reflected in the relative immune responses in PBMCs.

3.5.6 Cytokine Response to Subpopulations of LTA

The three fractions of purified LTA from each of the strains HN001, the DltD- mutant and IM126 that were analysed by NMR were also individually tested for cytokine inducing activity using the PBMC-CBA assay. The phosphate concentrations were used to standardise the amount of each LTA added into the PBMC assay, to ensure that approximately equivalent amounts of LTA were being compared. NMR analysis later revealed the average number of GroP units in each LTA, thus the average

concentrations of LTA could be estimated by taking into account the different amounts of phosphate in each molecule. It should be noted that the calculation of the number of GroP residues per LTA used the assumption that the LTA has two acyl chains per anchor (section 3.5.4.1). For each combined sample the average number of GroP units per chain was determined by calculating the weighted average of the chain lengths for each pool using the relative proportion of the total peak LTA represented by each fraction in the HIC chromatogram. Table 11 summarises the concentrations of each LTA equivalent to 100 µM PO_4. For example: HN001 Pool 1 has an average of 33 GroP units per LTA, which implies that there are 33 moles of phosphate per mole of this LTA, so for the 100 µM PO_4 sample of this fraction:

$$\text{Concn} = 100\ \mu\text{M } PO_4 \div (\ 33\ \text{mol} / 1\ \text{mol}\ ) = 3.0\ \mu\text{M LTA}$$

Using these values, the combined DltD- LTA sample (4.3 µM) was only slightly more concentrated than the HN001 combined LTA sample (3.1 µM) at the same concentration of phosphate, a ratio of ~ 1.4. Even if it is assumed that the DltD- LTA samples were twice as concentrated as the mutant, and the 100 µM PO_4 HN001 combined LTA is compared with the 50 µM PO_4 DltD- combined LTA (Figure 25), the TNF induction by DltD- LTA is still more than 6-fold (908 ÷ 134 pg/mL) higher than that induced by HN001 LTA. This suggests that much of the increased cytokine response is due to the altered structure of the LTA. The LTAs from HN001 and IM126 have similar numbers of GroP repeating units, and thus the estimated molar concentrations of LTA are similar at equal phosphate concentrations (3.1 vs. 2.8 µM LTA, respectively, at 100 µM PO_4), so comparisons between the combined LTA samples for these two strains is less affected by the small difference in effective LTA concentration.

The Pool 3 LTAs for each strain contain roughly half the number of phosphate groups per molecule than the Pool 1 and 2 LTAs; thus, for each strain, the 50 µM PO_4 Pool 3 samples are closest in effective LTA concentration to the 100 µM PO_4 samples of Pool 1 and Pool 2 (Table 11). For all three strains in general, the Pool 3 fractions were poorer inducers of cytokines than the Pool 1 or Pool 2 fractions at the same phosphate concentration. When, for example, the DltD- mutant Pool 3 at 25 µM PO_4 is compared with Pools 1 or 2 at 50 µM PO_4 (Figure 37A and B), the difference in cytokine induction

between Pool 3 and Pool 1 / Pool 2 is even more pronounced. All of the samples are well under the conservatively estimated CMC (221 μM PO_4 for DltD- LTA, Table 3), therefore the higher concentrations of cytokines induced by Pools 1 and 2 cannot be explained by the effective LTA concentrations.

The effective final concentrations of LTA in the PBMC experiments containing whole bacteria at 1×10^6 cfu/mL are given in terms of both phosphate and LTA (Table 11). These concentrations are three to four orders of magnitude lower than those of the purified LTA samples used (discussed in section 3.4.5).

	Average PGP Length (No. of GroP units)	**LTA-PO_4 Concn (μM PO_4)**	**Equivalent LTA Concn (μM LTA)**
HN001 Pool 1	33	100	3.0
HN001 Pool 2	35	100	2.9
HN001 Pool 3	18	100	5.6
HN001 Combined LTA	32	100	3.1
HN001 Whole Cells	32	0.016	0.0005
DltD- Pool 1	22	100	4.5
DltD- Pool 2	27	100	3.7
DltD- Pool 3	9	100	11.1
DltD- Combined LTA	23	100	4.3
DltD- Whole Cells	23	0.045	0.0020
IM126 Pool 1	40	100	2.5
IM126 Pool 2	39	100	2.6
IM126 Pool 3	19	100	5.3
IM126 Combined LTA	36	100	2.8
IM126 Whole Cells	36	0.065	0.0018

Table 11: Concentrations of LTA Estimated from the PGP Chain Length
Using the average number of GroP repeating units in each LTA as determined by NMR spectroscopy (section 3.5.4.1), the average molar concentration of LTA equivalent to 100 μM PO_4 was calculated for each fraction. For all three strains, the more hydrophobic Pool 3 fraction has fewer phosphate groups per LTA molecule; therefore Pool 3 has a higher molar concentration of LTA than the main peak fractions at the same phosphate concentration. For the combined LTA samples, the weighted average PGP lengths were calculated using the proportion of the total LTA-phosphate represented by each fraction. The concentration values for the whole cell samples represent the final concentration of cell-associated LTA in the PBMC experiments containing 1×10^6 cfu/mL. The PGP lengths are calculated assuming a diacyl-anchored LTA. If some triacyl-anchored LTA is present, then the equivalent LTA concentrations in the table will be slightly overestimated.

3.5.6.1 HN001 LTA

The concentrations of TNF, IL-1β, IL-8 and IL-10 secreted by PBMCs in response to each of the three LTA fractions from HN001 (Figure 20A) are shown in Figure 36. The patterns of cytokines induced by each of the three fractions, i.e., the relative ratios of TNF, IL-1β, IL-8 and IL-10, were quite similar. This may indicate that the three populations of LTA are inducing cytokine secretion via the same PRR(s), in similar ways, due to the structural similarity between the populations.

While these patterns were similar, there were some differences between the amounts of TNF, IL-1β, IL-8 and IL-10 cytokines secreted by PBMCs in response to each of the three separate populations of LTA from HN001. These differences were not as great as the inter-strain variations observed in this study, however. Pool 1 LTA from HN001 appears to induce greater amounts of all four cytokines measured here at the same concentration of LTA, than do the other two pools (Figure 36). It is difficult to say why the HN001 Pool 1 and Pool 2 samples exhibit similar cytokine induction at 100 μM LTA-PO_4 but different concentrations at 25 and 50 μM. The concentrations of cytokines induced by the 100 μM sample do not appear to have reached the apparent saturation level of the assay. It is possible though, that the assay may be saturated at a different point for different LTAs if, for instance, different receptors or combinations of receptors are involved in their immune recognition. The standard error values for the means are very large for the cytokine concentrations induced by the HN001 Pool 1 fractions, particularly for TNF. Thus, the apparently inconsistent dose relationship for the HN001 Pool 1 samples in Figure 36 may be a result of these large errors. Testing these samples using PBMCs from a range of different human donors may make this clearer, and will be part of ongoing work on this project. NMR analysis showed that Pool 1 and Pool 2 from HN001 are structurally similar, the main difference being the slightly shorter PGP polymer of Pool 1 (33 vs. 35 GroP units, Table 9). It seems unlikely that this small difference in chain length is responsible for the difference in immune response, as Pool 3 LTA from HN001 has a much shorter PGP chain (18 GroP) and was a poorer inducer of cytokines than either Pool 1 or Pool 2 (Figure 36). There is some variation in the pattern of peaks in the Gro+sugar region (~ 3.7 – 4.1 ppm) of the ^{1}H NMR spectra for HN001 LTA (Figure 30), while this pattern is much more consistent in the

^{1}H spectra of LTA from IM126 (Figure 32) and the DltD- mutant (Figure 31). This variation may be due to differences either in the substituents on the GroP units, and/or in the environment around the substituents, and further experiments are necessary to determine this. The increased cytokine induction by Pool 1 seems unlikely to be due to sugar substituents on the PGP polymer other than GlcNAc, as the peak dispersion in the Gro-sugar CHα region of the HSQC spectra seen for some of the samples (section 3.5.4.3), does not appear in the spectra for HN001 Pool 1 or Pool 2, suggesting that GlcNAc is the only sugar subtituent on the GroP units in these two samples. It seems, then, that the greater immune response to Pool 1 may be due to differences in the glycolipid anchor moiety, which could be only partially analysed in this study, and warrants further investigation. It seems reasonable that minor differences in the structure of LTA, such as those identified between Pools 1 and 2 from HN001, should have less impact on the immune activity than the major structural changes observed between LTA from the DltD- mutant and its parent strain HN001. It is quite possible, however, that the immune response to LTA is more sensitive to changes in some structural elements than others.

The LTA in the more hydrophobic Pool 3 from HN001 had a similar degree of D-ala subtitution, but a much shorter PGP chain, and approximately twice the number of GlcNAc residues per chain (three times the % GlcNAc) compared to the main peak fractions, Pools 1 and 2 (Table 9). The later eluting Pool 3 showed a limited cytokine inducing ability, particularly for TNF, at 100 µM LTA-PO_4, relative to the main peak fractions. At the 25 and 50 µM concentrations, Pool 2 LTA was only able to induce very low concentrations of cytokines, so it is difficult to compare Pool 3 with both main peak fractions at the lower LTA-PO_4 concentrations. As a consequence of the shorter PGP chain of Pool 3 LTA, the molar concentration of Pool 3 LTA added to the PBMCs was higher than for Pools 1 and 2 at the same phosphate concentration (Table 11). This would be expected, however, to produce a higher cytokine response, rather than the lower response seen here. These lower responses are unlikely to be due to micelle formation; the Pool 3 fractions at 100 µM PO_4 were at concentrations below the identified CMCs (Table 3), and the Pool 3 fractions at 200 µM PO_4 from HN001 (and the DltD- mutant) did not exhibit dimished cytokine responses compared to the 100 µM PO_4 samples (Figure 23).

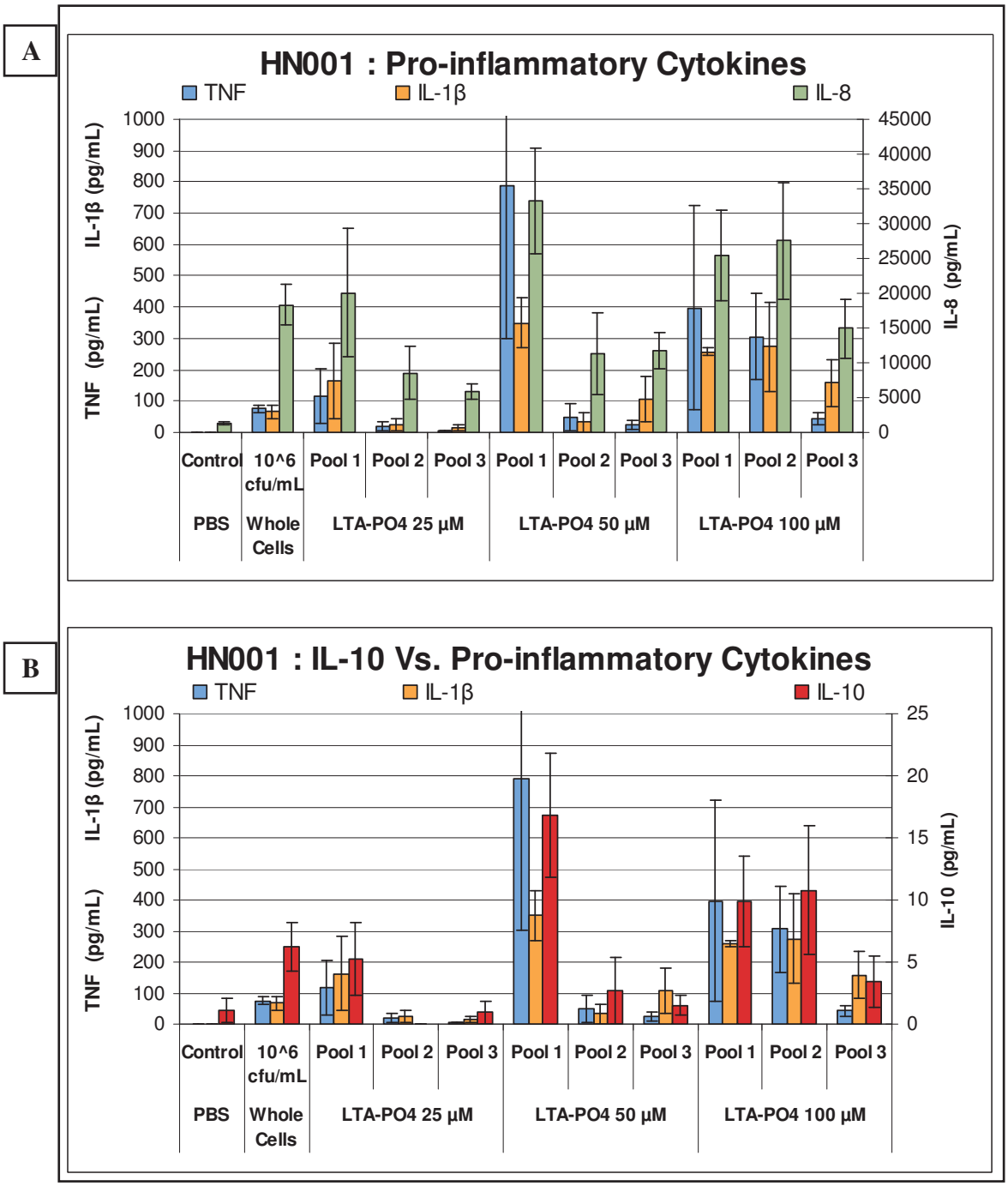


Figure 36: Cytokine Induction by Three Populations of LTA from HN001
PBS as a control, whole HN001 bacteria at 10^6 cfu/mL and separate fractions of LTA from HN001 at final concentrations of 25, 50 and 100 µM LTA-PO_4 were incubated with PBMCs for 24 hours. The HN001 whole cells sample contains ~ 0.016 µM LTA-PO_4 (section 3.4.5). The cytokine concentration values are means +/- SEM of three PBMC replicates for each sample. (A) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) and IL-8 (right-hand scale). (B) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) are compared with the anti-inflammatory cytokine IL-10 (right-hand scale).

If it is assumed that the membrane anchor moiety is the same for all fractions of LTA from HN001, then either the shortened PGP length and/or the greater GlcNAc substitution may be responsible for the weaker activity of Pool 3. There does not, however, seem to a be a general correlation between PGP length and immune potency evident in the literature. LTAs that are highly substituted with GlcNAc, such as those from *S. aureus* (15 %) and *B. subtilis* (25 %) have been reported to be highly potent stimulators of cytokine production, particularly when compared to *L. plantarum* LTA, which contains no GlcNAc but rather glucose and galactose substituents (Figure 35a vs. c) (Morath *et al.* 2002a; Ryu *et al.* 2009; Jang *et al.* 2011). These results are converse to the observations for LTA from HN001, where the LTA containing twice as many GlcNAc residues on average was shown to be a weaker inducer of cytokines. A synthetic LTA constructed with a single GlcNAc was shown to have similar IL-8 inducing activity in whole blood to the equivalent synthetic LTA with no sugar substituent (Deininger *et al.* 2007). These synthetic LTAs, however, contained only myristic acid (a saturated FA) in their anchor moieties, so they may be presented differently to immune cells, compared with the UFA-containing LTAs identified in this study. It is also uncertain how the position of the GlcNAc along this synthetic PGP chain compares with the distribution of sugar substituents in native LTA. There was considerable peak dispersion in the Gro-sugar CHα region of the HSQC spectra for this sample (section 3.5.4.3), that was not seen in the spectra for HN001 Pool 1 or Pool 2, which may be evidence of sugar substituents other than GlcNAc on the HN001 Pool 3 LTA. These would be much less abundant than the GlcNAc substituents detected here, but may nonetheless contribute to the immune response. In addition, there may be differences in the glycolipid anchor moiety unique to Pool 3 LTA, which need to be explored (section 3.5.4.5). Similar subpopulations of LTA were found in the Pool 3 fractions from both the DltD- mutant and IM126; this is further discussed in sections 3.5.6.2 and 3.5.6.3.

3.5.6.2 DltD- Mutant LTA

The concentrations of TNF, IL-1β, IL-8 and IL-10 secreted by PBMCs in response to each of the three LTA fractions from the DltD- mutant (Figure 20B) are shown in Figure 37. Once again, the pattern of cytokines induced for each of the three fractions from the DltD- mutant was similar, as for HN001. The differences in the cytokine

response between the fractions for the DltD- mutant are only seen at low concentrations of LTA in this assay (12.5, 25 μM PO_4) because at moderate concentrations (50, 100 μM PO_4), well below the CMC of 221 μM PO_4 (Table 3), the cytokine response appears to be saturated.

The most abundant LTA peak (Pools 1 and 2) was more heterogeneous for the DltD- mutant, having on average between 22 and 27 GroP units, than the main peak for HN001 or IM126 (Table 9). This is in contrast to both HN001 and IM126, where the two main peak fractions differed by only two or one GroP unit(s), respectively (Table 9). Pool 1 from the DltD- mutant was estimated to have FA anchors containing on average shorter acyl chains than those in Pool 2 (section 3.5.4.7). No D-alanine was detected, but the percentage of GlcNAc substituents on the PGP chain was 2 % for both Pools 1 and 2 (Table 9), or ~ 0.5 GlcNAc residues per LTA molecule. Even though their structures seem to be more dissimilar than LTAs from Pools 1 and 2 of the other two strains, the concentrations of cytokines induced by Pool 1 and Pool 2 from the DltD- mutant were quite similar (Figure 37). This suggests that the structural features that were found to differ between Pools 1 and 2 from the mutant, i.e., the average lengths of the PGP and FA chains, may have little or no involvement in the induction of cytokines, which is supported by a lack of consistent connection between chain lengths and immune activity of LTA in the literature.

The Pool 2 fractions from both HN001 and the DltD- mutant contain LTAs with similar average PGP chain lengths (35 and 27 units, respectively, Table 9); GlcNAc substitution (3 and 2 %, respectively, Table 9) and estimated FA chain lengths (integrals for the FA_CH2γ-ω peak of 17.5 and 16.9, respectively, Table 5). For the structural features identified in this study, the most obvious difference between the average structures of these two otherwise quite similar LTAs from HN001 and the DltD- mutant is the lack of D-alanyl esters on the DltD- LTA. It cannot necessarily be assumed that the DltD- mutant shares the same glycolipid(s) as the parent strain, and differences in this region may be important for immune recognition, so these structural details must be determined for both the WT and the mutant LTAs in future. Nevertheless, this strongly suggests that D-alanine substituents are important for the immune recognition of LTA from HN001.

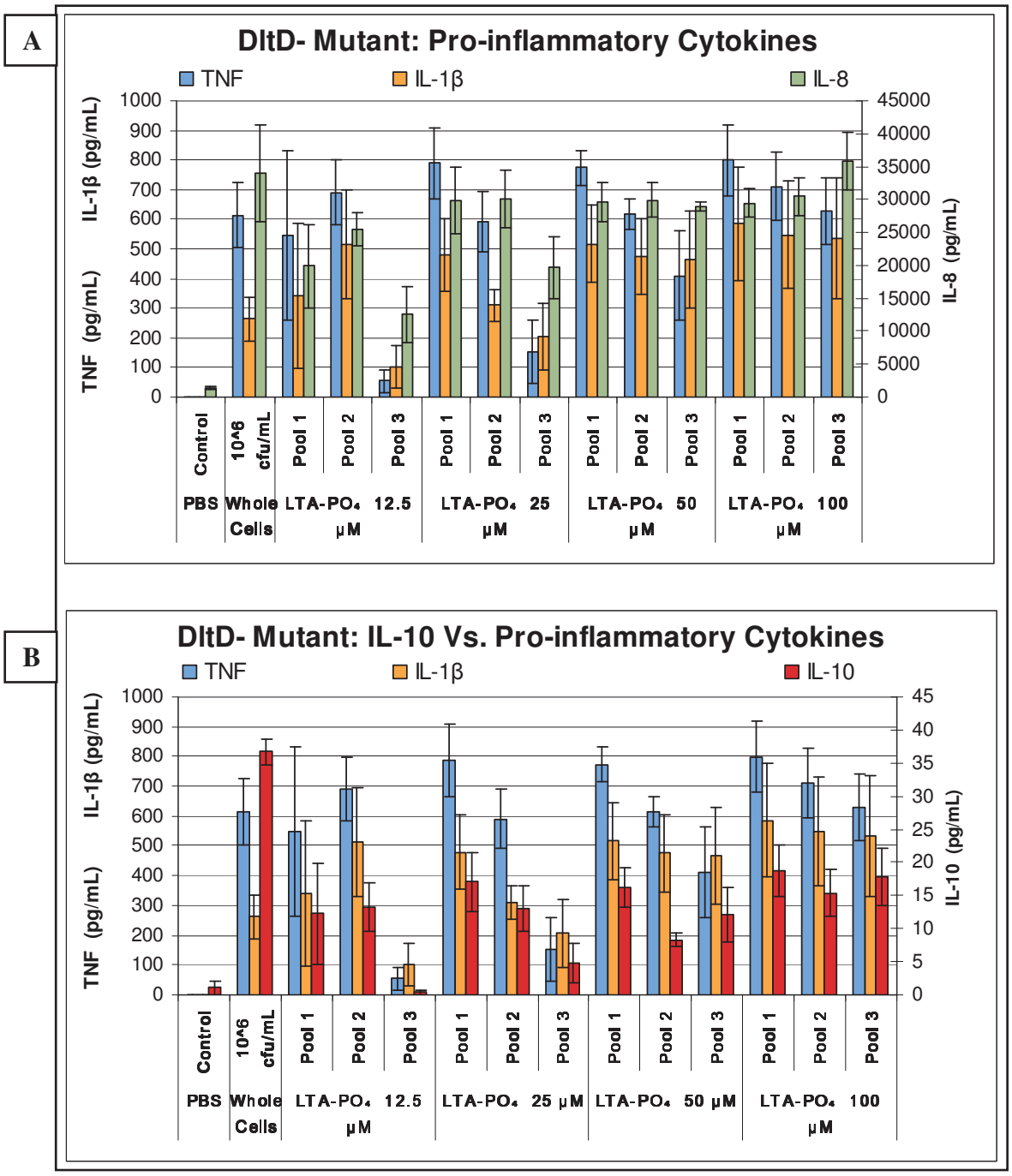


Figure 37: Cytokine Induction by Three Populations of LTA from the DltD- Mutant
PBS as a control, whole DltD- mutant bacteria at 10^6 cfu/mL and separate fractions of LTA from the DltD- mutant at final concentrations of 12.5, 25, 50 and 100 µM LTA-PO_4 were incubated with PBMCs for 24 hours. The DltD- mutant whole cells sample contains ~ 0.045 µM LTA-PO_4 (section 3.4.5). The cytokine concentration values are means +/- SEM of three PBMC replicates for each sample. (A) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) and IL-8 (right-hand scale). (B) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) are compared with the anti-inflammatory cytokine IL-10 (right-hand scale).

Pool 2 LTA from the DltD- mutant had no D-alanine substituents and was able to induce significant amounts of TNF, IL-1β, IL-8 and IL-10 at concentrations as low as 12.5 µM LTA-PO_4, while the structurally similar but 77 % D-alanylated Pool 2 from HN001 required at least 100 µM LTA-PO_4 to induce a comparable response (Figure 38A and B). HN001 Pool 2 LTA at 12.5 µM PO_4 induced similar levels of these cytokines to the PBS control. Taking into account the effective LTA concentrations in Table 11, the Pool 2 DltD- LTA is only slightly more concentrated than that of Pool 2 HN001 LTA: ~ 1.3-fold, at the same phosphate concentration. Thus, comparing the samples in Figure 38 in terms of molar concentration of LTA, Pool 2 LTA from the DltD- mutant at ~ 0.5 µM (12.5 µM PO_4) induced concentrations of TNF, IL-1β and IL-8 that were ~ 36, 21 and 3 times higher, respectively, than Pool 2 LTA from HN001 at ~ 0.7 µM (25 µM PO_4). This suggests that certain structural features of LTA, including the degree of D-alanylation, have a much greater impact on the immune stimulation compared to the average length of the PGP and FA chains.

The cytokine induction by Pool 3 LTA from the DltD- mutant was less at concentrations of 12.5 and 25 µM LTA-PO_4 than at 50 and 100 µM LTA-PO_4; whereas the amounts of cytokines induced by the Pool 1 and 2 fractions from the mutant were maintained at approximately similar levels for concentrations of 12.5 - 100 µM PO_4 (Figure 37A and B). For LTA Pools 1 and 2 from this strain, the difference of 5 units between the lengths of the PGP chains (Table 9) did not seem to impact much on the immune activities (Figure 37A and B). The DltD- Pool 3 LTA, however, had an average of only 9 GroP units per molecule (Table 9). As the PGP length value determined using NMR is an average, it is possible that this sample contains some LTAs that have even shorter PGP polymers. It is possible that there is a threshold for the PGP length of LTA required for immune recognition that is not met by some of the LTA molecules within the Pool 3 population. Synthetic LTA based on that of *S. aureus* but made with only six GroP repeating units has been shown to be at least as active as full length native *S. aureus* LTA (Morath *et al.* 2002b). This synthetic LTA, however, was shown by NMR analysis to be a homogeneous sample of uniform PGP length. Biological LTA samples, however, are defined by their average PGP length and are likely to be heterogeneous, which may explain differences between immune responses to artificial and native LTAs.

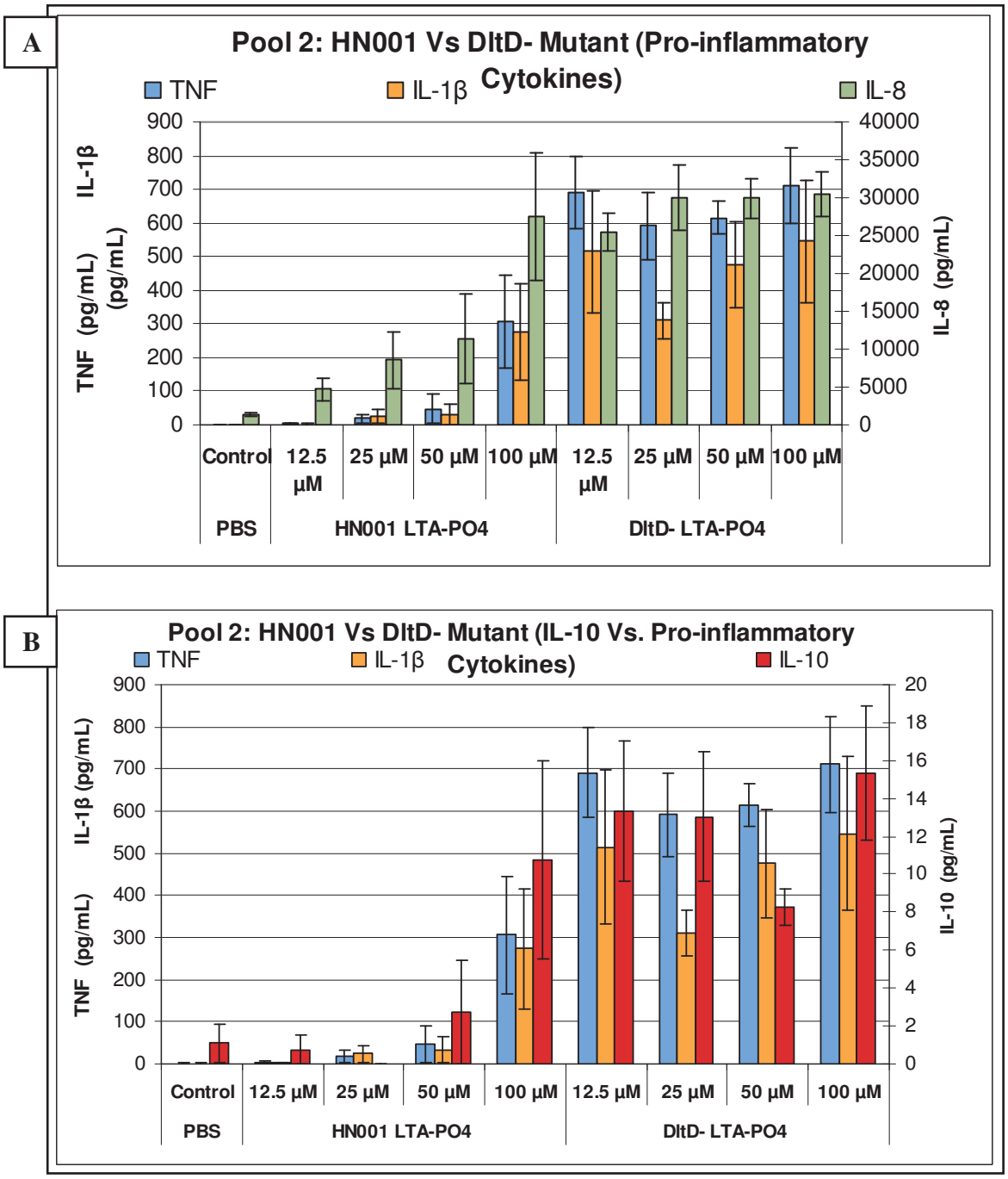


Figure 38: Cytokine Induction by the Pool 2 LTA fractions from HN001 and the DltD- Mutant
The PBS control, and the Pool 2 LTA fraction of LTA from both HN001 and the DltD- mutant at final concentrations of 12.5, 25, 50 and 100 µM LTA-PO_4 were incubated with PBMCs for 24 hours. The cytokine concentrations are mean values +/- SEM of three PBMC replicates for each sample. (A) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) and IL-8 (right-hand scale). (B) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) are compared with the anti-inflammatory cytokine IL-10 (right-hand scale).

As well as the shorter PGP chain length, Pool 3 LTA from the DltD- mutant has a higher GlcNAc substitution than both Pools 1 and 2 (8 % vs. 2 %, Table 9). As this is also seen for the Pool 3 fractions from HN001 and IM126, both of which contained more GlcNAc and induced lower concentrations of cytokines than Pools 1 and 2, it is

possible that the higher ratio of GlcNAc to GroP is responsible for the weaker cytokine response. However, as methods to specifically manipulate the degree of GlcNAc substitution of LTA have not been developed, there is no evidence in the literature to support such a conclusion. Both *B. subtilis* and *S. aureus* have LTAs which are highly pro-inflammatory and highly substituted with GlcNAc (Morath *et al.* 2001; Morath *et al.* 2002a). The different effects of GlcNAc substitution on these LTAs and those in this study may be due to the dissimilar compositions of their FA anchors (section 3.5.4.5). It should be noted that the possibility of sugar substituents other than GlcNAc may be a factor in this study (section 3.5.4.3). It is possible that Pool 3 LTA from HN001 contains a subpopulation that contains small amounts of glucose and/or galactose on the PGP chain, while Pools 1 and 2 are only modified with GlcNAc. The specific effects of different sugar substituents on LTA on the induction of cytokines have not been elucidated, and should be explored in future research on LTAs. A complete structural analysis of the glycolipid moiety of these LTAs should also be carried out, which may highlight other distinct features of the Pool 3 LTA samples. It is clearly difficult to attribute the lower cytokine concentrations induced by Pool 3 to differences in a single structural element of LTA. The results of this study seem to suggest that multiple features are involved in the interaction with PRRs, and that the overall structure of LTA is somehow involved in recognition.

3.5.6.3 IM126 LTA

The concentrations of TNF, IL-1β, IL-8 and IL-10 secreted by PBMCs in response to each of the three LTA fractions from IM126 (Figure 20C) are shown in Figure 39. The three fractions of LTA from IM126 shared a similar *pattern* of cytokine induction (Figure 39A and B), as that observed for the subpopulations of LTA from other two strains. There are some differences, however, in the magnitude of the cytokine response between some of the fractions. The differences are not clear-cut, due to the relatively low cytokine levels induced by IM126 LTA and the high standard errors observed for some samples. Consequently, it is difficult to draw conclusions about the differences between the separate fractions of LTA from this strain. Future testing of these fractions using a range of different donors may reveal clearer differences, and confirm that isolated LTA from IM126 is consistently a weak inducer of cytokines.

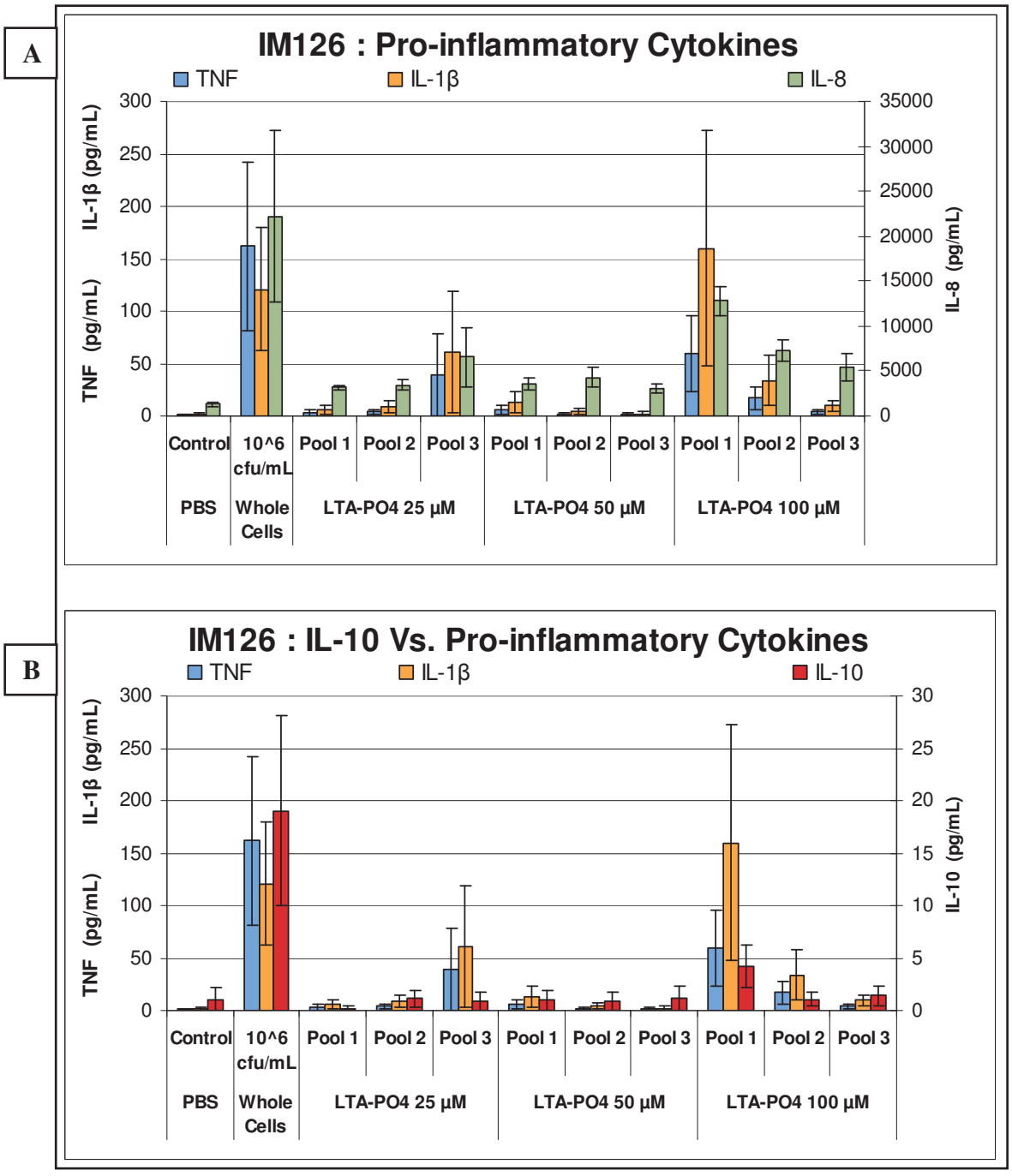


Figure 39: Cytokine Induction by Three Populations of LTA from IM126
PBS as a control, whole IM126 bacteria at 10^6 cfu/mL and separate fractions of LTA from IM126 at final concentrations of 25, 50 and 100 µM LTA-PO_4 were incubated with PBMCs for 24 hours. The IM126 whole cells sample contains ~ 0.065 µM LTA-PO_4 (section 3.4.5). The cytokine concentration values are means +/- SEM of three PBMC replicates for each sample. (A) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) and IL-8 (right-hand scale). (B) Pro-inflammatory cytokines TNF, IL-1β (left-hand scale) are compared with the anti-inflammatory cytokine IL-10 (right-hand scale).

There was little structural difference observed between Pool 1 and Pool 2 LTAs from IM126 in the present study (Tables 5 and 9). The slightly higher cytokine inducing activity observed at 100 µM LTA-PO_4 for Pool 1, compared to Pools 2 and 3 (Figure 39A, B) may not be significant, as one of the triplicate PBMC assays for this particular fraction contained much higher concentrations of cytokines than the other two.

As observed for the other two strains, the LTA in the more hydrophobic fraction, Pool 3, had a shorter average PGP chain (19 versus 39 – 40 units) and was also more highly subtituted with GlcNAc (13 %, ~ 2.5 GlcNAc per molecule) compared to the main peak fractions (2 - 3 %, ~ 0.8 - 1.2 GlcNAc per molecule, Table 9). Thus, differences in elution time from HIC could be related to differences in structure between the different fractions of LTA from IM126. The influence that these structures might have had on the immune response to each fraction, however, could not be ascertained, as the concentrations of cytokines induced by all samples of LTA from IM126 were relatively low (Figure 39). It is interesting, however, that this Pool 3 subpopulation of LTA seems to follow a consistent structural pattern in all three strains tested in this study. Further analysis of the cytokine inducing activity of these fractions using different approaches may help to explain this observation.

3.5.7 Structure-Function Analysis Excluding the Free D-Ala

The amounts of free D-alanine measured in the LTA samples in this study are higher than those normally reported in the literature (section 3.5.3). This is obviously not ideal for any structural analysis, and is a particular problem when comparing different LTA samples that seem to have lost dissimilar amounts of D-ala. Although the cause of this loss is not known, future studies should attempt to solve or minimise the problem. Until further experiments to determine the source of the free D-alanine in the spectra for HN001 and IM126 are completed, the unlikely possibility remains that the free D-ala did not originate from the LTA, but originated from the culture media or cells. For example, free D-ala could have associated non-covalently with the LTA and been carried through the purification process (although there is no other evidence to suggest that this occurred). Such a scenario seems unlikely, as the alanine found in media or cells is predominantly L-ala. Also, given the lack of free D-ala in the DltD- mutant LTA

samples, it seems likely that it originated from the LTA. If the free D-ala did originate from the LTA, it is not known at which point hydrolysis occurred. It is possible that the D-ala was hydrolysed from the LTA during or after chromatography, but before the immune cell assays were carried out, which is likely to affect the assays. Therefore, we must consider the possibility that the observed free D-ala did not contribute to the immune activity as a constituent of the LTA molecules.

The proportion of D-ala:GroP and non-substituted GroP units were re-calculated based only on the D-ala that was confirmed as being bound to the LTA, using the integrals for the Gro-D-ala peak at 4.25 ppm (section 3.5.4.2) and listed in Table 12. All of the LTA fractions from both HN001 and IM126 were still found to be substituted with significant amounts of D-alanine. The DltD- mutant samples contained no D-ala, so are unaffected by this re-calculation, but are included in the table for comparison.

Sample	PGP Length	D-Ala %	GlcNAc %	Non-substituted %	Free D-Ala (% of PGP)
HN001					
Pool 1	33	**37**	3	**60**	42
Pool 2	35	**24**	3	**73**	53
Pool 3	18	**39**	9	**52**	45
DltD- Mutant					
Pool 1	22	nil	2	98	0
Pool 2	27	nil	2	98	0
Pool 3	9	nil	8	92	0
IM126					
Pool 1	40	**55**	3	**42**	21
Pool 2	39	**53**	2	**45**	20
Pool 3	19	**54**	13	**33**	24

Table 12: Structural Elements of LTA Calculated Excluding the Free D-Ala Peaks
This table summarises the data in Tables 6, 7 and 8. The average chain lengths of the PGP head group of each LTA, as well as the average proportion of substituents to the PGP (either D-ala or GlcNAc) were calculated from the integrals of peaks in the ^{1}H NMR spectra. The values for the D-ala % represent the substitution based only on the D-ala proved to be chemically bound to the PGP at the time the spectra were recorded. The amount of free D-ala (not chemically bound to the LTA) in each sample, relative to the amount of GroP, is given in the right-hand column. The values that are different to those in Table 9 are shaded in grey.

As all of the LTA from HN001 was still substituted with significant amounts of D-ala, the comparisons of the HN001 WT LTA with the LTA from the DltD- mutant, which completely lacked D-ala, are essentially unchanged. The removal of the D-alanine from the LTA resulted in both mutant cells and altered LTA that induced a much larger

cytokine response in PBMCs than did the cells or LTA from the WT parent. This is one of the most important findings of this research project, and is not affected by the loss of D-alanine. Other significant findings, such as evidence of unsaturated fatty acids in the anchor of the LTAs in this study, and the appearance of GlcNAc substituents, which are not usually seen on the LTA from lactic acid bacteria, are unaffected by the lower D-alanine concentrations.

Including the free D-alanine peaks, the estimated D-ala substitution of ~ 75 – 80 % (Table 9) for LTA from HN001 and IM126 is similar to that reported for LTA from the other reported LTA from this species, *L. rhamnosus* GG (74 % D-ala, Figure 35b and Table 10). The re-calculated D-ala substitution values *excluding* the free D-alanine: ~ 30 % for HN001 LTA and ~ 54 % for IM126 LTA are quite different to that reported for *L. rhamnosus* GG. Other probiotic bacteria that exhibit immunomodulatory effects have, however, been shown to have LTAs with less than 50 % D-ala substitution, for example, *L. plantarum* NCIMB8826 (42 %) and *Lactococcus lactis* G121 (20 %), shown in Figure 35 and Table 10 (Grangette *et al.* 2005; Fischer *et al.* 2011). Similarly, LTAs from pro-inflammatory strains vary widely in their D-ala substitution, e.g., *S. aureus* DSM20233 (70 %), Group B *Streptococcus* COH1 (46 %) and *B. subtilis* DSMZ 1087 (25 %) (Morath *et al.* 2001; Morath *et al.* 2002a; Henneke *et al.* 2005). Thus, HN001 and IM126 would not necessarily lose their probiotic properties if the D-ala content of their LTA were reduced in contrast to being removed. This study has shown the effects of complete removal of the D-alanine from LTA, but the impact of specific LTAs with a range of different D-ala substitutions has not been examined in detail. Synthetic LTAs have been used to show the importance of different proportions of D-ala on the induction of cytokines, but these typically have very short PGP chains, so they may not accurately approximate the effect on native LTAs. Thus, the relationship between the nature and/or magnitude of immune responses to LTA and the degree of D-alanyl esterification is not consistent across gram positive bacteria, and the mechanism by which D-alanylation might modulate these immune responses to LTA has not been explained, and requires further investigation.

The values for the D-ala substitution of LTA given in the previous sections (Table 9) showed that LTA from both HN001 and IM126 were ~ 75 – 80 % substituted with D-ala, and that all of the fractions were substituted to with a similar degree. If the free

D-ala is excluded from the calculations, then the D-ala substitution on LTA from HN001 LTA is different to that on IM126 LTA. The LTAs in order of approximate average D-ala substitution are:

$$\text{IM126 (}\sim 54\ \%\text{)} > \text{HN001 (}\sim 30\ \%\text{)} > \text{DltD- mutant (0 \%)}$$

Calculated this way, the cytokine concentrations induced by isolated LTA from each strain seem to be inversely correlated with the D-ala substitution (Figure 25). This correlation seems consistent with the partial hydrolysis of D-ala residues occurring before the PBMCs were exposed to the LTA samples, although further evidence is required to show this. The Pool 2 LTA fraction from HN001 was 24 % substituted with D-ala, and seemed to induce slightly *lower* concentrations of cytokines than Pool 1 from HN001, which had 37 % D-ala substitution (Figure 36A and B), even though these two fractions were otherwise structurally similar. This structural difference may explain why the cytokine response to Pools 1 and 2 from HN001 differed; although it is not consistent with the above trend of cytokine induction potency increasing inversely proportional to D-ala substitution.

The isolated LTA in this study appeared to be less effective at inducing IL-10, relative to the pro-inflammatory cytokines, than the whole cells for each strain. It is possible that the partial hydrolysis of D-ala from the LTAs from HN001 and IM126 is responsible for this difference. This seems unlikely, however, as a similar observation was made for the DltD- mutant, which has no D-ala substituents on its LTA.

The three fractions of LTA from IM126 were calculated to have approximately equal levels of D-ala substitution, of 55, 53 and 54 % for Pools 1, 2 and 3, respectively, and as expected, the three fractions gave results consistent with the similarity in D-ala substitution (sections 3.5.4.8 and 3.5.6.3).

The D-ala substitution values in Table 12 do not seem to affect the identification of the Pool 3 fractions as a distinct subpopulation of LTA from each strain with weaker immune potency, when compared to the values including the free D-ala. In the case of HN001, the Pool 3 LTA has a similar D-ala substitution to Pool 1, and in IM126 all three pools contain very similar proportions of D-ala. The Pool 3 fraction from the

DltD- mutant also seemed to induce a weaker cytokine response compared to the more abundant fractions, and this could not be dependent on D-alanine.

The D-alanine substituents of LTA are able to be spontaneously redistributed along the PGP chain, independent of enzymes (Childs *et al.* 1985). Whether this has occurred in the LTA samples in this study after the partial hydrolysis of the D-alanine is not known. If, for instance, the hydrolysed D-ala residues were all lost from one end of the PGP chain, redistribution of the remaining D-ala residues may result in them being spaced further apart, which may alter a specific spatial motif involved in immune recognition of LTA. It is not known whether D-ala is involved in such recognition, but if this is the case, then the different degrees of D-ala substitution in Table 12, compared to those in Table 9, may be significant.

The origin of the free D-ala clearly needs to be clarified. Thus, it will be interesting to see if conditions can be found for purification of LTA from HN001 and IM126 that allow minimal loss of the D-alanine. Measurement of the cytokine induction by such intact LTAs from the same strains alongside the partially hydrolysed LTAs in the present study might help to increase understanding of the relationship between D-ala substitution and immune potency of LTA. Retesting the LTA samples in the PBMC-CBA assay alongside aliquots of the samples analysed by NMR may give clues as to whether the hydrolysis of D-ala occurred before or after these aliquots were removed.

Chapter 4 - Conclusions & Future Directions

4.1 Conclusions

Highly purified LTA from HN001, the DltD- mutant and IM126 induce the secretion of TNF, IL-1β, IL-8 and IL-10 in PBMCs, which is typical of immune responses to LTA reported in the literature. The cytokines IL-4, IFN-γ, IL-12 and IL-17 were also measured, but were not secreted by the PBMCs exposed either to the LTA samples or to the whole bacteria used in this study. The pattern of cytokine induction by each LTA was similar to that of its parent bacteria for each of the three strains, with the exception of lower levels of IL-10 induction by LTA relative to the other cytokines, suggesting that LTA is responsible for at least part of the cytokine response of PBMCs to the whole cells. The immune response to LTAs from HN001 and the DltD- mutant mimicked the response to their respective parent cells more closely than did the LTA from IM126. LTAs from all three strains appeared to induce the secretion of less of the anti-inflammatory cytokine IL-10, relative to the pro-inflammatory cytokines, than the whole cells. This was surprising, as IM126 cells induce higher concentrations of IL-10 than do HN001 cells, yet the isolated LTA from IM126 was a less potent inducer of IL-10 than HN001 LTA. These results suggest that there may be non-LTA factors in the cells that, either separately or together with LTA, are responsible for the immune response to whole cells. One likely reason for this discrepancy is the difference in presentation of LTA on the intact cells to immune receptors when compared to the free molecule.

LTA forms micelles at high concentrations, which has been shown in this work to inhibit the immune response in PBMCs. It is not known whether this effect occurs *in vivo*, although it seems unlikely that the concentrations of LTA *in vivo* would rise above the CMC. The CMCs were determined for the LTAs in this study and are similar to the value reported for LTA from *S. aureus*.

NMR analysis allowed structural characterisation of the purified LTAs. The structures of LTAs from the three strains studied, HN001, the DltD- mutant and IM126, are Type I, consisting of a polymer of glycerol phosphate, connected to a glycolipid anchor. The LTAs from both HN001 and IM126 are highly substituted with D-alanine, and those from all three strains contain GlcNAc substituents. D-alanine substitution is completely abolished from LTA after inactivation of the *dlt*D- gene in HN001. This

DltD- mutant produced LTA that had other altered structural elements, including shorter PGP chains and possible changes to the FA chain lengths. Substitution with GlcNAc, however, was similar to that seen in the WT. The identification of GlcNAc substituents on the LTA from the strains of *L. rhamnosus* in this study is interesting. GlcNAc is commonly found on the LTA from other gram positive bacteria, including *S. aureus* and *B. subtilis*; however, the lactic acid bacteria that have had their LTA structures characterised to date are most commonly found to contain non-acetylated sugar substituents, such as glucose or galactose, if any. It seems then, that the GlcNAc substituents may be involved in the responses of immune cells to both pro- and anti-inflammatory LTAs. A number of studies have collectively shown that reducing the D-alanyl ester content of LTA alters the immune response observed when the PGP chain is glycosylated, but has little to no impact when there are no sugar substituents (Morath *et al.* 2001; Grangette *et al.* 2005; Hasty *et al.* 2006; Perea Velez *et al.* 2007; Rockel *et al.* 2010; Jang *et al.* 2011). The results in this thesis are consistent with this observation, as removal of the D-alanyl esters from the LTA of HN001 altered the immune response to both the LTA and its cognate whole cell, and the LTAs from both HN001 and the DltD- mutant are substituted with GlcNAc. It is possible that the sugar substituents are involved in the recognition of LTA by immune receptors and that D-alanyl esters block or otherwise alter this interaction. Immune recognition of LTAs that have sugar substituents probably results in formation of immune complexes made up from different combinations of receptors and signalling proteins, compared with LTAs that lack sugar substituents. If this is the case, then the removal of D-alanyl esters may change the impact of the immune responses generated by different immune complexes.

The *dlt*D- mutation affects cell morphology, and growth characteristics of bacteria, so it is quite possible that some other MAMPs in the DltD- mutant of HN001 are altered compared to the WT organism, and/or that the presentation of these MAMPs to immune cells is different on the mutant cells. Thus it was important to test the immune response to isolated LTA from this mutant. The results showed that the structure of the LTA was indeed changed, and that it was the altered LTA, and not merely the other changes to the mutant, that was responsible for at least part of the modified immune response to the cells.

Differences between the average PGP and/or FA chain lengths of LTAs may affect the immune response, but these do not appear to be as important as the D-ala and sugar substituents on the chain, and probably other features of the anchor such as the degree of saturation of the fatty acyl chains, in the strains used in this thesis and in the literature. It is possible that the incorporation of D-ala has some effect on the biosynthesis of LTA, as the LTA from *dlt*- mutants both in this study and in the literature are often different in chain length and sugar substitution compared to the WT strains. The importance of the distribution of specific chain lengths within heterogeneous populations of LTA is not yet known. Jang *et al*. (2011) suggest that the differences between LTAs from pathogenic and non-pathogenic/probiotic bacteria that are most important for immune recognition are the saturation and chain length of the lipids, and the sugar substituents on the PGP chain. To verify this, further exploration through the characterisation of LTAs from different strains with a wide range of specific immunomodulatory profiles is required. LTAs from *L. rhamnosus* HN001 and IM126 were shown to contain unsaturated fatty acids, and may possess a third fatty acyl chain, similar to *L. plantarum* LTA, which may be the reason why these strains induce low pro-inflammatory responses compared with those of *S. aureus*. HN001 and IM126 LTAs, in contrast, contain N-acetylated sugar substituents, which have been found on the LTAs of *S. aureus* and other pro-inflammatory strains, but not on other lactobacilli. These structural differences may explain the distinct immunobiological activities observed for the strains in this study, compared to those observed for *L. plantarum*, *S. aureus*, and other strains of gram positive bacteria.

For *L. rhamnosus* HN001, and some other strains, the degree of D-ala substitution on LTA is important for the immune response. Complete abolishment of D-ala from HN001 has a large impact, but smaller differences in D-ala substitution between subpopulations of LTA from HN001 and IM126 seem to have a less measurable impact. Partial hydrolysis of D-ala on LTA was observed in this study, which was not reported by other researchers also using similar mild methods designed to prevent this. One consequence of the hydrolysis may have been that the purified LTAs were not structurally identical to the LTAs on cells, making immunobiological comparisons between them less straightforward. The observed immune responses to the isolated LTA appeared to be more consistent with calculations of the D-ala % excluding the free D-ala. Therefore the reasons for the hydrolysis should be investigated with a view to

eliminating this problem, allowing fair comparisons between isolated LTAs and those on bacteria. The impact of abolition of D-ala esters from the LTA from HN001 appears to be contrary to that of elimination of D-ala on LTA from all other strains reported to date. It has been suggested that the LTA D-alanylation system of bacteria could be a target for new antibacterial drugs. However, this should be considered carefully, as this study has shown that the removal of D-alanine from LTA does not always have the same impact on the immune response induced by different strains of bacteria.

In discussing their observations of *L. plantarum*, Grangette *et al.* (2005) state: "Therefore, perhaps in the absence of biologically active LTA, unmasked peptidoglycan of the Dlt- mutant strain may exert an inhibitory effect on the pro-inflammatory NF-κB cascade through NOD2 signalling.". Although this may be the case for *L. plantarum*, it cannot explain the *increased* immune response to the DltD- mutant of HN001, which has LTA that is similarly reduced in D-alanyl ester content. Both Grangette *et al*. and this study observed cytokine induction by isolated LTAs that paralleled that of the whole cells. Perhaps it is indicative of a difference in structure of the respective peptidoglycans from *L. plantarum* and *L. rhamnosus,* which is reflected in their immune potency. *L. plantarum* has WTA molecules covalently attached to its peptidoglycan, and the D-alanyl ester substituents of WTA are derived from LTA. As a result, the Dlt- mutation would also have altered the WTA, in contrast to *L. rhamnosus*, which does not have WTA. Some researchers have posited that the immunomodulatory activity of purified LTA is entirely due to contaminating lipoproteins. If the activity measured for LTA was due entirely to this contaminant, it would be difficult to explain why the removal of D-alanines from the LTA reduced the potency of this contaminant in *L. plantarum* LTA samples, yet enhances the potency of *L. rhamnosus* LTA samples. This would imply that HN001 contains lipoproteins that are very different to those from *L. plantarum*. Although possible, specific lipoproteins inducing this immune activity have not yet been identified. A mutant of *S. aureus* with an inactivated lipoprotein diacyl-glycerol transferase, and therefore lacking lipoproteins, was shown to produce LTA that induced the secretion of lower concentrations of IL-1β than LTA from the WT organism (Rockel *et al.* 2010). It is conceivable, however, that such a mutation may have caused structural alterations to the LTA, which was not structurally characterised.

The Pool 3 samples are clearly different to main population of LTA, and may serve a unique purpose in the cell. Even though this subpopulation represented only 12 – 14 % of the total LTA-PO_4, it may be important for the specificity of the immune response. A similar separate population was not apparent in the HIC chromatogram of LTA from *S. aureus*, which induces very high concentrations of cytokines, so perhaps the Pool 3 subpopulation of LTA identified in *L. rhamnosus* is able to modulate the cytokine response in some way. Pool 3 LTA samples, however, induced the production of lower concentrations of anti-inflammatory IL-10 compared to Pools 1 and 2. Perhaps the Pool 3 subpopulation competes with more potent LTAs for receptor binding without triggering a cytokine response. It might be possible to manipulate probiotic bacteria in some way to control the biosynthesis of this subpopulation of LTA, to understand its role and eventually generate more desirable immunomodulatory effects.

It is becoming evident that multiple structural elements of LTA are able to influence the immune response to bacteria, which may explain the inconsistency with which LTAs from different strains appear to depend on particular parts of the molecule for their activity, e.g., the D-alanine substituents. This may also be a reason that not all LTAs seem to induce immune responses through the same PRR(s). It has been shown that different heterodimers of TLR proteins are required for the recognition of different types of MAMP. Possibly, LTAs with different structures are recognised by different combinations of PRRs and their co-receptors. The dependence of immune responses to LTA on TLR2 is currently under debate. *S. aureus* LTA has been shown by one group to induce cytokines in a CD14 dependent, but TLR2- and TLR4-independent manner (Hattar *et al.* 2006), contrasting with previous work that showed *S. aureus* LTA induced cytokines via TLR2, independent of TLR4 (Schroder *et al.* 2003). Bunk *et al.* (2010) showed that a mutant of *S. aureus* lacking lipoproteins produced LTA that required TLR2, TLR6, CD14 and needed to be immobilised then internalised by immune cells, in order to induce an immune response. It is important to note that the conditions under which immune experiments are done are important. Often, researchers attempt to determine the dependence of LTA on a particular PRR, such as TLR2, by using host cell lines transfected with the PRR of interest. Such cell lines may lack other PRRs or other immune factors required for the response. Given that the specific immune response to specific LTA structures may depend on different PRRs or combinations of

PRRs, the use of such limited models may not give a true indication of the situation *in vivo*.

The lipid moiety of LPS from gram negative bacteria is the principal component of this molecule recognised by immune cells. Therefore LPS must be shed from the bacteria, or released after lysis by host cells such as APCs, in order to be efficiently recognised. By estimating the amount of LTA per cell for the strains in the present study, it has been shown that isolated LTA in solution is highly inefficient at inducing cytokines, compared to LTA on cells. Immune recognition of LTA may differ from that of LPS, and this study suggests that LTA may be more dependent on the presentation of the molecule on the cell surface in the context of other MAMPS. This may explain why the role of LTA in the immune response to gram positive bacteria has not been unequivocally defined, in contrast to the well-defined importance of LPS in gram negatives. The use of LTA alone as a bioactive ingredient or supplement may therefore be limited; however, it is possible that LTA combined with (an)other MAMP(s), and perhaps immobilised in some way to mimic the scaffold on the cell surface, may be therapeutically useful.

4.2 Future Directions

4.2.1 For the Current Project

To allow for donor to donor variation, the immune cell experiments in this study need to be repeated with several different PBMC donors.

The cause of D-ala hydrolysis in the NMR samples should be identified, and reduced or eliminated as far as possible to enable a true determination of the structural features of LTA. The storage conditions should be more closely examined; the effects of temperature, storage, buffering, etc., on the stability of the D-alanyl esters of LTA should be determined. Trials with higher grade solvents (i.e., N-butanol, N-propanol) and buffer components (i.e., sodium citrate, ammonium acetate) may identify potential sources of contaminants or D-alanine-hydrolysing compounds. To narrow down the source of the free D-ala in the NMR samples, an experiment could be performed where

a solution of purified LTA is spiked with free D-alanine, then re-purified using HIC. If the total quantity of D-alanine in the re-purified samples (as measured using NMR or enzymatic methods) was not increased compared to a non-spiked sample, this would confirm that the free D-ala in the current samples appeared post-chromatography.

The structural analysis of the LTA molecules in this study was incomplete. Further NMR and MS experiments are required to fully characterise the glycolipids of each LTA fraction in HN001, the DltD- mutant and IM126. The constituent sugars of the glycolipid, whether di- or tri-saccharide, as well as the fatty acid composition, must be determined to clearly show the structural differences between these LTAs. In particular, the number and location of the double bonds within the FA chains, and the existence of a third fatty acyl chain (and potentially a fourth as in *L. monocytogenes*, which has a second DAG on its LTA), may be important for specific immune recognition. Although GlcNAc substituents to the PGP chain were identified in all LTA fractions, additional sugar analysis by NMR, MS and/or HPAEC-PAD is necessary to identify the presence or absence of any substituents that were not detected.

Additional controls could be added to the PBMC assay. Standard *S. aureus* LTA tested alongside the *L. rhamnosus* LTAs in the same set of PBMC experiments would allow a direct comparison with a highly pro-inflammatory LTA. This would be conditional on the availability of a commercial *S. aureus* LTA free of LPS contamination and purified using the modern non-destructive butanol-extraction methods. The LPS inhibitor, polymyxin B, has been used by some researchers to confirm the absence of LPS-induced stimulation. The LAL assay provides an excellent test for LPS, and is widely accepted. Testing a selection of LTA samples both with and without the addition of polymyxin B in the PBMC assay would further validate this. Treatment of samples with peroxide to inactivate potential lipoprotein/lipopeptide contaminants has also been carried out by some laboratories; however, the structural integrity of LTA samples after such a treatment would always have to be checked. Mutants lacking lipoproteins have been constructed by researchers to allow the investigation of immune responses to purified LTA free of this contaminant (Rockel *et al.* 2010). It may be possible to develop similar mutants of HN001 and IM126, although such a change may alter other characteristics of the cell, as the DltD- mutant in this study did.

The anchor moiety of LTA is thought to be important for immune recognition, and the separated glycolipid of LTA has been shown by some groups to have immune activity. Studies where the isolated glycolipid and PGP chain have been tested for immune activity often used alkaline hydrolysis to separate the glycolipid from the hydrophilic chain. This may be useful to determine the structural requirements for induction of cytokine responses by LTA; however, de-acylation of the LTA with alkali is also likely to result in loss of D-alanine residues from the PGP chain. Thus, this method should not be used to examine the immune properties of the isolated PGP chain.

Biological LTA samples are inherently heterogeneous, making structural analysis difficult, and preventing the attribution of immune responses to specific LTA structures. Construction of synthetic LTAs based on the heterogeneous mixture of LTA found in *L. rhamnosus* would allow homogeneous samples of specific structures to be tested using the PBMC-CBA assay. This may be the only way to isolate the immune impact of variation in each structural feature, as modification of native LTAs, by chemical methods or by construction of mutant strains, seems more often than not to alter more than one aspect of the LTA.

The LTAs from IM126 and HN001 appear to be structurally similar, yet the immune responses to these two LTAs are very different. It would be interesting to see whether a *dlt*D- mutant of IM126 would have similar impacts on the bacteria and its LTA, compared to HN001. Discovery of other strains where the removal of D-alanyl esters enhances cytokine induction may help to explain why the DltD- mutant of HN001 produced such an unexpected change in immune profile, which was in contrast to other Dlt- mutants reported in the literature. While construction of Dlt- mutants allows the elimination of most or all of the D-alanyl esters from LTA, limited work has been done to examine the effects of different degrees of D-ala substitution. LTA from different strains often have different amounts of D-ala, but this is rarely the only structural difference between them. It may, therefore, be useful to measure the relationship between the proportion of D-ala substitution of LTA and the immune response to both the bacteria and the pure LTAs for a specific LTA structure. However, care is required as chemical methods employed to alter the D-ala substitution may damage other parts of the molecule, e.g., the lipid hydrolysed in alkaline conditions. A system similar to that used in *Lactococcus lactis*, where the *dlt* operon was placed under control of an

inducible promoter, could be used to tune the degree of D-alanylation on LTAs in strains of interest (Giaouris *et al.* 2008).

The importance of TLR2 in immune responses to LTA has been questioned by some. It would be useful to test the dependence on TLR2, and other PRRs such as TLR1, TLR6, CD14 and CD36 of immune responses to LTA from HN001, its DltD- mutant and IM126. Blocking antibodies could be used to compete with LTAs for PRR binding sites, or immune cells from knockout mice deficient in specific PRRs (e.g. TLR2 -/-) could be substituted for the PBMCs. Approaches such as these may reveal different dependencies of different LTAs on specific PRRs and their co-receptors, which is a potential mechanism for the strain specific recognition of LTAs.

4.2.2 LTA in General

The focus of many recent structural studies of LTA seems to be the hydrophilic polymer and its substituents, but future exploration of LTA should also include the detailed analysis of the glycolipid component. In particular, the saturation of the fatty acids and the number of acyl chains, as well as other features such as methyl and cyclopropyl substitutions, all of which may be important for immune recognition of LTA. It may be useful to alter the structure of the lipid moiety of LTA, either by construction of mutants with altered lipid, glycolipid or LTA biosynthesis, or by creation of synthetic LTAs with altered lipids but identical hydrophilic polymers. Such experiments are needed to clearly show the impact of different lipid anchors on the immune recognition of LTA.

The development of techniques to manipulate the saccharide substituents of LTA would assist in determining the involvement of specific sugars in the immune recognition of LTAs. Protocols for removal of sugar substituents by chemical or enzymatic means could be developed. Construction of mutants which produce LTA either lacking or containing alternative sugar substituents may be helpful, although this may require additional knowledge of the LTA-glycosylation pathways first. The use of synthetic LTAs could be extended to examine the importance of sugars while controlling the overall structure of the molecule.

Further analysis of the physical interactions of LTA with PRRs, in particular the TLRs, may help us to understand strain specific immune-responses to LTA. TLR2 is implicated in the recognition of LTA; however, this PRR is thought to be responsible for recognising a structurally diverse range of ligands (Kang and Lee). Further structural analysis of these TLR-ligand interactions is necessary to understand how these different complexes are formed, and how each gives rise to a specific immune outcome. The X-ray crystal structures of the TLR2/TLR1 and TLR2/TLR6 heterodimers, in complex with triacyl and diacyl lipopeptide ligands, respectively, have been determined (Jin *et al.* 2007; Kang *et al.* 2009). The crystal structure of LTA from *Streptococcus pneumoniae* bound to TLR2 has also been determined, and the diacyl lipid moiety of the LTA was found to be located in the same hydrophobic pocket as the diacyl-glycerol part of the lipopeptides (Kang *et al.* 2009). The authors report that the pneumococcal LTA-bound TLR2 was unable to form heterodimers with either TLR1 or TLR6, in contrast to the lipopeptide-bound structures. It should be noted, however, that the LTA from *S. pneumoniae* has a very different structure to that of the Type I LTA found in *S. aureus* and *L. rhamnosus* (Draing *et al.* 2006), so the same may not be true for all LTAs. Interestingly, Bunk *et al.* (2010) reported that the immune response to LTA from *S. aureus* was dependent on both TLR2 and TLR6, suggesting that the interaction may be similar to that with diacyl lipopeptide. Elucidation of ligand-bound structures may confirm this, and may answer the question of whether triacylated LTAs such as that of *L. plantarum* are recognised by a TLR2/TLR1 heterodimer. It is important then, to investigate the interactions of structurally different LTAs with a range of TLRs. If LTA interacts with TLR2 through the lipid anchor moiety, then the hydrophilic PGP chain may be free to interact with other PRRs or their co-receptors. Further research into the different immune complexes that are formed during recognition of various LTAs may help to explain how the structures of LTAs relate to strain-specific immune responses. Structural methods including X-ray crystallography and NMR will help with this, although X-ray crystallography is notoriously difficult for membrane-bound molecules such as these, owing to the inherent problems with obtaining diffraction quality crystals. A complete understanding of the structure of LTA may assist in prevention and treatment of infections by gram positive pathogens, and may also provide a potential basis for selection of new probiotics. Scientific evidence is required to substantiate reported health benefits of strains in order to satisfy the strict regulatory requirements for commercialisation of probiotics. Such evidence is usually provided by clinical trials;

however, these are both expensive and time-consuming, so is not practical to screen large numbers of strains in this way. New probiotics with highly specific immunomodulatory profiles could be selected based on the structure of their LTA, allowing more efficient selection of new strains to be included in clinical trials, and aiding in the development of probiotics to treat or prevent specific health conditions. Elucidation of the structural requirements for LTA and other MAMPs to modulate the innate immune system may also lead to the development of new immunomodulatory drugs, targeting receptors such as the TLRs.

The current knowledge of the functions of LTA, and the control of LTA biosynthesis, is limited. Two populations of LTA with dissimilar structures were identified in *Listeria monocytogenes*. Growth at 37 ºC increased the amount of the less abundant LTA, while the amount of the main LTA was maintained, when compared to cultures grown at room temperature. It was posited that this second LTA may assist the bacteria in evading host immune defences, triggered by the warmth of the host (Dehus *et al.* 2010). Further research into the potential functions of different subpopulations of LTA may reveal possible links between the environment and the composition of LTA. Manipulation of the proportions of subpopulations of LTA in probiotics may be possible, either by altering the culture conditions – pH, temperature, nutrients; or other means such as genetic modification, allowing the enrichment of bacteria with therapeutically useful subspecies of LTA, potentially creating improved probiotics. It may also be possible to induce bacteria to synthesise LTA containing specific glycolipids, by the construction of mutants, or perhaps by feeding bacteria with a particular glycolipid, or altering the growth conditions in other ways. In this way, probiotics could be tailored to produce a specific immune response.

The search for immunomodulatory molecules in bacteria was initially aimed at identifying the source of the highly inflammatory immune responses that are induced by prominent pathogens. The discovery that the innate immune system is also able to recognise non-pathogenic and commensal bacteria and produce a range of different strain-specific responses has changed the focus of this search. The identification of LPS as “the gram negative endotoxin”, and its proven potency as an immunobiological stimulant, led to a search for a similar major endotoxin in gram positive bacteria. While LTA has certainly been shown to be important for the immune recognition of gram

positive bacteria, it may not stand alone as "the gram positive endotoxin". It is becoming clear that the immune recognition of these microorganisms may be more complicated than first thought. Further investigation of other MAMPs in addition to LTA is necessary. Relationships between the responses to LTA and other MAMPs may be important in the overall immune response to lactic acid bacteria.

Gram positive MAMPs other than LTA have been shown to induce innate immune responses; the most abundant and most studied is peptidoglycan (Strober *et al.* 2006; Le Bourhis *et al.* 2007); but others such as lipoproteins, flagellins, nucleic acids (Kawai and Akira 2010) and in some strains, exopolysaccharide (EPS) (Sylvie *et al.* 2001; Liu *et al.* 2011), are though to be important. The structures of peptidoglycan are more widely conserved across different gram positive species compared to those of LTA (Vollmer 2008; Weidenmaier and Peschel 2008; Silhavy *et al.* 2010). Thus, while peptidoglycan has been shown to be recognised by PRRs of the innate immune system, most notably the NOD-like receptors, LTA seems more likely to be a determinant used by the immune system to distinguish between closely related organisms. Peptidoglycan has been shown to act in synergy with LTA to bring about septic shock (De Kimpe *et al.* 1995; Middelveld and Alving 2000; Thiemermann 2002; Wang *et al.* 2003). Protection against chemically-induced IBD (inflammatory bowel disease) by probiotic strains of LAB in mice was found to be dependent on both TLR2 and NOD2 (Foligne *et al.* 2007b), supporting a model in which the recognition of both a TLR2 ligand(s) such as LTA and a NOD2 ligand(s) such as peptidoglycan produce an immunomodulatory effect that cannot be generated by either MAMP alone. Watanabe *et al.* (2004) showed that NOD2, activated by the muramyl dipeptide (MDP) component of peptidoglycan, negatively regulated TLR2-dependent induction of inflammatory cytokines. MDP has also been shown to enhance induction of pro-inflammatory IL-8 by either LTA or LPS, although it exhibits little IL-8-inducing activity alone (Yang *et al.* 2001). Interestingly, it has been shown that LTA and LPS together do not synergistically interact, suggesting that such responses to MAMPs may have evolved to recognise specific species, as there are no bacteria that contain both LTA and LPS. There is cross-talk between many pathways in the innate immune system, which may contribute to the overall host response to any given strain (Kawai and Akira 2011; Kumar *et al.* 2011), adding a further layer of complexity to the possible immune

outcomes. It is possible that the combination of MAMPs presented by a strain increases the *specificity* of the immune response above that due to the individual components.

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