The structure of Lipid A and composition of the oligosaccharides of the lipopolysaccharides of the bacterial species: *Veillonella parvula*

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Abstract

Veillonella parvula is a Gram-negative bacterium known to be an abundant commensal coloniser of the human gut and mouth; it is linked to progression of childhood immune system but has also been found to be an opportunistic pathogen. Lipopolysaccharides (LPS) are glycoconjugates found in the outer membrane of almost all Gram-negative bacteria and are composed of three components: a glycolipid (lipid A), an oligosaccharide (the core region) and a repeating oligosaccharide unit (O-antigen).

Both Lipid A (the toxic component of LPS) and the polysaccharide side chains (the nontoxic but immunogenic portion of LPS) act as determinants of virulence in Gram-negative bacteria. O-antigens have adhered properties and these are resistance to phagocytes, protection toward to antigens and antigenic variation property. Lipid A act as an immune stimulator, which induces the biological responses of a specific organism

The LPS activates the host immune system, the lipid A through the Toll-like receptor 4/myeloid differentiation factor 2 (TLR4/MD-2) receptor complex in a structure dependent manner and the polysaccharide side chains, act as virulence determinants of the Gram-negative bacteria. Therefore, the structural elucidation of the LPS found in gut microbiota population is particularly interesting to understand the role of gut bacteria LPS in the activation and/ or suppression of the immune system response.

Successful separation and purification of the individual components from LPS (O-antigen, core- oligosaccharide and lipid A) of *Veillonella parvula* was achieved through a variety of chemical approaches, such as acetylated alditols derivatives, acetic acid hydrolysis and alkaline hydrolysis. Elucidation of the lipid A structure from a clinically isolated strain of *V. parvula*, grown within laboratory conditions, was achieved and gave insights into the high structural heterogeneity of lipid A from a gut bacterium. The most abundant species comprises a *bis*-phosphorylated hexa-acylated species with a variety of *iso*-branched acyl chains.

Additionally, the saccharide composition of the LPS of two separately grown strains of *Veillonella parvula* have been characterised and compared to give an insight into the complexity of the carbohydrate components of LPS from a gut microbiota species.

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Abbreviations

AA	Acetylated Alditols
AMG	Acetylated Methyl Glycosides
DMSO	Dimethyl sulphoxide
DMSO	Dimethyl Sulfoxide
DNAse	Deoxyribonuclease I from bovine pancreas
DOC	De-oxycholate
EPS	Exopolysaccharide
Gal	Galatose
GalN	Galactosamine
GC-MS	Gas Chromatography Mass Spectrometry
Glc	Glucose
GlcN	Glucosamine
Kdo	3-deoxy-D-*manno* octulosonic acid
LOS	Lipooligosaccharide
LPS	Lipopolysaccharides
MAPK	Mitogen-Activate Protein Kinase
MALDI -TOF	Matrix-assisted laser desorption/ionization Mass Spectrometry
MD-2	Myeloid Differentiation Factor 2
$NaBD_4$	Sodium borohydride
NMR	Nuclear Magnetic Resonance
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen associated molecular pattern
PMAA	Partially Methylated Acetylated Alditols
PS	Polysaccharides
Rha	Rhamnose
Rib	Ribose
RNAse	Ribonuclease A

SDS	Sodium Dodecyl Sulphate
SEC	Size-exclusion Chromatography
TFA	Trifluoroacetic acid
TLR4	Toll-like receptor 4

Chapter 1: Introduction

Gut Microbiota

The intestinal tract is colonised by 10^{13}-10^{14} organisms composing the gut microbiota. These organisms give metabolic functions to the human host but are also sustained and influenced by the host diet, immune system and presence of antibiotics (Sender, Fuchs and Milo, 2016).

Research into the microbiota has increased in the last ten years, nevertheless there are many questions remaining, such as: how do bacteria adapt and adjust to an environment as complex as the human gut? How do bacteria compete or work symbiotically together, between and/or within species? How do bacteria gain immune tolerance from the host immune system? (Eloe-Fadrosh and Rasko, 2013) (d'Hennezel *et al*., 2017). A better understanding of the interactions occurring between the gut microbiota and the human health could influence our current views on gut pathologies and also human nutrition, catalysing nutritional recommendations and polices dependent on age, geography, diet and health of individuals (Waldor *et al*., 2015).

90 % of the bacteria constituting the gut microbiota belong to the Firmicutes and Bacteroidetes phyla (Goodman and Gardner, 2018). The latter comprises Gram-negative bacteria while the former phylum is composed of both obligate and facultative anaerobic bacteria of which most are mesodermal and stain as Gram-positives (Kim, Covington and Pamer, 2017). However, within the Firmicutes phylum there is a class, still poorly investigated, known as *Negativicutes* consisting of bacteria which stain as Gram-negatives, such as *Veillonella* and *Acidaminococcaceae* (Figure 1) (Vesth *et al*. 2013).

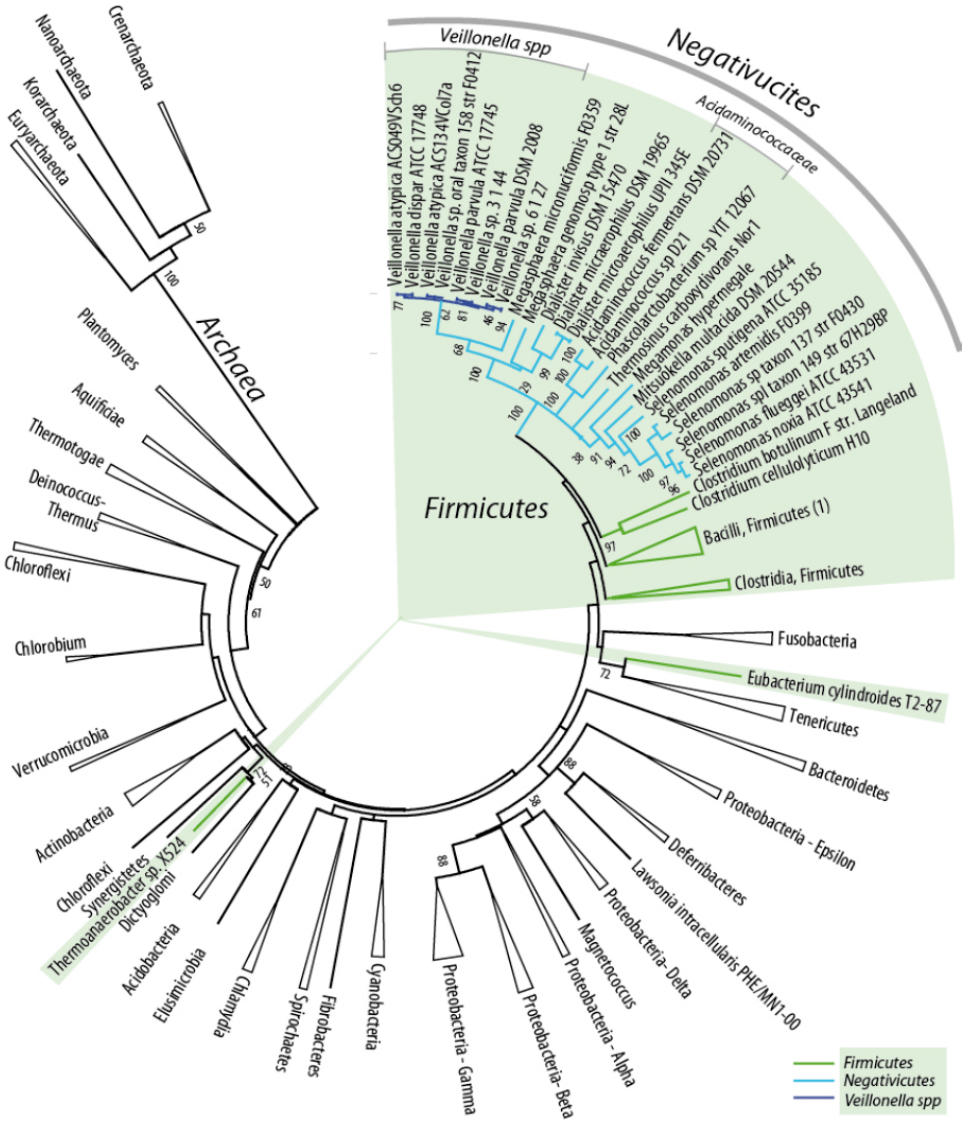


Figure 1: Diagram of a phylogenetic neighbour-joining tree (based on 16S rRNA genes), taken from Vesth et al. 2013

Gram-Negative and Gram-Positive Bacteria

Bacterial cells can be classified as Gram-negative or Gram-positive based on a staining technique which reflects the differences in the cell envelope structure and chemical composition (Silipo and Molinaro, 2011). Both Gram-positive and Gram-negative bacteria have a phospholipid bilayer which encloses the cytosol (cytoplasmic membrane) (Silipo and Molinaro, 2011). Enclosing the cytoplasmic membrane is a peptidoglycan layer which is a rigid envelope that gives

the shape and osmotic strength to the bacteria. The peptidoglycan is a carbohydrate mesh formed by cross-linked chains of *N*-acetylglucosamine and *N*-acetylmuramic acid (Silipo and Molinaro, 2011). In Gram-positive bacteria, the peptidoglycan layer is a thick layer whereas Gram-negative bacteria possess a thin layer and an additional phospholipid bilayer known as the outer membrane (OM) which is responsible for the increased permeability of hydrophobic compounds and higher molecular weight hydrophilic compounds (Delcour, 2009). This OM is rich in lipopolysaccharides (LPS), covering up to 75 % of the bacteria cell surface (Silipo and Molinaro, 2011). Additional extensions to the bacteria cell wall may be present, such as flagella, pili, a capsular polysaccharide and/or exopolysaccharide (EPS) (Harvey *et al*., 2013).

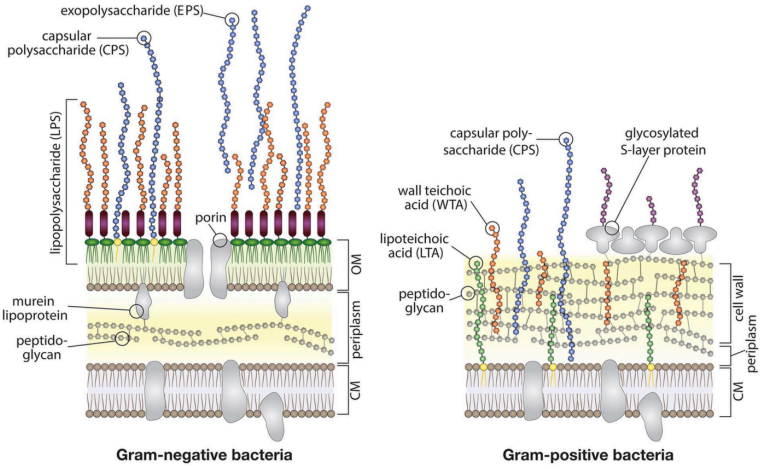


Figure 2: Illustration of the structural differences between Gram-negative and Gram-positive cell walls, taken from Whitfield et al. (2015).

Lipopolysaccharides (LPS)

Lipopolysaccharides (LPS) are amphiphilic macromolecules found in the outer membrane of almost all Gram-negative bacteria. These heat stable compounds make up 75% of the Gram-negative outer membrane (OM) and provide, among others, a fundamental role in cellular rigidity (Whitfield et al., 2015). The LPS monolayer is highly ordered and has low fluidity which is due to the electrostatic interactions with environmental divalent cations (eg. Ca2+ and Mg2+). Due to the external location, LPS are involved in vital host-bacterium interactions like recognition,

adhesion, colonisation, virulence, elicitation of the animal and plant host immune system. Furthermore, LPS are also crucial for the viability and survival of Gram-negative bacteria as they assist in the resistance to antibacterial compounds and cellular stress factors (Silipo et al., 2010).

Structure

In general, LPS are composed of a common general architecture, which is made up of three diverse biochemical components: a glycolipid portion (Lipid A), an oligosaccharide part (core) and a polysaccharide (known as O-antigen or O-chain or O-polysaccharide) (Silipo *et al*., 2010). Bacteria which possess LPS with the O-antigen region are known as smooth-type LPS (S-LPS) due to the "smooth" appearance associated with the colony on agar plates (De Castro *et al*., 2010). Whereas other bacteria possess rough-type LPS (R-LPS), which lack the O-antigen and gives a rough appearance to the bacteria colony. R-LPS, otherwise known as LOS (lipooligosaccharide), gain the antigenic properties from the core sugar chain (Steimle *et al*., 2016). Although, there is heterogeneity within each LPS molecule, it has been discovered that LPS structure can change within species (Raetz, 1990). The composition of LPS is highly heterogeneous and can be altered in response to various challenges such as exposure to different stress conditions or changes in growth medium (Klein and Raina, 2019). studies of the structure of LPS from H.pylori have been identified as altering its LPS structure during chronic gastric infection (Maldonado et al., 2016). Another study showed that Francisella bacterial species alter their LPS in response to growth temperature; at low temperatures a mannose sugar residue is added to the non-reducing end of the lipid A glucosamine disaccharide, as well as alteration in the acyl chain positions (Yi et al., 2012).

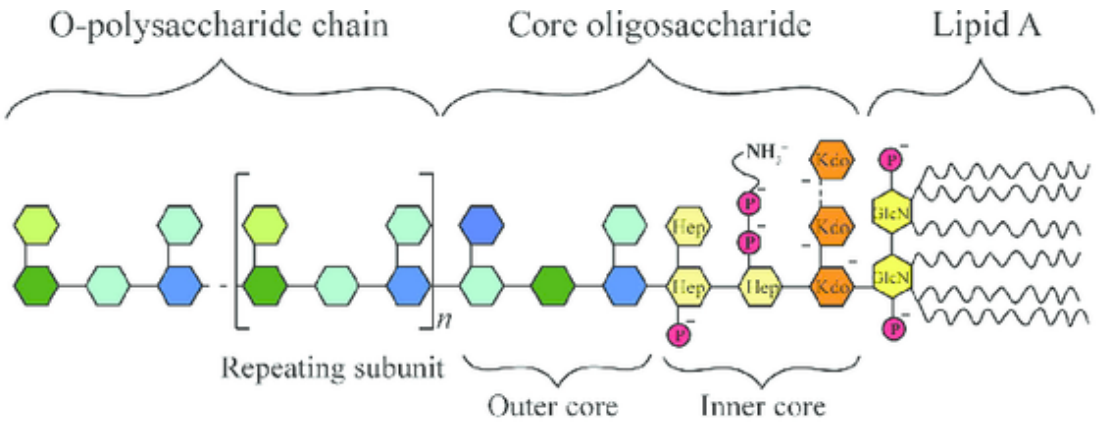


Figure 3: Schematic representation of LPS structure. The inner core consisting of heptose (hep), Kdo (3-deoxy-D-manno-oct-ulosonic acid). The backbone sugars of lipid A is a disaccharide of Glucosamine (glcN). Taken from Wang et al. 2016

Lipid A

Lipid A possesses a highly conserved structure consisting of a β-(1→6)-linked D-glucosamine disaccharide backbone substituted with a number of amide- and ester-linked 3-hydroxy fatty acids at the positions 2,2' and 3,3' respectively. The acyl chains that are directly linked to the sugar backbone are defined primary and some are further acylated at the hydroxyl groups by secondary acyl chains. The acyl chains of the lipids affect the interactions between neighboring lipid A molecules and therefore plays an important role in the physiology of the membrane of the bacteria cell (Molinaro *et al.*, 2015). Furthermore, the sugar backbone is generally α-phosphorylated at position O-1 of the reducing glucosamine (GlcN I) and at position O-4' of the non-reducing glucosamine (GlcN II) (Raetz and Whitfield, 2002). These phosphate groups can also be substituted with other functional groups for example, phosphate, ethanolamine, ethanolamine phosphate, ethanolamine diphosphate, GlcN, 4-amino-4-deoxy-L-arabino-pyranose and D-arabino-furanose (Erridge *et al*., 2002) (Silipo *et al*., 2010). The structure of lipid A is of particular interest in the field of biochemical research as it has been identified as the toxic and immunomodulatory component of LPS (Rietschel *et al*., 1994). In 1985, Galanos *et al*. produced synthetic lipid A and discovered it exhibits identical biological properties to *Escherichia coli* (*E. coli*) lipid A. This research provided evidence that lipid A is responsible for the endotoxic activity of LPS, which is recognised by the innate immune system receptor complex, which is made up of the Toll-like receptor 4 (TLR4) and myeloid differentiation factor-2 (MD-2) (Galanos

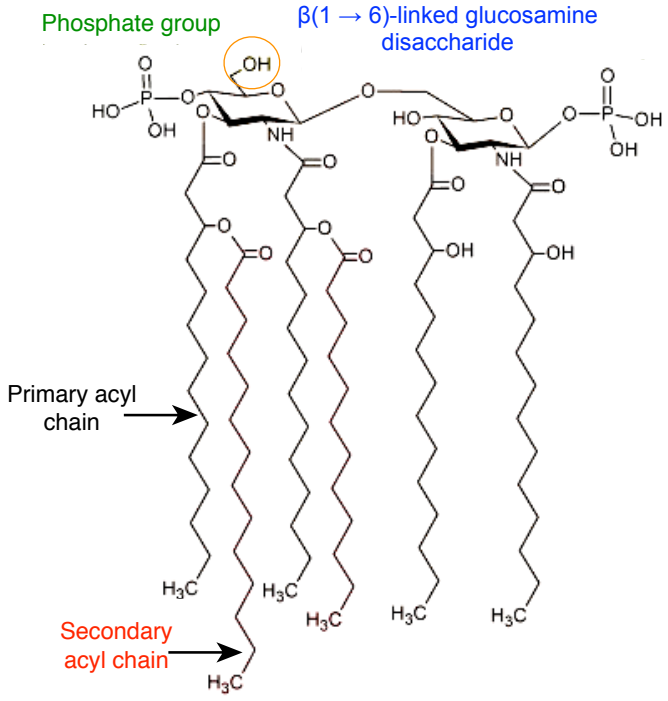


Figure 4: The structure of E. coli lipid A to show a structural representation of Lipid A from Gram-negative bacteria. The hydroxyl group (circled in orange) links the lipid A to the core region. Also highlighted are the primary acyl chains linked to the sugar residues and the secondary chains linked to the primary ones. Adapted from Steimle et al. (2016).

et al., 1985) (Fujihara *et al*. 2003) (Miyake, 2003). This receptor complex is present on the cell surface of many different cell types including the immune cells such as macrophages and dendritic cells. The activation of this receptor by lipid A induces an intracellular signalling cascade that results in the production and release of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF- α), interleukin-1β (IL-1 β), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Jain and Darveau, 2010).

Core Oligosaccharide

Core oligosaccharide is a complex component of the LPS molecule since it can be characterised by up to fifteen monosaccharides which can be organised giving either a linear or a branched structure. In the core LPS portion two different regions can be distinguished on the basis of the monosaccharide composition termed: inner core and outer core. The inner core region is directly linked to the lipid A, is well conserved and consists of uncommon sugar residues such as heptoses (L-*glycero*-D-*manno*-heptose and, less commonly, D-*glycero*-D-*manno*-heptose) and Kdo. The lipid A molecule is linked to the polysaccharide portion LPS through a 3-deoxy-D-

manno-oct-ulosonic acid (Kdo) (Figure 5) residue at position 6' of the non-reducing GlcN residue (Erridge *et al*., 2002). This link between the lipid A and the kdo of LPS is reasonably well conserved across Gram-negative bacteria. The inner core region of LPS has been found to interact only with TLR4, and not with both the MD-2 and TLR4 complex and Cochet and Peri (2017) suggested that the inner core play a role in stabilising the interaction but not essential for the endotoxic activity (Chochet and Peri, 2017).

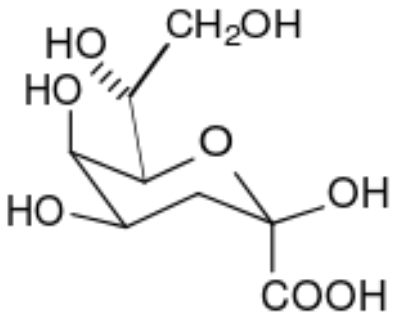


Figure 5: Structure of Kdo Residue taken from: Ghalambor et al. 1966

Some gram-negative bacteria have LPS lacking the O-antigen which are named rough (R)-form LPS or lipooligosaccharide (LOS) (Steimle et al., 2016) , therefore for these bacteria, the outer core region is the most exposed portion of the LPS, often branched, and is characterised by a higher structural variability than the inner core region. It is typically characterised by common hexoses such as glucose, galactose, *N*-acetyl glucosamine. The extent of the structural variability of the core oligosaccharides is limited within each species and genus (Erridge *et al*., 2002).

O-Antigen (smooth LPS)

The O-antigen is a polysaccharide of up to 8 repeating residues and can consist of up to 50 residues long. Depending on the bacterial strain, the O-antigen region varies in residues, sequence, chemical linkage, substitution and ring formation leading to nearly limitless diversity (Erridge *et al*., 2002).

The O-antigen is the outer most part of the LPS molecule and therefore, is the interface between the bacterium and its environment (Raetz and Whitfield, 2002). Although the o-antigen is non-toxic, the polysaccharide chain is the immunogenic portion of LPS by presenting epitopes for immune system. The long chains of the o-antigen polysaccharide can act as a physical barrier against the interaction between the antibodies and epitopes on the surface of the bacteria and thereby prevent the activation of the complement system and lytic effect. Therefore, the prevention of complement system activation by the o-antigen polysaccharide also protects the bacteria from uptake by phagocytes (Lerouge and Vanderleyden, 2002).

Moreover, several studies have found that some commensal and pathogenic bacteria (such as *Helicobacter pylori*, *Neisseria gonorrhoeae*, *N. meningitidis* and *Haemophilus influenza*) have been found to possess O-antigen composed of sugars such as *N*-acetylneuraminic acid (Neu5Ac) or L-fucose which resemble those of human glycosphingolipids. This adaptive ability of bacteria to mimic host glycoconjugates contributes to the increased resistance from attacks by the hosts immune system (Alexander and Rietschel, 2001).

Lipid A and Immune Response

Toll-like receptors (TLRs) are expressed on the surface of mammalian cells which recognise specific pathogen-associated molecular patterns (PAMP) (Kawai and Akira, 2009). Activation of these TLRs initiates signal transduction pathways activating inflammatory cytokines, B7 co-stimulatory molecules and histocompatibility complex class II, which are necessary requirements of the acquired immune system (Kikkert *et al*., 2007).

TLR4 was first discovered as the principle receptor for LPS through experimental evidence using mutations in the TLR4 gene leading to LPS hypo-responsiveness in mouse models (Qureshi *et al*., 1999). This was confirmed by research with TLR4 knock-out led to the same results (Hoshino *et al*., 1999). Further research focused on the TLR4 receptors discovered that transfection of TLR4 receptor protein into TLR4 defective cells was not sufficient to produce response to LPS leading to the discovery of a further important molecular component: MD-2 which forms with TLR4 a protein complex for LPS/lipid A receptor (Shimazu *et al*., 1999).

The mechanism of the TLR4/MD-2 complex activation begins with LPS-binding-protein (LBP) delivering LPS to another protein CD14 (Correia *et al*., 2001). Correia *et al*. (2001) reported that LPS is brought into proximity of the TLR4/MD-2 receptor when it is in complex with CD14. Research by Kim and Kim, (2017) outlining the total cascade of TLR4/MD-2 receptor activation supports the essentiality of the interaction between CD14, LBP and LPS to bring the LPS into proximity of the TLR4/MD-2 receptor.

Lipid A binding to the CD14/MD-2 receptor complex causes dimerization of the TLR4 monomers leading to activation of the adaptor protein Myeloid Differentiation Factor 88 (MyD88) (Takeuchi *et al*. 2000), the family of IL-1 receptor-associated kinases (IRAKs) and the adapter TNF receptor-associated factor 6 (TRAF6) (Horng, Barton and Medzhitov, 2001). The activation of these proteins then triggers multiple intracellular signalling pathways, importantly the nuclear factor-kappaB (NF-κB) inducing cascade and the mitogen-activate protein kinase (MAPK) cascades (Zhang *et al*. 1999) both leading to the amplification of the transduction signal with the consequent massive production of inflammatory proteins thus eliciting the inflammatory process (Figure 5) (Arancibia *et al*., 2007).

The immunogenic potential of the LPS is dependent on the lipid A structure with the *E. coli* lipid (Figure 4) recognised as the most active agonist for human cells with its *bis*-phosphorylated hexa-acylated lipid A, with an asymmetric (4 + 2) acyl group distribution. A moderate agonistic activity is seen for the lipid A from *Salmonella* which expresses generally *mono*-phosphorylated hexa-acylated or hepta-acylated lipid A forms (Silipo and Molinaro, 2011). An example of a weak agonist is the lipid A from *H. pylori* with a *mono*-phosphorylated tetra-acylated lipid A (Steimle et al., 2016). All these examples of alterations within the distribution and length of acyl chains, as well as in the phosphate content, affect the interaction with the hydrophobic cavity of the TLR4/MD-2 receptor complex (Steimle et al., 2016).

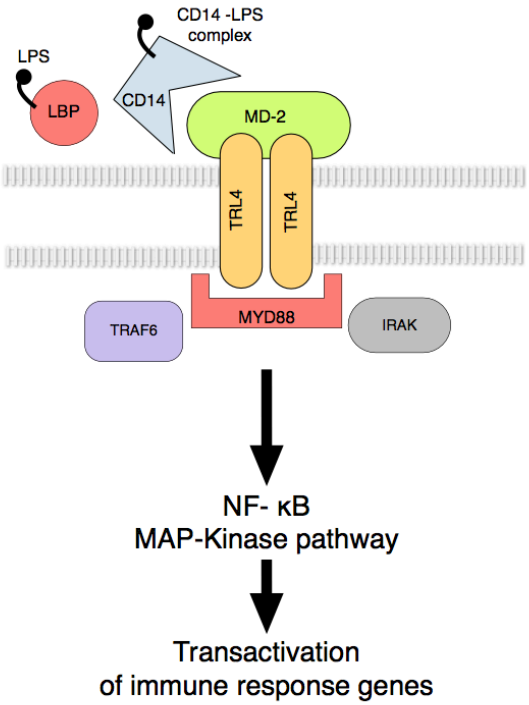


Figure 6: Diagram of the signalling pathways of receptor complex TLR4/MD-2. Lipid A component of LPS binds to LPS-binding-protein (LBP) which transfers the LPS to CD14. CD14 binds to TLR4/MD-2 and causes dimerisation of TLR4 proteins resulting in intracellular activation of the proteins MyD-88, TRAF6 and IRAK. These proteins activate a variety of pathways and create a cascade of reactions resulting in a cellular immune response.

C-type lectins

The saccharide part of the LPS (core and O-antigen) have also been discovered to play an important role in the activation of the host immune system.

Phagocytic immune cells known as dendritic cells (DCs) express a C-type lectin called "DC-specific ICAM-grabbing nonintegrin" (DC-SIGN) (Zhang et al. 2006). These DC-SIGN ligands bind to specific sugar residues found on the surfaces of bacteria and viruses in a structure-dependent manner causing an immune response. Several studies found a specific epitope within the core region of the LPS which is necessary for the binding to DC-SIGNs and mutations within the saccharide composition can influence the ability of the host immune response to phagocytose the bacteria (Klena *et al.,* 2005) (Zhang *et al.*, 2006). Resistance to this phagocytosis has been

seen in bacteria which possess LPS with an O-antigen saccharide component through a shielding mechanism of the conserved core structures which be targeted by the immune system proteins (Burns *et al.,* 1998) (Cortes et al. 2002).

Other C-type lectins, such as Mannose-Binding Lectins have also been found to interact in a structure dependent manor with the core oligosaccharides of bacterial LPS (Man-Kupisinska *et al.* 2018). Further research on the interaction and host immune system activation between LPS from *Klebsiella* and the C-type lectins SP-D indicated that's the oligosaccharide structure of the LPS influences the binding between these molecules (Sahly *et al*. 2008).

Veillonella Genus

The genus *Veillonella* belongs to the *Negativicutes* class and is characterised as comprising anaerobic, non-motile, small spherical cocci about 0.3 microns in diameter (Rogosa, 1964). *Veillonella* species are abundant in the human microbiome and are present as a part of the normal flora of the gastrointestinal tract, oesophagus, throat, vagina and oral cavity of healthy subjects (Bogert *et al*., 2013).

Conversely, a wide range of microbiology research has shown presence of *Veillonella* in oral (Mashima and Nakazawa, 2015), prosthetic joint (Marchandin *et al.,* 2001) and pulmonary infections (Pustelny *et al*., 2015), endocarditis (Greaves and Kaiser, 1984), and meningitis (Bhatti and Frank, 2000). There is published evidence which indicates the active role in which *Veillonella* plays in disease; in 2006, Kolenbrander found that in oral infections a rise in *Veillonella* species results in proliferation of lactic acid producing *Streptococci* and *Actinomyces*, leading to dental plaque and infections (Kolenbrander, 2006). In 2015 Pustelny *et al.* investigated the relationship between *Veillonella* and the bacterium *P. aeruginosa* in contribution to the pathology of Cystis Fibrosis (CF). This research established that *Veillonella* supports *P. aeruginosa* growth within the CF lung where high *P. aeruginosa* colonization is correlated with clinical deterioration of the patient (Pustelny *et al*., 2015). In 1970 Bladen *et al*. observed *Veillonella* assists in the development of plaque by Gram-positive filamentous diphtheroid. This development of plaque

was observed when inoculated in lactate medium, which supports growth of *Veillonella* but hinders that of the diphtheroid (Bladen *et al*., 1970). The basis of these interactions between bacterial species can be correlated to the unusual metabolism of the seven species of the *Veillonella* family. In fact, *Veillonella* are unable to breakdown carbohydrates, however they successfully thrive on lactate, pyruvate, malate or fumarate, which are by-products of metabolism from other bacteria species and also from the diet of the host (Kolenbrander, 2006). Therefore, *Veillonella* is important for the metabolic function in the breakdown of bacterial by-products and promoting a mutualistic community for the development of other bacterial species (Ng and Hamilton, 1971).

Metabolic cooperation between *Streptococcus* species and *Veillonella* has been subjected to much research for oral infections. Mashima and Nakazawa (2015) discovered different *Veillonella* species had different effects on different *Streptococcus* species. Further supporting this, research by Bogert *et al.* in 2013 suggested combinations of streptococcal and *Veillonella* strains produce an increased immune response in comparison to those observed with mono-stimulations. Mashima and Nakazawa (2015) highlighted that several factors may influence these interactions between species including signalling molecules. This emphasised the deficiency in our knowledge of the signalling mechanisms between species and the requirement to elucidate these mechanisms of interactions.

Lipopolysaccharides from Veillonella spp

The first evidence that LPS was the endotoxin of *Veillonella* bacteria was seen in 1968 by Gewurz *et al.* who discovered complement system activation by LPS extracted from *Veillonella*. Later Kikkert *et al.* (2007) found *Veillonella parvula* species can activate both TLR4 and TLR2. This assay was carried out using purified bacterial substances and measuring the IL-8 levels in a TLR-transfected human embryonic kidney (HEK) cells. Although *V. parvula* did show stimulation of both TLRs, the level of stimulation was low compared to other Gram-negative periodontal bacteria (Kikkert *et al*., 2007). In 2009, a more in-depth research conducted by Matera *et al*. found that *Veillonella* LPS induced cytokine production in human cell lines and that this event was TLR4

dependent. The study used purified LPS from *V. parvula* species and tested cytokine production on peripheral blood mononuclear cells (PBMC). Additionally, this study also revealed that *V. parvula* LPS is able to activate the p38 MAPK pathway also resulting in cytokine release (Matera *et al.,* 2009).

The previous studies showed that *V. parvula* LPS induces 10 – 100 - fold less cytokine production compared to *E. coli* LPS (Matera *et a*l., 2009). The low immune stimulation by *Veillonella* LPS indicates that this bacterium and/or their LPS play a different role in its interaction with host cells. Several research studies have suggested that *Veillonella* is an important bacterium within our healthy microbiota and contributes towards development of the early childhood immune system. Various studies show that the absence of *Veillonella* is correlated with development of diseases, such as asthma (Arrieta *et al*., 2015), bronchiolitis (Hasegawa *et al*., 2016) and autism (Strati *et al*., 2017). These studies show that *Veillonella* has a negative correlation with disease, however these findings were through investigation of the total gut microbiota and not solely *Veillonella*.

Structure of Veillonella LPS: The History

In 1970 the first compositional analysis of *Veillonella* LPS was performed, discovering the main monosaccharides as Kdo, a heptose, glucose, ribose, galactose, glucosamine and galactosamine (Hofstad and Kristoffersen, 1970). In 1984, Tortorello and Delwich further investigated the LPS fatty acid composition revealing the occurrence of tridecanoic acid, 3-hydroxytridecanoic acid, and 3-hydroxypentadecanoic acid.

Importantly, Tortorello and Delwich (1984) detected that alterations in the growth conditions of *Veillonella* bacteria affect the LPS extraction yield and chemical changes in the LPS structure.

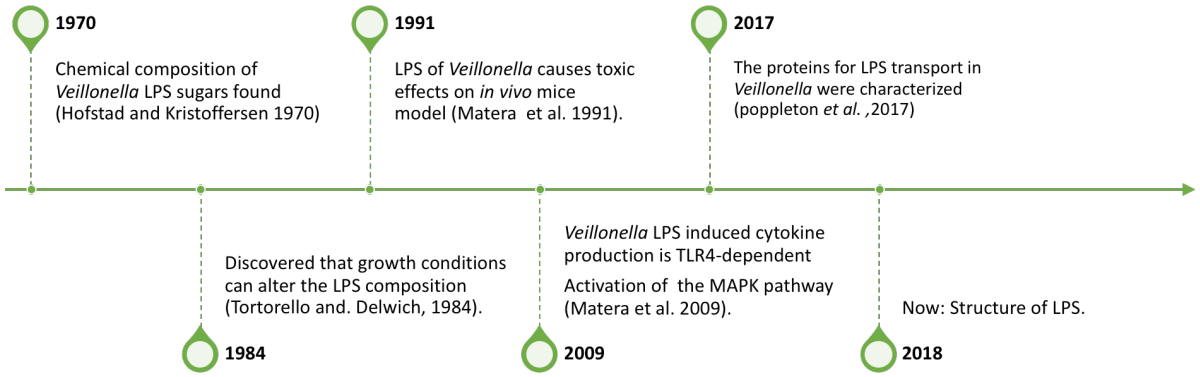


Figure 7: A schematic of the literature review of the information known about the LPS from Veillonella species.

One of the principle hindrances in the structural study of *Veillonella* LPS is the need for fastidious anaerobic growth conditions and the slow-growing rate of the species (Marriot, Stark and Harkness, 2007). Secondly, the growth of bacteria within the laboratory can be either planktonic or biofilm-forming, the former being the easier, more commonly used method where a homogeneous population of bacteria can be grown bacterial cells in suspension and extracted by centrifugation. Conversely, culture of biofilm forming bacteria is more representative of natural 'in host' conditions where bacteria are under 'stressed' situations by co-inhabitation with other bacteria and create a biofilm for survival (Kumar *et al.,* 2017) (Donlan, 2002).

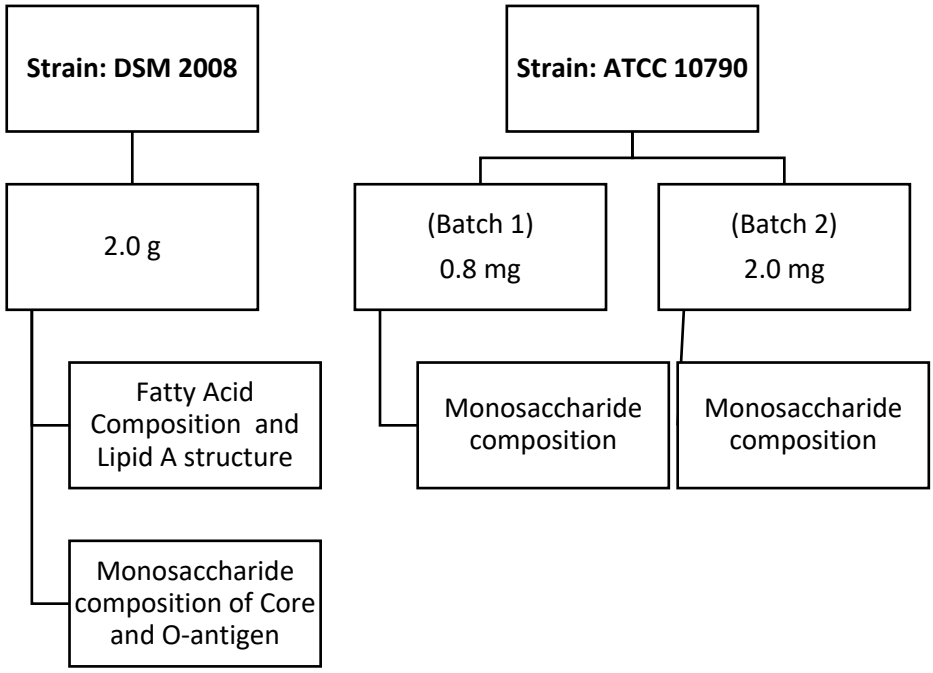
Aims and Objectives of the Project

It is clear from a review of the literature that there is still a lack of structural understanding of the *Veillonella parvula* LPS. A detailed structural analysis of this LPS would provide further information into the biological importance of these bacteria in human health. Furthermore, it will lead to increased knowledge of the structure-function relationship between LPS and immunological activity.

The aims of the project are to fully characterise the carbohydrate moieties of extracted LPS from two separately grown *Veillonella parvula* cells through Gas Chromatography Mass

Spectrometry (GC-MS) analysis and NMR techniques. Furthermore, the aim is to make a full characterisation of the lipid A component through GC-MS and MALDI-TOF MS analysis.

During this study three batches of *Veillonella parvula* were obtained and the LPS was extracted and studied. Two batches were grown in the same lab, of the strain named: ATCC 10790 and were grown in "anaerobose basa" broth in anaerobic, planktonic conditions. The third batch of *Veillonella parvula* LPS was studied from *Veillonella parvula* strain DSM 2008. DSM 2008 cells were also grown planktonically and anaerobically. Both ATCC 10790 and DSM 2008 are the same strain of bacteria, however the DSM 2008 bacteria are known to have been isolated from a hospitalised patient. For the purpose of this study the two strains were analysed separately and will be defined as either '*V. parvula* ATCC 10790' or '*V. parvula* DSM 2008' (Scheme 1).



Scheme 1: Diagram of the three batches of Veillonella parvula cells analysed during this project. With the name of the strain, the starting dried cell weight and the analysis completed with each batch.

Chapter 2: Techniques for the Structural Characterisation of Lipopolysaccharides

Extraction and Isolation of LPS from Bacterial Cells

In order to structurally characterise LPS, it is essential to extract it from intact microbial cells. There are several procedures to achieve the extraction of LPS depending on the occurrence or not of the O-antigen moiety which renders the *smooth*-type LPS more hydrophilic than the *rough*-type LPS. One of the best known ways to isolate *smooth*-type LPS is the hot phenol-water method (Westphal and Jann, 1965) consisting of suspending the dried cells in 90% phenol/water 1:1 *v/v* at 68 °C. The hot water/phenol method also requires the additional step of enzymatic digestion with DNAase, RNAase and Proteases to remove nucleic acids and proteins followed by purification by dialysis (Westphal and Jann, 1965). For the *rough*-type LPS (LOS), an extraction method with Phenol/Chloroform/Petroleum ether (PCP) (2:5:8, v:v:v) is typically employed (Galanos *et al*., 1969). After removal of Chloroform and Petroleum ether, the LOS is precipitated from the phenol phase by adding drops of water. The PCP procedure results in a sample that is generally free of cell contaminants allowing for a following easier purification of the extracted LPS (Galanos *et al*., 1969).

Gel Electrophoresis

The detection of LPS typology and purity can be achieved through polyacrylamide gel electrophoresis (PAGE) with sodium deoxycholate (DOC) or sodium dodecyl sulphate (SDS) as denaturing agents; both disaggregate the LPS micelles (Peterson and McGroarty, 1985) thus giving them the possibility to migrate through the gel. For the identification of the LPS, silver nitrate gel staining procedure (0.1 % $AgNO_3$) is used. The presence of *smooth*-type LPS is determined by the observation of a 'ladder-like' pattern on the gel, which is due to the migration of the O-antigen polysaccharide repeating units. The LPS molecules devoid of this polysaccharide part will migrate to the bottom of the gel, due to the lower molecular weight of the LOS molecule

as it does not possess an O-antigen, (Tsai *et al*., 1982) the next band would be the core plus one repeating unit and so forth (Palva and Mäkelä, 1980).

Purification

Various and long purification methods have been applied to the extracted LPS to successfully characterise both the saccharide and lipid A components.

Enzymatic Digestion and Dialysis

Enzymatic digestion with DNAase, RNAase and Proteases can be applied to remove contaminants, such as proteins, DNA and RNA. Following enzymatic digestion, the sample is then subject to dialysis against distilled (milliQ) water to purify the digested LPS. Dialysis is also a useful technique in the purification of the sample from salts and other impurities from chemical modification reactions, which are described later.

Low pressure Liquid Chromatography

Size exclusion chromatography (SEC), also known as gel filtration/gel permeation chromatography/molecular exclusion is frequently used for purification of LPS. SEC separates a mixture of molecules by their hydrodynamic volume through the interaction of components in the sample (mobile phase) with a porous matrix resin (stationary phase). The sample passes through the resin and the smaller molecules (impurities) enter the pores and the larger molecules (LPS or degraded LPS portions) flow around the resin beads. Consequently, the sample takes a more direct pathway and therefore elutes before the small molecules (Neue and Phoebe, 1997). Various chromatography resins use this behaviour and can be applied for different techniques within the purification process of LPS (Holst, 2000) (Silipo *et al*., 2012).

Structural Characterisation of Carbohydrates

Gas chromatography Mass Spectrometry (GC-MS) is a useful analytical technique for the detection of complexes of carbohydrates. For the use of this technique it is mandatory that the sugars are converted into appropriate derivatives, so they are in a volatile form for their detection (Ruiz-Matute *et al.*, 2011). To identify monosaccharide type and glycosylation patterns there are a variety of derivatisation techniques that can be employed to highlight different features of the sugar residues within the original sugar oligosaccharide/polysaccharide sequence.

Derivatisation Methods

Acetylated *O*-Methyl Glycosides (AMG)

A qualitative analysis is performed by treatment of the oligo/polysaccharide with MeOH/HCl to cleave the glycosidic bond and form methyl glycosides. Subsequent acetylation with acetic anhydride in pyridine produces the peracetylated *O*-methyl glycosides (AMG) (De Castro *et al.* 2010). By comparison of the retention times of the GC analysis and the fragmentation pattern of the obtained mass spectra, it is possible to identify the type of monosaccharide residues. One drawback to the AMG method is the production of isomers, which cannot be distinguished apart: pyranose and furanose either α and β anomers could lead to miss-quantification (Di Lorenzo, 2014).

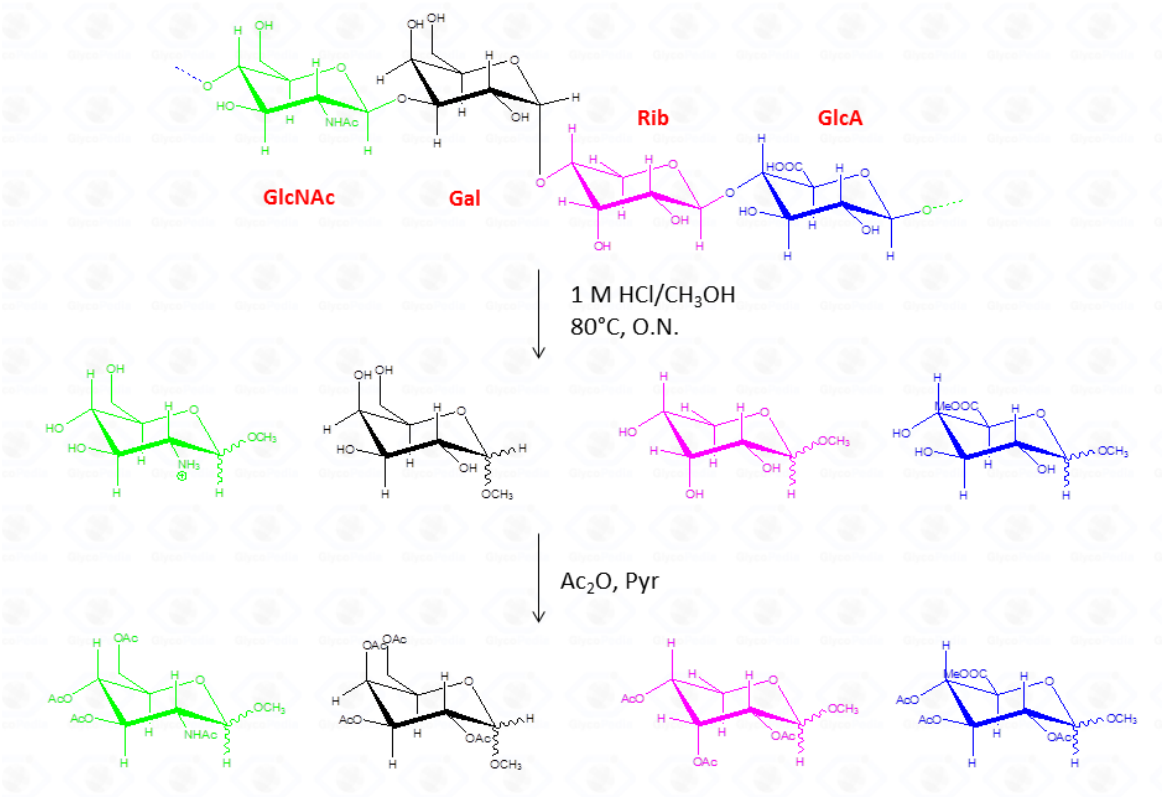


Figure 8: Reaction step of the peracetylated O-Methyl Glycosides (AMG) method. Taken from (De Castro, 2014).

Acetylated Alditols (AA)

An alternative approach of derivatisation is production of acetylated alditol (AA) derivatives, which is a useful analysis of basic and neutral monosaccharides. These monosaccharide derivatives are created using trifluoracetic acid (TFA) for acid hydrolysis followed by reduction of carbonyl moiety with Sodium tetrahydridoborate ($NaBH_4$) (Di Lorenzo, 2014). An advantage of the acetylated alditol derivatives is the production of "only one peak per residue" in the mass spectra (excluding ketoses and aldoses as the reduction step can yield the same alditol) (Ruiz-Matute *et al.* 2011), which is a very useful aspect for quantification analysis. An unfavorable aspect of this method is that only neutral or basic species can be detected, therefore acidic monosaccharides cannot be detected (De Castro *et al.,* 2010).

Partially Methylated Acetylated Alditols (PMAA)

Partially Methylated Acetylated Alditols (PMAA) is a crucial derivatisation method which involves longer and more extensive reaction steps than either the AA or the AMG methods. First the compounds are methylated with Iodomethane (CH_3I) in strong alkaline conditions and then permethylated compounds are hydrolysed in acidic conditions and then reduced with $NaBD_4$ (Figure 9) (De Castro *et al.,* 2010). Use of $NaBD_4$, a deuterated reagent, is to mark where the hydrolysis of the sugar ring occurred. The resultant compounds have a free hydroxyl group which can be acetylated and then used as indicators of the position involved in the glycosidic bond or cyclisation. When injected in the GC-MS, these compounds can be easily analysed following their substitution groups (acetyl and methoxyl groups) because the molecules break through ionisation preferably leaving the methoxyl group with a positive charge (Di Lorenzo, 2014).

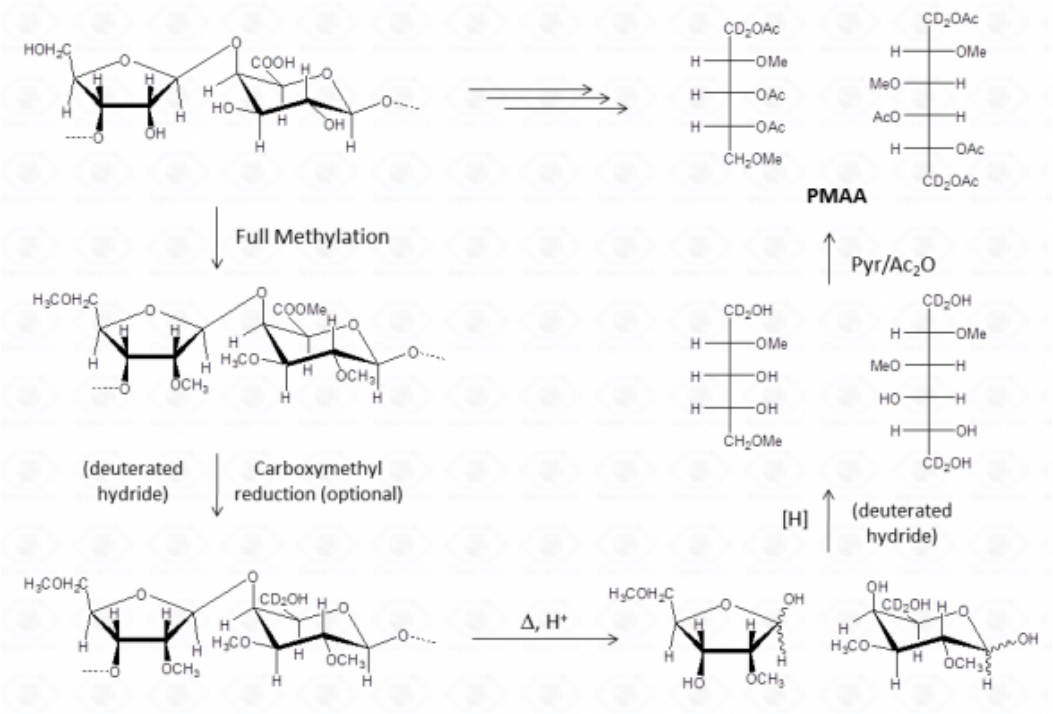


Figure 9: Reaction step of the Partially Methylated Acetylated Alditols method. Taken from (De Castro, 2014)

Absolute Configuration

For the analysis of the absolution configuration, the distinction between enantiomers is performed by solvolysis with an enantiomerically pure alcohol as 2-(+)-octanol or 2-(+)-butanol. After the acetylation and injection to GC-MS, a comparison between the retention time of the acetyl 2-(+)-octyl glycosides and one of a standard mixture of *O*-2-(±)-octyl-glycosides standard monoses in D or L configuration allows the assignment of the monosaccharide configuration (Leontein *et al*., 1978).

For a full analysis of a complex mixture of sugars, the composition can be derived using a combination of these methods; Table 1 gives a comparison of the methods used (De Castro, 2014). Furthermore, a comparison through GC-MS injection against known standards enables identification of the exact monosaccharide residues

Table 1: Comparison of derivatization methods used to analyse carbohydrates on Gas Chromatography - Mass Spectrometry

DERIVATISATION METHOD	ADVANTAGES	DISADVANTAGES
Acetylated Alditols (AA)	• Negative and basic monosaccharides analysed • One residue gives rise to one peak on MS spectra • Acetylated alditols are stable and once prepared they can be stored and used for several years	• Acidic monosaccharides not detected • Ketose gives rise to two different diastereoisomers alditols producing two peaks on MS • A reactive reducing end in the reaction steps causes unwanted derivatives and lower yields. • Very low abundance of molecular ion seen on MS
Acetylated Methyl Glycosides (AMG)	• Less reaction steps than AA • No side reactions from aldehyde group as seen in AA • Suitable for most types of sugar residues.	• One residue will produce more than one peak on MS due to rings and isomers formed • Not often suitable for ketose analysis • Anhydrous conditions are essential during methanolysis steps • Very low abundance of molecular ion peak
Partially Methylated Acetylated Alditols (PMAA)	• Interpretation of the MS is simple • Enables determination of substitution patterns of all residues	• Similar disadvantages as AA • Many reactions required, of which of some to be carefully carried out in anhydrous conditions • Reaction steps are different for the detection of uronic acid. • Humidity and consequent under-methylation can produce false results

Acetic Acid Cleavage of the LPS

This procedure is used to cleave the acid-labile glycosidic bond between the Kdo residue and the non-reducing glucosamine of the lipid A. This method comprises a centrifugation step which produces a water phase containing the oligo-/polysaccharide moiety of the LPS (that is then generally studied by NMR spectroscopy), and a precipitate, namely the lipid A component that can be analysed by MALDI-TOF mass spectrometry or even NMR.

The deoxy functional group of kdo is positioned close to the glycosidic carbon atom which renders the link to GlcN II very sensitive to acid hydrolysis however this reaction can result in the production of unwanted products such as the loss of phosphate groups (Lindberg *et al.* 1975). ^{31}P NMR can be performed to confirm the occurrence of dephosphorylation (Helendera *et al*. 1997). Furthermore, another disadvantage of the acid hydrolysis method can lead to the production of Kdo reducing units with a heterogeneity of various conformations (α and β anomers of pyranose and furanose rings, condensed or anhydro forms) which can render the analysis by by NMR spectroscopy particularly difficult (Volk et al., 1972) (Di Lorenzo, 2014).

Therefore, it is usful to perform de-lipidation of the lipid A by means of alkaline treatment to determine the primary structure of the core oligosaccharide portion.

Alkaline hydrolysis of LPS

The alkaline treatment lies in a first *O*-deacylation of the LPS, and a successive *N*-deacylation, which leads to the isolation of a polysaccharide containing the glycosidic portion of the lipid A. *O*-deacylation reaction removes ester-linked (*O*-linked) fatty acids whereas *N*-deacylation causes the removal of amide-linked acyl chains. The latter reaction is very strong and could cause loss of information about the presence of acetyl groups and pyrophosphates substituents.

The obtained product is typically full of salts, therefore it is then desalted by size exclusion chromatography and structurally investigated by NMR.

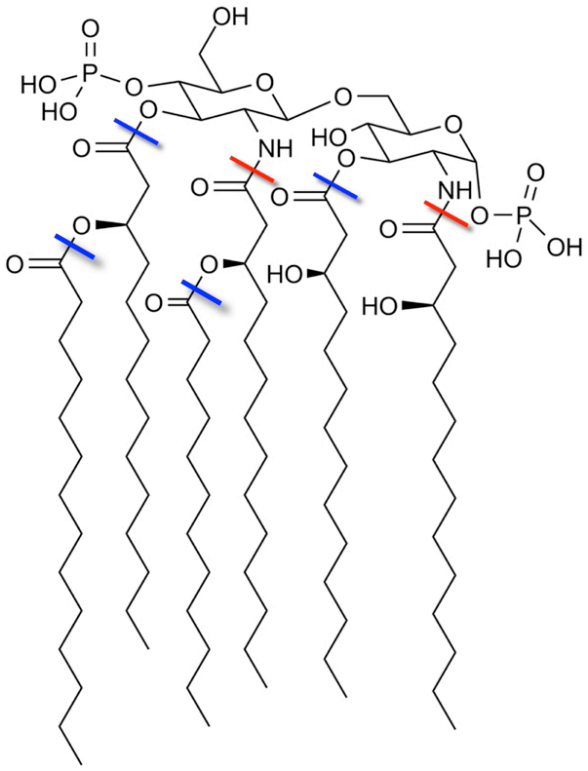


Figure 10: Scheme showing the bonds broken through O-deacylation (blue) and N-deacylation (red) reactions. Structure portrayed is Lipid A of Escherichia coli.

Basics of NMR

Nuclear Magnetic Resonance (NMR) analysis provides detailed information of oligo-polysaccharides structure. The interpretation of the NMR spectra however is not simple due to

the similar chemical environment possessed by the protons on the carbons, resulting in similar chemical shifts. Fortunately, protons are affected by the neighbouring protons, known as scalar-through-bond or *J*-coupling, which produces splitting of peaks on NMR spectra and this enables identification of different sugars (Zwahlen, 2002). Scalar-coupling is effective when protons are separated by two or three bonds, the protons are not isochronous (do not have the same chemical shift) and the protons are not bound to a nitrogen or oxygen. A proton with no neighbouring protons, results in a singlet, if it has one neighbour it is a doublet, with two neighbours it is a triplet etc (Bruice, 2011). Karplus rule defines that amplitude of the splitting of the NMR peaks is proportional to the cosine of dihedral angle between the two protons (Reich, 2010). Therefore, the splitting constant gives information of what environment the proton is neighbouring (Vilén, 2013). Within sugar residues, stronger coupling is seen when the hydrogens are in a *trans* arrangement, but weaker coupling is observed when there is a *cis* arrangement. Use of the coupling constant can help to determine the disposition of the hydrogens, or hydroxyl groups in sugars resides. Furthermore, the coupling constant can be used in the determination of the stereochemistry of the glycosidic linkages (Taylor and Drickamer, 2006). As an example, the anomeric configuration can be determined by observation of the chemical shift of the anomeric protons and carbons and the $^{3}J_{H1,H2}$ and $^{1}J_{C1,H1}$ values.

The starting point of the analysis is through ^{1}H NMR experiments that give most resonances in the region between ~ 3.4 and ~ 4.0 ppm relative to ring proton signals. The anomeric protons are found between 4.4 – 5.5 ppm and are useful in estimating the number and nature of monosaccharides composing the oligo-/polysaccharide under investigation (Vilén, 2013). In the high field region of the ^{1}H-NMR spectrum it is possible to find Kdo diagnostic signals, namely the $H3_{ax,eq}$, which usually resonate around 1.8- 2.0 ppm, whereas the methyl groups of deoxysugars are found around 1.1 ppm (Di Lorenzo, 2015).

Due to the complexity of the 1D spectrum, it is usually vital to study several two-dimensional NMR experiments, in order to assign all the ^{1}H and ^{13}C resonances in the oligo-/polysaccharide. Homonuclear NMR experiments, e.g. ^{1}H-^{1}H COSY (2D correlation spectroscopy), ^{1}H-^{1}H TOCSY (total correlation spectroscopy), ^{1}H- ^{1}H NOESY (nuclear Overhauser effect spectroscopy) and ^{1}H-

^{1}H ROESY (Rotating frame nuclear Overhauser effect spectroscopy) and heteronuclear experiments, such as ^{1}H-^{13}C HSQC (Heteronuclear single-quantum correlation spectroscopy), ^{1}H-^{13}C HMBC (Heteronuclear multiple-bond correlation spectroscopy) and ^{1}H-^{13}C HSQC-TOCSY are all employed for carbohydrate analysis. Since LPS derived-oligo-/polysaccharide may be decorated by phosphate groups, ^{31}P and ^{1}H-^{31}P HSQC experiments are also typically recorded..

Structural Characterisation of Lipid A

Chemical compositional analysis

The determination of the lipid A fatty acid content is usually achieved through GC-MS analysis of their methyl ester derivatives. This derivatisation method is achieved through treating the extracted LPS with MeOH/HCl followed by extraction of the lipids by *n*-hexane (Rietschel, 1976). Through interpretation of the mass spectrum produced the nature of acyl chains can be identified, the level of saturation and the hydroxylation pattern (De Castro *et al.,* 2010). This classical chemical analysis determines the composition of the fatty acids but can be used in support of the analysis performed using MALDI-TOF MS and Electrospray Mass spectrometry (ESI-MS).

MALDI-TOF Mass Spectrometry

The lipid A may be obtained directly from the LPS and LOS using the protocol stated above, that consists in a selective hydrolysis of the linkage between Kdo and lipid A. The glycolipid part is recovered through precipitation or by direct extraction with a specific solvent mixture (e.g. chloroform/methanol/water in different proportions). On the other hand MALDI MS analysis of lipid A directly on cells or after a precise micro-extraction of it from an aliquot amount of cells, is also employed to reach the lipid A structure assessment (Di Lorenzo F, 2017). The complete chemical characterisation of lipid A requires the determination of:

1. the sugar backbone, usually a GlcN disaccharide
2. the ester and amide linked fatty acids
3. the acyl chains distribution
4. the location of the polar head groups (phosphate groups).
5. phosphate substituents and their location (if present)

MALDI spectra can be executed in positive and negative mode. In particular, in the positive-ion mode it is possible to visualize also peaks corresponding to oxonium ions, necessary to differentiate the fatty acid distribution between the two GlcN of lipid A (Di Lorenzo, 2014).

Chapter 3: Methods and Materials

Solvents and Reagents

REAGENT	COMPANY	NUMBER
Acetic acid	Sigma-Aldrich	33209
Acetic anhydride	Sigma-Aldrich	320102
Acetone	Merck	1.00012.2500
Ammonium bicarbonate	Sigma-Aldrich	09830
Chloroform	Sigma-Aldrich	372978
Deoxyribonuclease I from bovine pancreas	Sigma Aldrich	9003-98-9
Deuterium Oxide	Sigma-Aldrich	151882
Dimethyl Sulfoxide	Sigma-Aldrich	34869
Ethanol	VWR chemicals	20821.330
Formaldehyde	Sigma-Aldrich	F1835
Hexane	VWR chemicals	24577.323
Hydrogen chloride – methanol solution	Sigma-Aldrich	17935
Iodomethane	Sigma-Aldrich	67692
Light Petroleum Ether	VWR chemicals	23835.365
Methanol	Sigma-Aldrich	322415

Phenol (solid)	Sigma-Aldrich	16016
Phenol	Sigma-Aldrich	16018
Proteinase K	Sigma-Aldrich	P6556
Ribonuclease A from bovine pancreas	Sigma Aldrich	9001-99-4
Sephedex G10 resin	GE Life Sciences	17001001
Silver Nitrate	Sigma-Aldrich	10220
Sodium borohydride	Sigma-Aldrich	452882
Sodium deoxycholate	Sigma-Aldrich	D6750
Sodium hydroxide	Sigma-Aldrich	30620
Sodium metaperiodate	Sigma-Aldrich	S1878
Superdex 30 resin	GE Life Sciences	17090501
Trifluoroacetic acid	Sigma-Aldrich	302031
Tris base	Sigma Aldrich	77-81-6
Alcian Blue	Sigma-Aldrich	A5268
Hydrochloric Acid	Carlo Erba	403872
Acetetic Anahydride	Fluka	45830
Pyridine	Sigma-Aldrich	270970
Potassium hydroxide	Sigma- Aldrich	P1767

Equipment

EQUIPMENT	INFORMATION
Gas Chromatography-Mass Spectrometer	Agilent Technologies gas chromatograph 7872A equipped with a mass selective detector 5977B and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm internal diameter, flow rate 1 mL min−1, He as carrier gas).
NMR	Bruker 600 DRX spectrometer equipped with a cryoprobe
Electrophoresis kit	BioRad mini-protean Tetracell (1653369) with glass plates (1653308, 1653310) and comb (1653354). Run with the BioRad “powerpac” basic.

Dialysis	Spectra-Por-3 Dialysis membrane (132724) MWCO: 3,500. (132754) MWCO: 12-14,000
Dialysis Pure-a-Lyzer	Pur-A-Lyzer™ Mega Dialysis Kit, MWCO 1 kDa, cap, 10 mL displacement. Sigma Aldrich: PURG10010-1KT

Software

SOFTWARE PROGRAMS	
Documenting and Presenting	Microsoft Office: Word, Excel, PowerPoint
GC-MS Analysis	Automated Mass spectral Deconvolution and Identification System (AMDIS)
NMR analysis	Bruker TopSpin 2.1

Growth of Veillonella parvula

Veillonella parvula strain ATCC 10790 was grown in anaerobose basal broth (OXOID), under anaerobic and planktonic conditions. The bacteria cells were grown within the laboratory of Professor Ricca in the Department of Biology at Federico II University, Naples. The first batch of *Veillonella* cells provided were 800 mg of dried cell mass, the second batch had a dried cell mass of 2 grams.

The bacterial cells from the strain *Veillonella parvula* DMS 2008 (Veillon and Zuber 1898), were grown in anaerobic, planktonic conditions. Provided by a collaboration with Professor Beloin in the Department of Microbiology at The Pasteur Institute. The strain was obtained from the intestinal tract of a hospitalised patient and then grown within laboratory conditions. The starting weight of dried *Veillonella* cells was 2 grams.

Extraction of LPS from Bacterial Cells

PCP extraction

LPS can be extracted using phenol 90%/chloroform/light petroleum ether in a 2:5:8 ratio (v/v/v). The dried cells were suspended in the mixture, stirred at room temperature (RT) for 30 minutes and then centrifuged. The supernatant was collected retained and the steps are repeated twice more with the pellet. The removal of solvents was performed using rota-evaporation and the LPS was precipitated from the phenol phase by adding drops of water (Galanos *et al*. 1969) (De Castro *et al*. 2010).

Water/phenol extraction

Dried cells or PCP residual (from method described above) were suspended in water/90 % phenol (v/v) mixture at 68 °C (Westphal *et al*., 1965). Three extractions were performed with hot water and centrifugation, each time collecting the supernatant. The phenol phase was also reserved, and both phases were dialysed (Spectra/Por® cut-off 12–14 kD, Ravensburg, Germany) against milliQ H_2O for 3-5 days.

Following this, the extracted materials were purified with enzymatic treatment of DNase (DN25-Sigma Aldrich®, St. Louis, MO, USA), RNase (R5503-Sigma Aldrich®) (37 °C, 5 h), and protease (P4630-Sigma Aldrich®) (56 °C, 16 h). Dialysis (Spectra/Por® cut-off 12–14 kD) was then repeated on the digested material against milliQ water.

Table 2: Starting dried weight of Veillonella cells and weight of extracted LPS

	DRIED CELL STARTING WEIGHT	EXTRACTED LPS	EXTRACTION METHOD
Veillonella parvula, ATCC 10790	800 mg	21 mg	Hot phenol/water
	2 g	86 mg	Hot phenol/water
Veillonella parvula, DMS 2008	2 g	50 mg	PCP

Typology analysis of LPS: DOC PAGE

Gel Preparation:

A 14 % DOC PAGE was prepared, using the system of Laemmli (Laemmli, 1970). Briefly, the separating gel (14 % in polyacrylamide) is prepared in a 0.75 mm spacer glass plate, overlaid with isopropanol and removed once polymerisation was complete. The stacking gel (5 %) was prepared and loaded directly on top of the separating gel with a comb to form wells for sample loading.

Table 3: Tabulated concentrations for the solution preparation of a 14% DOC PAGE.

SEPARATING GEL (14 %)	PREPARATION OF THE STACKING GEL (5 %)
1.15 mL milliQ H_2O	1.5 mL milliQ H_20
1.25 mL TRIS buffer (pH 8.8)	125 μl DOC (2%)
250 μl DOC (2%)	335 μl Acrylamide (30%)
-	30 μl Bromophenol Blue (%0.1)
2.33 ml Acrylamide (30%)	12.5 μl Ammonium Persulfate (10%)
25 μl Ammonium Persulfate (10%)	2.5 μl TEMED
2.5 μl TEMED	625 μl TRIS buffer (pH 6.8)

Sample Preparation:

8 μL of sample (1 mg/mL) was mixed with 2 μL sample buffer (Bromophenol Blue 10 mg/mL) and incubated for 10 minutes at 100 °C. The samples were loaded into the gel and run for about 45 minutes at 150 V.

Staining Procedure:

The gel is removed from the glass plates and washed in fixing solution (Ethanol 40 %, acetic acid 5 %, H_2O 55 % v/v/v) for a minimum of 2 hours. This is then replaced with 100 ml of oxidising solution (7 % Na_2O_2 in fixing solution, stated above) for 10 minutes and then washed for 10 minutes, 3 times with H_2O. 100 μL of silver nitrate solution (0.1 % $AgNO_3$ v/v) is prepared and added to the electrophoresis gel for 30 minutes. The gel is washed in developing solution (3 % Na_2CO_3 w/v, 20 μL Formaldehyde) until sufficient visualisation of the banding patterns on the gel. The gel is rinsed in a stopping solution (1 % acetic acid *v/v*) before being stored in H_2O (Kittelberger and Hilbink, 1993).

To identify the presence of exopolysaccharides and other charged sugars such as GAGs, an alternative staining can be applied to the sample DOC-PAGE of Alcian Blue. Solution of 0.5 % Alcian blue in 1 % acetic acid is prepared and applied to the gel for 16 hours before continuing with the first staining step of fixing solution (Al-Hakim A. *et al*., 1990) (Tsai *et al*. 1982).

Compositional Analysis

Carbohydrates:

Determination of monosaccharides composition was carried out through producing sugar derivatives and GC-MS analysis, as described in Leontein and Lönngren (1978). Monosaccharides were identified as acetylated *O*-methyl glycosides derivatives (AMG) by methanolysis of ~1 mg of dried sample using 0.3 mL of 1.25 M HCl/MeOH (1:1 *v/v*), (85 °C, 16 h) and acetylated with acetic anhydride in pyridine (85 °C, 30 min). The AMG were isolated using chloroform/water extractions. The organic phases were collected, dried and diluted in 500 μL of acetone to be analysed by GC-MS.

Monosaccharides were also identified as Partially Methylated Acetylated Alditols (PMAA). 1 mg of sample is dissolved in ~0.5 mL of DMSO with small amount of crushed NaOH and stirred for 1 hour. The sample was then treated with 0.3 mL Iodomethane (CH_3I) for 16 hours. The sample was then extracted 5 times with water and $CHCl_3$ (1–2 ml), with the organic phases collected and dried under airflow. The sample was dissolved in 0.3 mL of 2 M TFA (90 minutes, 110 °C). Neutralization of the sample was achieved using MeOH. Sample was dissolved in 2 mL of EtOH and a small amount of $NaBD_4$ was added (stirred for 16 hours, RT). The reaction was stopped using 2 M HCl and dried and neutralized using MeOH. Acetylation of the sample was performed with acetic anhydride in pyridine (85 °C, 30 min). Chloroform/water extractions were then performed to isolate in the organic phases the PMAAs which were diluted in 500 μL of acetone to be analysed by GC-MS.

Fatty Acids:

For the analysis of fatty acids, an aliquot of the LPS fraction (0.5 mg) was taken. The sample was dried and methanolized with 1 M HCl/CH_3OH, incubated at 85 °C for 16 h. The fatty acids were extracted with hexane, which was dried under airflow and dissolved in 500 μL acetone and analysed by GC-MS.

The analyses by GC-MS were all executed on an Agilent Technologies gas chromatograph 6850A equipped with a mass selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm internal diameter, flow rate 1 mL min^{-1}, He as carrier gas). The temperature program was employed for the lipid analysis and carbohydrate analysis was: 140 °C for 3 min, 140 °C → 280 °C at 10 °C min^{-1}.

Isolation of the oligo-/polysaccharide Portion (O-antigen and core) and Lipid A

Oligo-/polysaccharide

The extracted LPS sample was dissolved in acetate buffer solution (100 mM, pH 4.5) and incubated at 100 °C for 2 hours. Precipitation of the lipid A occurred and the supernatant,

containing the polysaccharide components was recovered through centrifugation (8500 rpm, 4 °C, 30 minutes). The supernatant was purified using a size-exclusion liquid chromatography column (Superdex 30, GE Healthcare, 0.75cm^{2}x75cm, rate: 10mL/hour, fraction volume: 1.6mL, eluent: milli-Q H_2O), each fraction was characterised by 1D NMR spectroscopy.

Lipid A

The lipid A precipitate was recovered as a pellet after centrifugation, washed with H_2O and then freeze-dried before being prepared for analysis with MALDI-TOF Mass Spectrometry.

Isolation of oligosaccharide portion by Alkaline Hydrolysis

O-de-Acylation and *N*-de-Acylation

A 15 mg aliquot of dry (over phosphorus anhydride under vacuum), purified LPS were subject to alkaline hydrolysis in anhydrous conditions by incubation with methylhydrazine (1.5 ml, 37°C, 1.5 h). Ice-cold acetone was added until precipate of the O-deacylated LPS was seen. Centrifugation (3000 RPM, 4°C, 30 minutes) was then performed and the pellet recovered, and freeze-dried (Holst, 2000).

The O-deacylated LPS sample was then treated with KOH (4 M, 1.5ml), kept at 20-22°C under nitrogen for 15 minutes and then incubated at 120°C (16 hours). Neutralization was performed with HCl (4 M) until pH 6 was reached. Extraction with chloroform and milliQ water followed by centrifugation 3 times (3000RPM, 4°C, 30 minutes) was used to separate sample (now water soluble) from the salts.

The de-acylated oligosaccharide was recovered (from the water supernatant phase of centrifugation) and freeze-dried. For successful analysis of the sample a desalting purification was required through low pressure liquid chromatography (Sephadex G-10, 16 mL/h), eluted with 10 mM ammonium bicarbonate. (Holst, 2000) (Silipo *et al*., 2012).

NMR analysis

For structural analysis of the extracted and isolated oligo-/polysaccharide ^{1}H-NMR spectra were performed at 278 K with a cryoprobe-equipped Bruker 600 DRX spectrometer and dissolving each sample in 500 μL of deuterated water (D_2O). Spectra were analysed using Bruker Top-Spin software.

MALDI-TOF Analysis

The analysis of the lipid A was performed using MALDI-TOF MS and MS^2 analysis on a ABSCIEX TOF/TOF™ 5800 Applied Biosystems mass spectrometer, equipped with an Nd:YLF laser with a λ of 345 nm, a <500 ps pulse length, and a repetition rate of up to 1000 Hz (Di Lorenzo, 2017), (Silipo A *et al*., 2004). The matrix was the trihydroxyacetophenone (THAP) (91928-Sigma Aldrich®) dissolved in methanol/0.1% trifluoracetic acid/acetonitrile (7:2:1, v/v/v) at a concentration of 75 mg mL^{-1}

A micro extraction method of LPS was performed as described in (Di Lorenzo, 2017) and loaded onto a trihydroxyacetophenone (THAP) (91928-Sigma Aldrisch®) matrix dissolved in methanol/0.1 % trifluoracetic acid/acetonitrile (7:2:1, *v*/*v*/*v*) at a concentration of 75 mg mL^{-1} (Silipo A *et al*., 2004), (Sturiale *et al*. 2011).

Chapter 4: Compositional study of Oligo-/Polysaccharide components from the LPS of Veillonella parvula DSM 2008

Extraction with PCP method

LPS was extracted from dried bacterial cells using the PCP method (Galanos *et al*. 1969). LPS was extracted by precipitation by adding water, the precipitate was then subject to purification by dialysis (12–14,000 MWCO). A 14 % DOC-PAGE and silver nitrate gel staining showed a polydisperse "ladder-effect" indicating the presence of a *smooth*-type LPS.

Compositional Analysis of LPS saccharide domain

Monosaccharide analysis through AMG derivations and analysis of the mass spectra fraction pattern enabled the detection of ribose, glucose, galactose, heptose, glucosamine, galactosamine and Kdo residues. Further structural information was provided by the analysis of the PMAA derivations of the PCP precipitate extract (Table 4).

Table 4: Results of sugar composition from PPMA derivation method of the PCP precipitate of Veillonella parvula DSM 2008

NATURE	LINKAGE
Pentose (Ribose)	To be further investigated
Hexose (Glc, Gal)	t-Hex*p* t-Hex*f* 2-Hexp 3-hexp 4-Hexp 3-Hexp 6-Hex*p* 6-Hex*f* 2,3-Hex*p* 2,4-Hex*p* 2,6-Hex*p* 3,4-Hex*p*
HexN (GlcN/GalN)	6-Hex*p*N
Heptose	2,7-Hep
	2,4-Hep
Kdo	To be further investigated

An acid hydrolysis treatment was performed on the PCP precipitate in order to gain identification of the saccharide composition and structure of the LPS extracted. After centrifugation, the water-soluble supernatant was analysed by ^{1}H NMR spectroscopy. The ^{1}H-NMR spectrum showed signals typical of a mixture of oligo-/polysaccharide, however indicated that the product was not pure enough and required further purification steps. The use of a Superdex® 30 column (GE Healthcare Life Sciences) eluted with 50 mM ammonium bicarbonate successfully produced 6 distinct fractions containing different sized oligosaccharides.

Analysis of each fraction using ^{1}H-NMR revealed a successful separation of the O-antigen moiety and the core oligosaccharide portion. The anomeric region of the ^{1}H-NMR can be seen to contain many signals highlighting the complexity of the saccharide in the LPS (Figure 11).

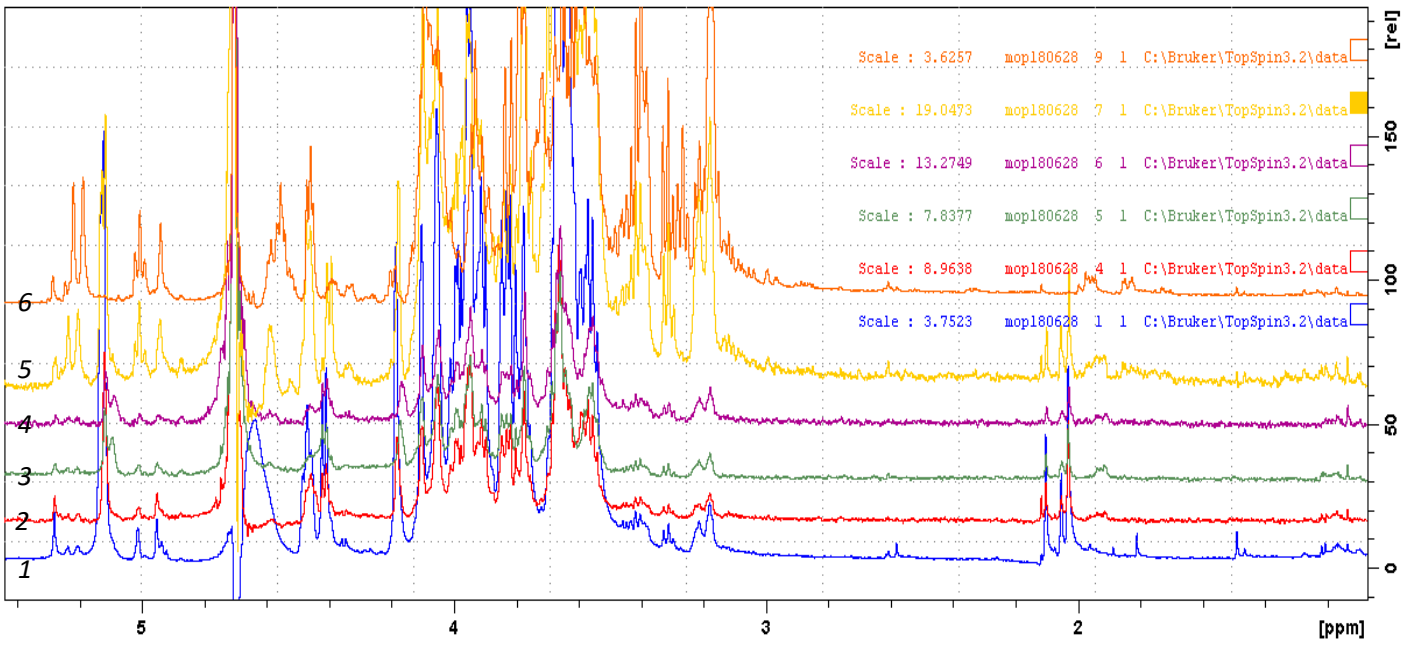


Figure 11: Following acid hydrolysis of the PCP procedure extracted LPS, fractionation of the oligosaccharides was achieved through Superdex® 30 chromatography. Six fractions were produced and analysed through ^{1}H-NMR. Fractions 1 and 2 identified as containing the O-antigen region, fraction 6 contained the core region, fractions 3, 4 and 5 are a mixture containing both the core and O-antigen moieties.

Core- oligosaccharide

From the Superdex 30® column fractionation (Figure 11), the final eluted fraction (fraction 6) was identified using ^{1}H- NMR to contain the core region. This hypothesis arose from the presence of signals at 1.9/2.0 ppm which are identified as the H-3 methyl protons of the Kdo unit. A PMAA derivatisation was performed on an aliquot of this fraction and enabled the composition of the sugars of the core region of the LPS from *Veillonella parvula* DSM 2008 showing a high variety of hexoses variously substituted, also hexosamine residues were found (Table 5).

O-Antigen Polysaccharide

The first two fractions eluted from the Superdex 30® chromatography (Fractions 1 and 2) were identified as containing the O-antigen saccharide component of LPS. This was known through the ^{1}H-NMR spectra being typical of a polysaccharide. The first two eluted fractions were subject to AA and PMAA derivatisations to make the sugar compositional analysis. Both AA derivations and PPMA derivations were compared to standards and identified the presence of glucose and galactose residues for the hexose residues (Table 5).

Additionally, some preliminary ^{31}P NMR analysis of fractions 1 and 6 (of Figure 11) indicated the presence of phosphates which will be further examined through 2D NMR experiments and MALDI-TOF MS investigation.

Table 5: The saccharide composition of the Core and O-antigen regions of extracted LPS using the PCP method. The composition if the core was found using Partially Methylated Acetylated Alditols and for the O-antigen a use of both Partially Methylated Acetylated Alditols and Acetylated Alditols derivatives. Analysis was through GC-MS.

CORE	O-ANTIGEN
t-Hex (Glc)	2-pent (to be further investigated)
3-Hex*p*	*t*-Hex (Glc)
2-Hex*p*	3-Hex (Gal)
2-Hex*f*	4-Hex*p*/5-Hex*f* (4-Glc)
4-Hexp/5-Hexf (4-Glc)	3,6-Hex
6-Hex*p*	
2,3 Hex*p* (Gal)	
2,6 Hex*p*	
2,4-Hex*p*	
6-HexN	
2,7-Hep	
2,4-Hep	

Future steps will be the study of the 2D NMR spectra that will be recorded for each Superdex® fraction, in order to characterise the structure of each eluted component.

Alkaline treatment of LPS

The extracted LPS sample was subject to *O*-de-acylation and *N*-de-acylation reactions in order to isolate the full saccharide region of the investigated LPS. Following purification using size exclusion chromatography to remove salts, the fully deacylated product was subject to ^{1}H-NMR

analysis. The spectrum showed an impure sample, but anomeric signals were clear to detect the presence of an oligosaccharide.

The sample was then purified using a Pure-A-Lyzer dialysis kit, 1 kDa for 3 hours and ^{1}H-NMR of the dialysed and freeze-dried sample confirmed the purification through the loss of many background signals (Figure 12). Through analysis of the ^{1}H-NMR, defined signals were identified as belonging to glucosamine residues of the lipid A (5.0-5.5 ppm), as well as signals from the Kdo units (1.8/2.0 ppm): potentially 2 Kdo were identified.

The combination of the data belonging to the *O*-de-acylation/*N*-de-acylation step with those from fraction 5 of the Superdex 30® column separation (which has the potential to define the linkage region between the O-antigen and the core oligosaccharide), the full LPS structure can be understood. Further 2D NMR will need to be performed to confirm this.

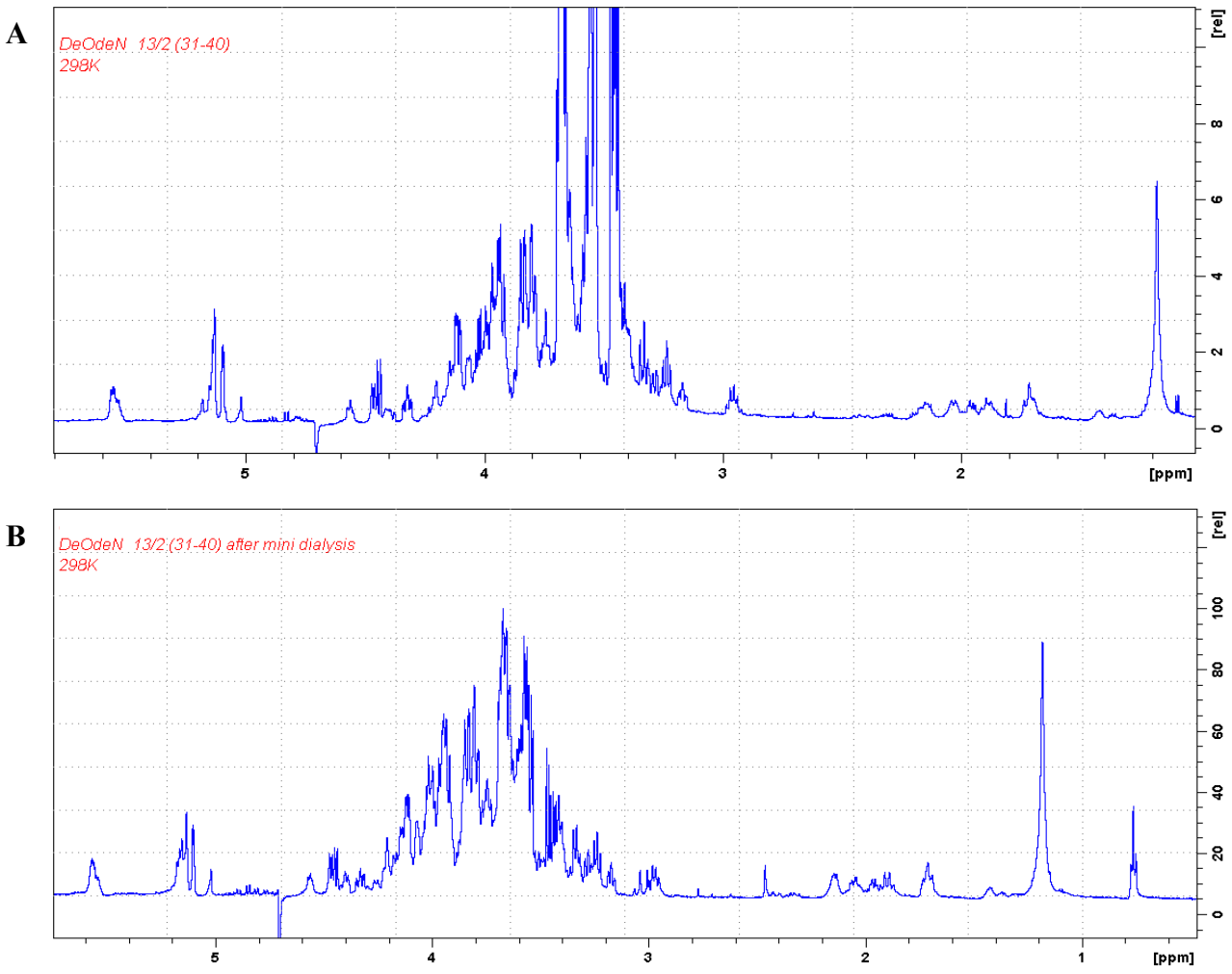


Figure 12: ^{1}H NMR of PCP extracted LPS from Veillonella parvula DSM 2008 after de-acylation procedure. a) spectra after SEC to remove salt impurities. b) after dialysis to further purify sample

Discussion

Veillonella parvula is known to be an abundant commensal coloniser of the human gut and mouth; it is linked to progression of childhood immune system but has also been found to be an opportunistic pathogen. Furthermore, *Veillonella parvula*, is found in the *Negativicutes* which is a class of the Firmicutes phyla (Poppleton *et al.,* 2017). *Negativicutes* are a phylogenic phenomenon as they ancestrally are Gram-positives, although bacteria, such as *Veillonella* species, possess two systems of membrane and express LPS therefore they stain as Gram-negatives (Antunes *et al.,* 2016) (Poppleton *et al.,* 2017). Increasing the understanding of the

chemical structures of LPS found from gut microbiota bacteria species is particularly interesting to enable to form a link between how and if the structures of the LPS play a vital role in the activation/modulation/suppression of the immune system response (Matera *et al*., 2009) (Arrieta *et al*., 2015). Moreover, in this case, it could also improve the knowledge of the evolution of bacterial cell envelope being *Veillonella* a bacterium at halfway between Gram-positives and Gram-negatives.

In this present research it was uncovered that *Veillonella parvula* DSM 2008 (isolated from the gut of a hospitalised patient) possesses *smooth*-type LPS. As well as characterisation of the lipid A component, *described in Chapter 5*, the saccharide portion of the LPS has been isolated and preliminary structural analyses have been performed. The results were obtained from a combination of chemical analyses coupled with GC-MS spectrometry and ^{1}H-NMR spectroscopy.

The study of the composition of each distinct region of the LPS is extremely interesting as they both play important and separate biological roles. The composition of the O-antigen has been found to play many roles for the survival and virulence of bacteria species. The structure of the O-antigen affects the TLR4 signalling outcome (Lebeer *et al*, 2010). The O-antigen composition is also important for the survival and 'hiding' from the host immune system. A publication by Moran *et al. (*1996) found that specific monosaccharides within the O-antigen enables the LPS to mimic carbohydrates found on host cells therefore the bacteria form a "camouflage" from attacks from the immune system. Possibly, *Veillonella*, found in this phylogenetic phenomenon class, adapted to express LPS with an O-antigen as a protective mechanism from immune system within human intestinal tract and therefore can inhabit the human host without harm.

Here, the O-antigen was found to be composed of repeating units of pentose and hexose residues. Through performing both AA and PPAM chemical derivations and comparison with standards, the pentose sugars of the O-antigen were identified as a 2-linked ribose residue and the hexose residues are mixture of glucose and galactose. From the number of anomeric signals found in the ^{1}H-NMR spectrum it can be hypothesised that the O-antigen could be made up of a

particular trisaccharide repeating unit. In summary, the O-antigen of *Veillonella parvula* DSM 2008 strain has a complex structure, additionally it is known that the O-antigen of bacterial species are heterogeneous therefore further investigation using additional 2D NMR techniques need to be employed.

Confirmation of the core oligosaccharide sugar composition of LPS is extremely interesting biologically as it has been identified that there are specific sugar residues within the core region which directly bind to C-type lectins and activate phagocytosis of the bacteria by the human immune system (Zhang *et al*., 2006). In the present study, for the core region, the presence of the Kdo was found: a structural element in almost all core regions of bacterial LPS (Holst, 2007) which is comparable to the LPS from phylogenetically close bacteria (Helander *et al.* 1992). Here, the composition of the core was found to be a mixture of glucose, galactose, glucosamine, galactosamine, heptoses and Kdo residues. The results of the PPAM analysis provides information about the ring structure and the branching of the sugars, showing that many of the hexose residues are 3-substituted. Further confirmation of the presence of Kdo was obtained by ^{1}H-NMR analysis due to the presence of the typical methylene signals in the range 1.9-2.2 ppm.

Further Work:

A more detailed investigation of the full structure of the core and O-antigen region of the LPS from *Veillonella parvula* will be the next step of the present work. This can be achieved through further investigation with AMG derivative standards by GC-MS analysis. A full completion of the compositional saccharide analysis would aid the prospective 2D NMR experiment investigation.

Chapter 5: Lipid A Structure of Veillonella parvula DSM 2008

Veillonella parvula DSM 2008

Fatty Acid Composition

The lipid A portion of LPS has been analysed starting from both the PCP and hot phenol/water extracts, as previously discussed in Chapter 4. The lipid A fatty acid composition has been achieved by the analysis of their methyl ester derivatives which showed the occurrence of hydroxytridecanoic acid (C13:0(3-OH)), hydroxytetradecanoic acid (C14:0(3-OH)), hydroxypentadecanoic acid (C15:0(3-OH)), undecanoic acid (11:0), dodecanoic acid (12:0), tridecanoic acid (13:0), hexadecanoic acid (C16:0) and heptadecanoic acid (C17:0). Also, unsaturated species were identified as tetradecenoic acid (C14:1) and pentadecenoic acids (C15:1) as minor species.

MALDI-TOF analysis *V. Parvula* DSM 2008

Full structural elucidation of the lipid A was obtained through MALDI MS and MS^2. Three methodologies were applied to gain the overall results: Negative-ion MALDI MS and MS^2 analysis executed directly on the dried bacterial cells, the same analysis after a microextraction method of lipid A (Figure 13) (Di Lorenzo, 2017), and the MALDI MS and MS^2 analysis of the mild acid hydrolysis precipitate (see Chapter 4) which supported the results.

From all the spectra the high heterogeneity of the lipid A is clearly visible, both in terms of phosphorylation and acylation pattern in accordance with the fatty acid compositional analysis. It is possible to identify four distinct clusters of peaks relative to *bis*-phosphorylated tri- to hexa-acylated species and each of these clusters is also characterized by the occurrence of species lacking one phosphate unit ($\Delta m/z$=80).

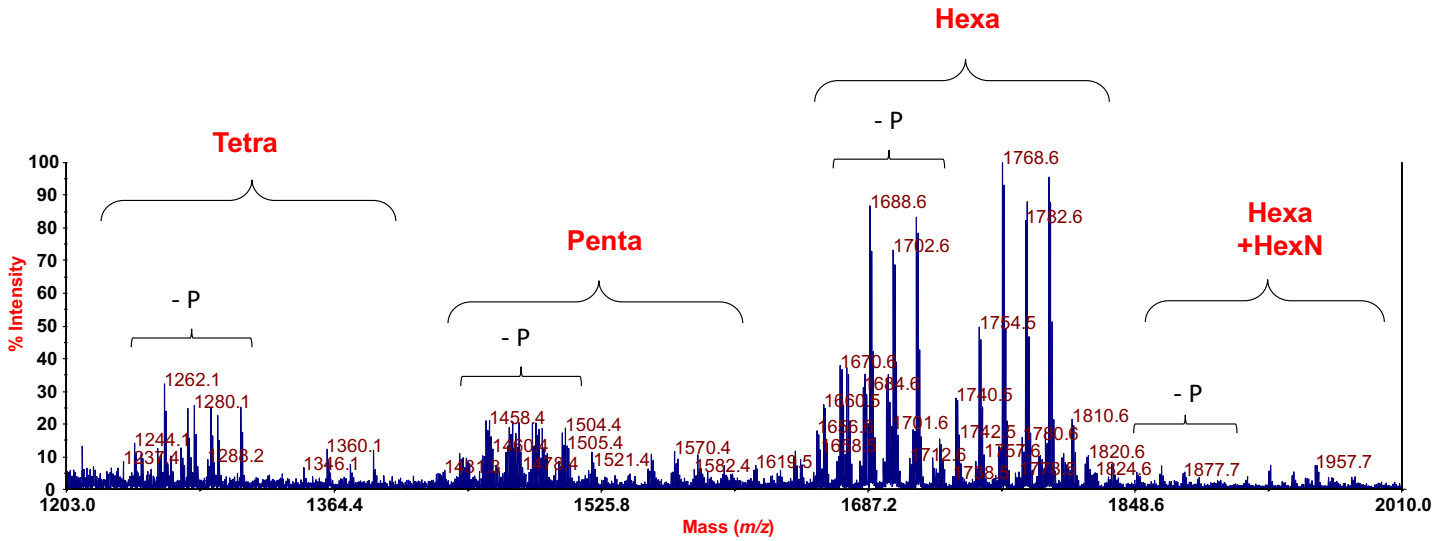


Figure 13: MALDI-TOF Mass Spectrum recorded in negative ion mode using the micro-extraction method of the PCP precipitate from Veillonella parvula DSM 2008. (communications with Di Lorenzo, 2018)

Mono- and *bis*-phosphorylated hexa-acylated lipid A species are the most dominant in the spectrum which was identified by the cluster at around *m/z* 1688.6 and 1768.6 respectively. In detail, the peak at *m/z* 1768.6 matched with a *bis*-phosphorylated GlcN disaccharide backbone carrying two primary ester-linked C13:0(3-OH), two amide-linked C15:0(3-OH) acyl chains, while one C11:0 and one 13:0 were the secondary acyl substituents. The species identified at *m/z* 1768.6 was attributed to a *bis*-phosphorylated lipid A carrying two primary ester-linked C13:0(3-OH), two amide-linked C15:0(3-OH) as previously described, while two 13:0 were the secondary acyl chains in this species (Figure 14). The slightly less intense cluster of peaks at around *m/z* 1688.6, as stated above, is attributed to the related hexa-acylated species lacking one phosphate group ($\Delta m/z$=80).

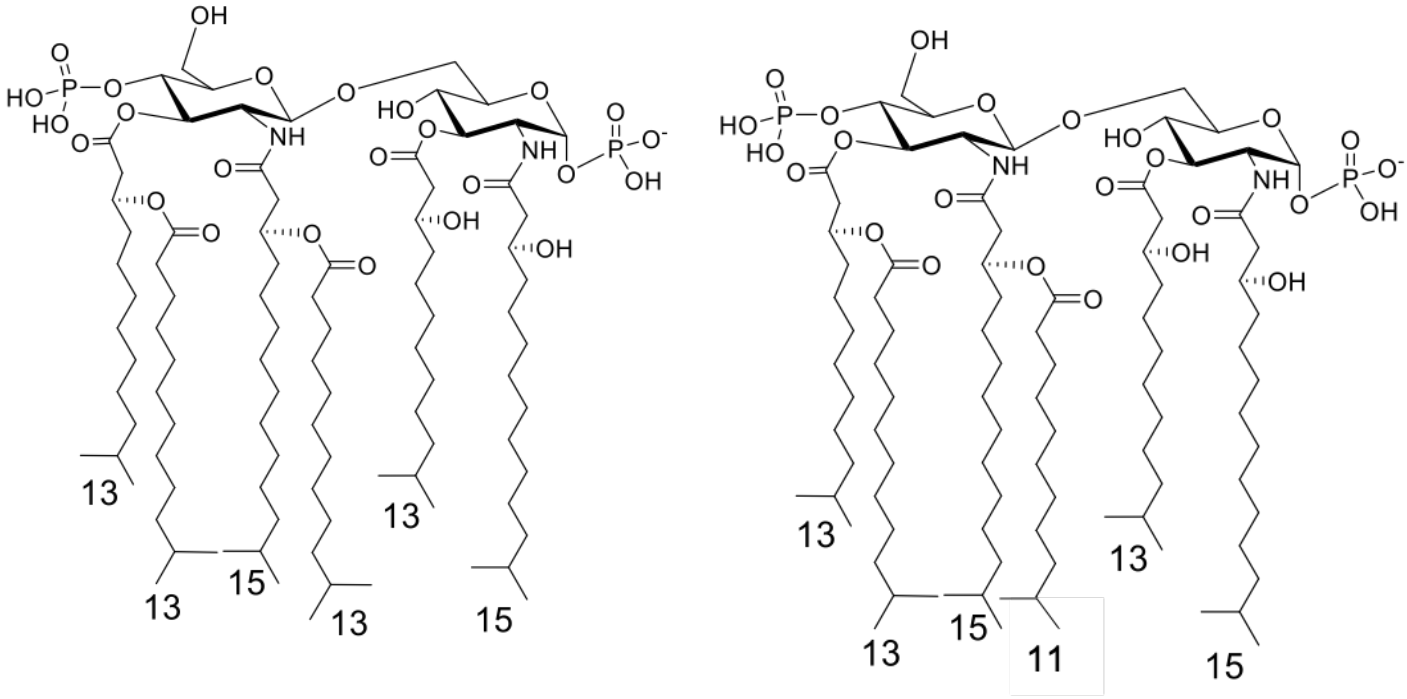


Figure 14: Sketch of the bis-phosphorylated hexa-acylated lipid A species identified using MALDI-TOF MS. A) Species at m/z 1796.6 with two ester linked C13:0 (3-OH) and two amide linked C15:0 (3-OH) acyl chains and further substitutions on both the primary acyl chains of the non-reducing GlcN by tridecanoic acids (C13:0) B) Species at m/z 1768.6 also with two ester linked C13:0 (3-OH) and two amide linked C15:0 (3-OH) acyl chains but with secondary substitutions by C13:0 and C11:0

In order to characterise the location of the secondary acyl substituents, with respect to the GlcN disaccharide backbone, several negative ion MALDI MS^2 experiments were recorded. Herein, the MALDI MS^2 spectrum of precursor ion at *m/z* 1796.6 will be described as example (Figure 15). The spectrum showed a main peak at *m/z* 1566.4 relative to a fragment avoid of one C13:0(3-OH), whereas other peaks at *m/z* 1352.3 and 1138.3 were relative to lipid A fragments lacking one C13:0(3-OH) and one C13:0 (*m/z* 1352.3), or lacking C13:0(3-OH) and two C13:0 (m/z 1138.3). The peak at *m/z* 1370.3 was very important in terms of the structural characterisation as it was relative to a fragment devoid of an entire ester-linked moiety made up of one C13:0(3-OH) and one C13:0, indicating the occurrence of the acyl chain C13:0 as a secondary substituent of the primary ester-linked C13:0(3-OH). Similarly peak at *m/z* 925.1 was attributed to a lipid A species derived from the loss of one entire unit of C13:0(3-OH) and C13:0, plus the sequential loss of one primary C13:0(3-OH) and the other secondary C13:0. Finally, the important peak at

m/z 710.0 derived from the cleavage of the glycosydic linkage (Y_1) (Domon and Costello, 1988) indicated that the acyl chains linked to the reducing GlcN units were only the primary C13:0 (3-OH) and C15:0 (3-OH), thus confirming that the secondary acyl chains only decorate the non-reducing GlcN residue.

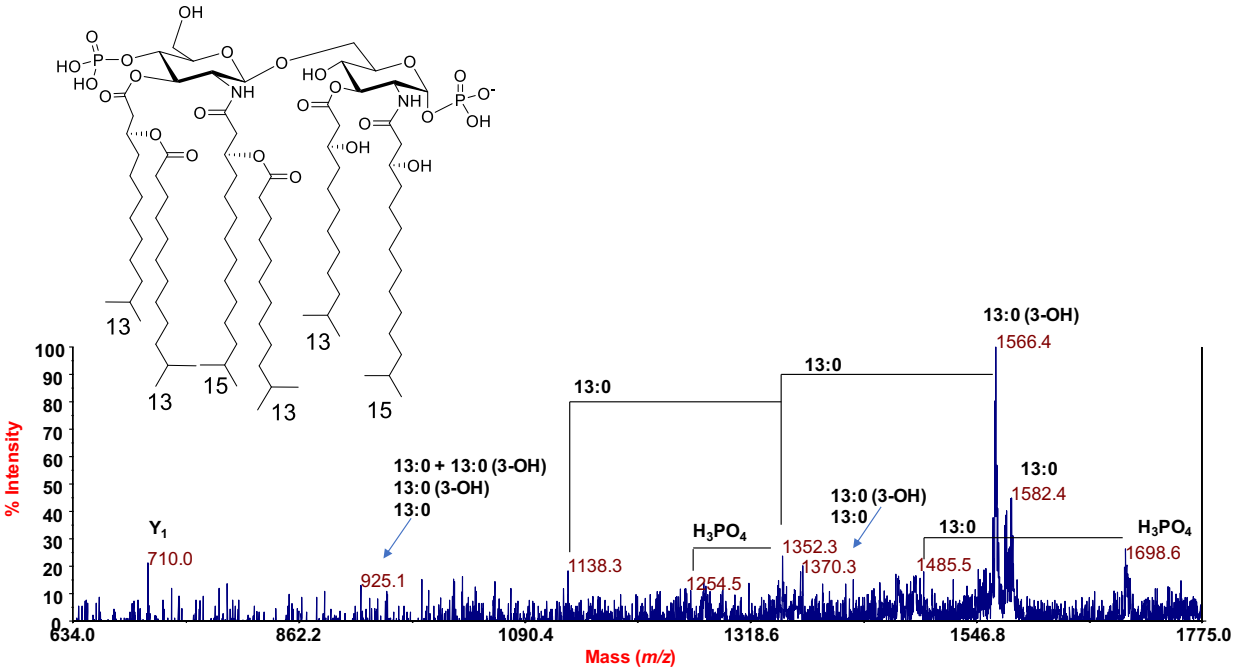


Figure 15: MS^2 spectrum recorded in negative ion polarity of precursor ion at m/z 1796.6. In the inset the proposed bis-phosphorylated hexa-acylated lipid A structure.

A less intense lipid A species identified at *m/z* 1877.7 and 1957.7 matched with *mono*- and *bis*-phosphorylated hexa-acylated species which are further decorated by a hexosamine residue attached at position 4' of the non-reducing GlcN via phosphodiester bridge (Figure 16). The distinction of whether this hexosamine is glucosamine or galactosamine remains undetermined as both species were identified in the compositional analysis.

In conclusion, one of the main species composing the *Veillonella parvula* lipid A is the one sketched in Figure 15.

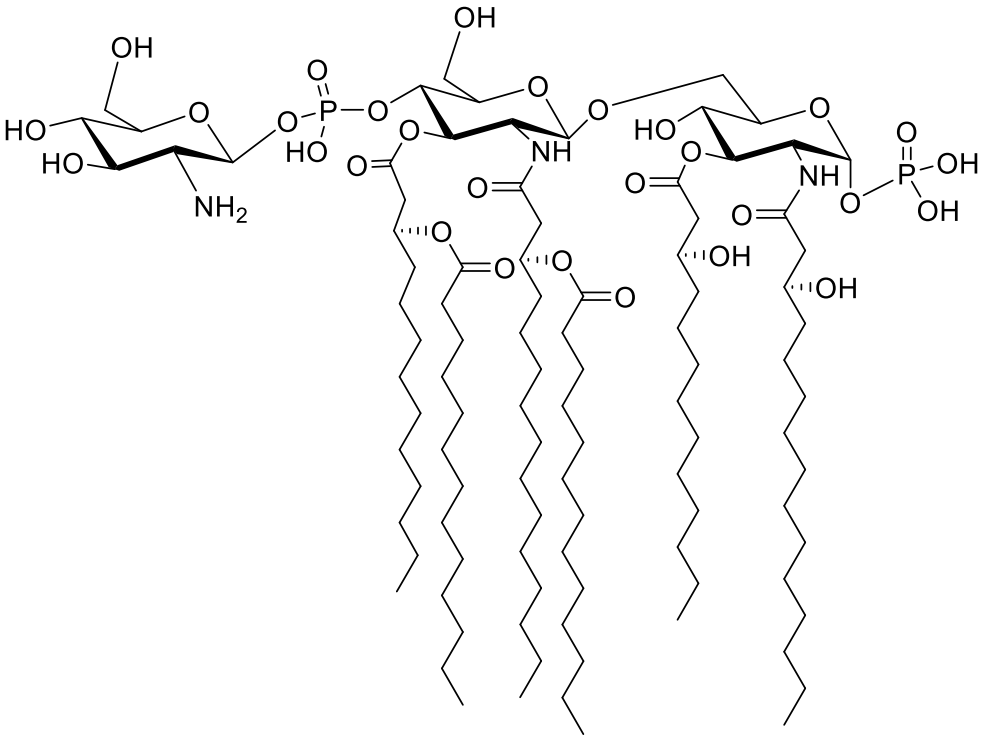


Figure 16: Image of hexa-acylated species with a hexosamine residue linked to the non -reducing end of the GlcN I residues of the lipid A disaccharide. This was identified using MALDI TOF MS analysis with a m/z 1957.7. The glucosamine decorating the lipid A backbone in position 4' of GclN I is tentative.

Discussion

Lipid A has been identified as the toxic component of the LPS through activating the TLR4/MD-2 complex, leading to an immune response. Much research into lipid A structure-activity relationship has shown that the number of acyl chains, phosphorylation pattern and the presence of further charged groups on the GlcN disaccharide are major determinants modulating the toxicity of the lipid A (Molinaro *et al.* 2015).

In this study, the production of methyl esters derivatives of the fatty acids in the extracted LPS were identified using GC-MS which gave a preliminary indication of the high heterogeneity of the acyl chains within the lipid A species. The methyl ester derivatives indicated, among others, the presence of odd-numbered acyl species. The presence of these latter acyl chains is particularly unusual compared to the known published lipid A structures; however, a few

publications of the lipid A from other microbiota bacteria, such as *Bacteroides fragilis,* have also seen this trend (Wollenweber *et al.,* 1980). There is also evidence that many Gram-positive bacteria of the microbiota synthesise predominantly odd-numbered branched-chain fatty acids in their bacterial membrane lipids, such as *B. subtilis* and *S. aureus* (Kaneda, 1991). This would potentially correlate with the ancestral tree of *Veillonella*, which is found within a class of the Gram-positive phyla Firmicutes (Poppleton *et al.,* 2017).

The chemical structure of the lipid A from *Veillonella parvula* was seen to change in number of acyl chains, with the predominance of hexa-acylated lipid A species but also penta-acylated and tetra-acylated species (Figure 15). Moreover, the MALDI TOF mass spectra investigation found that the lipid A species also have alterations in the phosphate content as species containing only one phosphate group were identified. The lipid A typically has an overall negative charge, which is supported by the presence of the phosphate groups therefore, a loss of phosphate will reduce the negative charge. The human immune system can recognize and attack through charged interactions between the negative lipid A and positively charged anti-microbial proteins (Maeshima and Fernandez, 2013). In *Bacteroides thetaiotaomicron*, another bacterium in the gut microbiota, the loss of a phosphate group on the GlcN head group of lipid A creates resistance to high levels of associated antimicrobial peptides (Cullen *et al.,* 2015), therefore it is possible to suppose that the loss of a phosphate group on *Veillonella* could reduce or prevent the host immune system attacks on the bacteria (Steimle et al., 2016) (Maeshima and Fernandez, 2013). Additionally, phosphate groups of the lipid A are able to form interactions with some of the protein residues in the TLR4/MD-2 receptor complex (Maeshima and Fernandez, 2013). Alterations in the number of phosphate groups of the lipid A may affect the ability of the LPS to activate the TLR4 signaling pathway. Moreover, it has been demonstrated in the case of *Bordetella pertussis* LPS that the TLR4 activation increases when both the GlcN composing the lipid A sugar backbone are modified with an addition of glucosamine residues (Shah *et al.*, 2013). Interestingly, the presence of a side chain of a hexosamine residue was also observed here in *Veillonella parvula* DSM 2008 (Figure 16). The addition of groups like amino-sugars can further alter the charge and assist the LPS/bacteria to evade the host immune system (Steimle *et al*.,

2016). This could be a mechanism by which the bacterium alters itself to “hide” from the host, an important role when inhabiting the human gut.

In conclusion, whether and how all these structural peculiarities affect the immunological properties of *Veillonella* LPS will be further investigated in a future detailed structure to function relationship study. Nevertheless, it is obvious from the high heterogeneity of the lipid A species found in this study on *Veillonella parvula*, and supported by previous literature data on gut microbiota, that there is a delicate balance to be maintained between a bacterial species being commensal or pathogenetic which is highly reliant on the chemical structure of the lipid A.

Chapter 6: Characterisation of saccharide domains of the LPS from Veillonella parvula strain ATCC 10790.

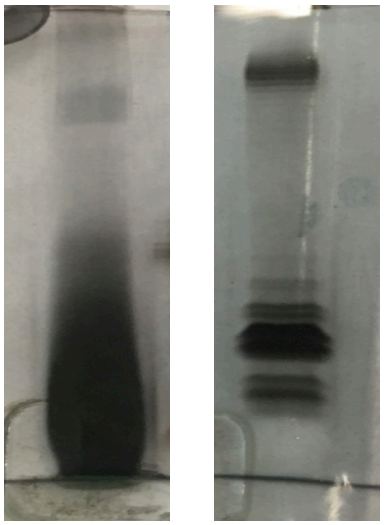
Veillonella parvula ATCC 10790 (Batch 1)

Isolation of LPS

A first attempt of extraction of the LPS from *Veillonella parvula* ATCC 10790, grown in planktonic and anaerobic conditions, was performed with a very small starting weight of dried bacterial cells (800 mg), but was used to give an insight into the carbohydrate composition of the LPS. A first method of extraction was executed using the PCP method (Galanos *et al.* 1969) however no precipitation after centrifugation was formed when water drops where added to the phenol phase. Therefore, the hot water/phenol extraction method was applied (Westphal and Jann, 1965).

A 14 % DOC PAGE analysis with silver nitrate gel staining was performed on the dialysed and lyophilised water phase obtained by the above extraction, producing a weak 'laddering' effect demonstrating the presence of high molecular-weight species in the upper part of the gel.

The sample was purified with DNase, RNase, and Proteinase K followed by extensive dialysis (MWCO 12-14.000 Da) against milliQ water for three days. The sample was recovered and freeze dried, producing only 21 mg of extracted material. A 14 % DOC PAGE analysis of the enzymatically treated sample was run and stained with both Alcian Blue (used to stain acidic polysaccharides) and silver nitrate. The Alcian Blue stain was positive with a dark blue banding indicating the occurrence of some acidic polysaccharide contaminant (not shown). The silver nitrate staining did not produce a ladder effect but a dark staining across the electrophoretic pattern on the gel (Figure 17).



Veillonella parvula LPS *E. Coli LPS*

Figure 17: 14% DOC PAGE run at 150 V for 1 hour. Blue banding was seen with Alcian Blue and laddering effect seen with silver nitrate staining.

Chemical Analysis of LPS carbohydrate component

Monosaccharide compositional analysis was achieved through AMG derivations of the extracted, purified sample to give the monosaccharide composition of the full LPS. Table 6 gives the results obtained by analysing GC-MS spectra; it is worth noting that it was not possible to identify the occurrence of Kdo, namely a key component of the LPS structure.

Table 6: Monosaccharide detected from AMG analysis of LPS before and after enzymatic digestion of extracted LPS from Veillonella parvula (Batch 1)

Monosaccharide (**before enzymatic digestion**)	**Monosaccharide**s (after **enzymatic digestion**)
Deoxyhexose (Rha)	Deoxyhexose (Rha)
Pentose (Rib)	Pentose (Rib)
Deoxy-hexosamine	-
Hexose (Glc)	Hexose (Glc)
-	Hexosamine (GlcN)

Veillonella parvula ATCC 10790 (Batch 2)

Isolation of LPS

A second tentative of structural investigation was performed starting from bacterial cells with a dried weight of 2 grams that were washed with distilled water, ethanol and acetone. To confirm no saccharides were lost in this cell wash, a proton NMR spectrum was recorded.

The hot phenol/water extraction method (Westphal and Jann, 1965) was used. Both the water and phenol phase were subject to dialysis (MWCO 12-14.000 Da) and freeze dried. 86 mg of sample was weighed for the water phase which was then attempted to confirm the presence of LPS though a 14 % DOC PAGE and silver nitrate gel staining, however the gel produced inconclusive results (Figure 18).

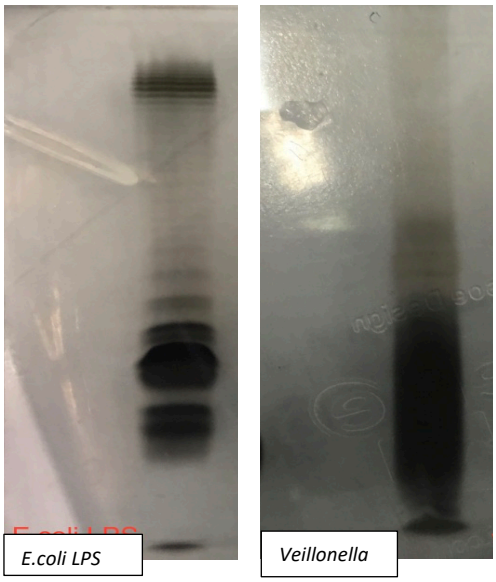
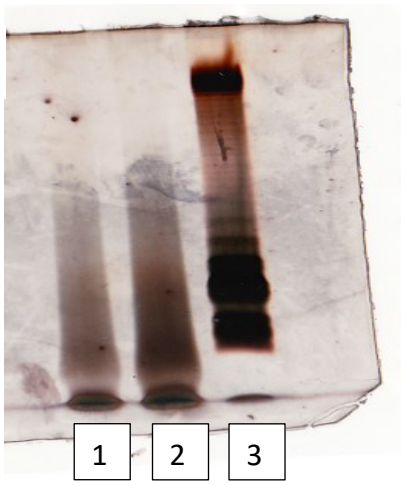


Figure 18: A 14% DOC PAGE run at 150 V for 1 hour with 1 mg/ml concentration of LPS. V. parvula LPS was extracted using hot water/phenol method from Batch 2 and E. coli LPS is used as standard.

The water phase from the hot phenol/water extraction was then subject to enzymatic digestion using DNAase, RNAase and proteinase K, followed by extensive dialysis against milliQ water for four days. After the purification with enzymatic treatment, 41 mg of sample was collected. 14% DOC page with silver nitrate gel staining was performed (Figure 19a). No distinct ladder-effect was seen on the gel, only a thick banding and the same results were seen when repeated with further dilutions of *V. parvula* LPS (Figure 19b).

a)



b)

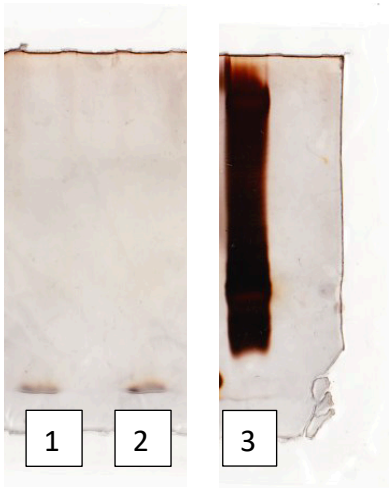


Figure 19: 14 % DOC PAGE run at 150 V for 1 hour. Then stained with silver nitrate A) E. coli LPS standard giving laddering effect compared to the dilutions of Veillonella LPS (1 mg/ml of Batch 2 extracted with hot phenol/water), producing inconclusive results. 1: Veillonella 1mg/ml, 2: Veillonella 500ug/mL, 3: E. coli LPS b)Further dilutions of enzymatically treated Veillonella LPS: 1: Veillonella 0.33 mg/ml, 2: Veillonella 0.16 mg/ml, 3: E. Coli LPS.

Chemical Analysis of Extracted LPS

Compositional Analysis with GC-MS

Monosaccharide content analysis though AMG derivations of the extracted LPS before enzymatic purifications revealed the presence of deoxy-hexose (Rha), pentose (Rib), deoxy-hexosamine, hexose (Glc), hexosamine (GlcN) and heptose residues (traces) (Figure 20). However, following enzymatic digestion purification it was not possible to perform compositional analysis as the quality and quantity of the remaining sample was so poor which is mainly due to the low starting material and also due to loss of sample during the various reaction steps involved in the analysis.

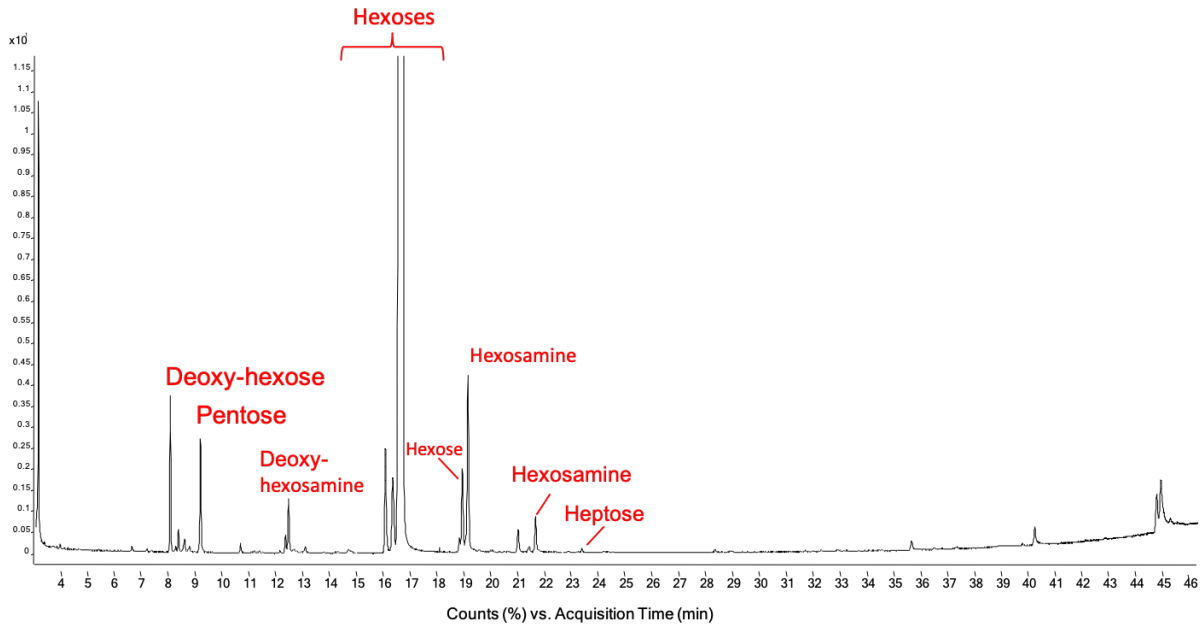


Figure 20: Annotated chromatogram of monosaccharides from a GC-MS analysis of AMG derivations of extracted LPS from hot water/phenol of Veillonella parvula (Batch 2)

NMR analysis of Polysaccharide portion

To improve the analysis of the structure of the LPS saccharide, the lipid A portion was cleaved through mild acid hydrolysis with an acetate buffer treatment (see Experimental Section).

Separation through centrifugation produced two phases: a water phase and a pellet, the former containing the saccharides component whereas the latter containing the lipid A portion. Analysis though a proton NMR spectrum was inconclusive due to contamination of the sample by exopolysaccharide glucans.

Discussion

In this current research study, the first attempt of extraction of the LPS from *Veillonella parvula* ATCC 10790, grown in planktonic and anaerobic conditions, was performed with a very small starting weight of dried bacterial cells (800 mg) (Batch 1), but was used to give an insight into the carbohydrate composition of the LPS.

Extraction of LPS was only possible with hot/phenol water method indicating that the LPS from *Veillonella parvula* contains an O-antigen, therefore being a *smooth*-LPS. The DOC PAGE with silver staining was unsuccessful in the verification of the presence of the O-antigen, which is usually seen by a ladder effect on the gel (Tsai *et al*., 1982). Furthermore, the results of the Alcian Blue were positive highlighting the presence of contaminating sugars such as EPS (Al-Hakim A. *et al.,* 1990) (Tsai *et al.,* 1982). The AMG results of this extracted component show a highly heterogeneous mixture of various sugar monosaccharides within the core and O-antigen of the LPS. After purification with enzymatic treatment and dialysis, a clearer compositional analysis was made.

A second batch of the same bacteria cell culture strain ATCC 10790 with a dry cell mass weight of 2 grams was analysed. Comparing the compositional analyses of these two batches, the carbohydrate content is comparable with large peaks representative of hexose (Glc) and also pentose and deoxyhexose residues. However, the second batch gave further signals which were identified as deoxy-hexosamine and heptose residues in traces. This showed that more starting material gave a more successful analysis as a higher concentration of potential LPS was extracted.

It is important to highlight that in the AMG results of both these batches of extracted *Veillonella parvula* LPS (strain ATCC 10790), the Kdo could not be found. Kdo may not be found within this analysis could be due to quantity of "contaminating" monosaccharides within the sample. Here the purity of the sample could be one of the reasons in which Kdo may not be identified. To overcome the problem of purification the extracted LPS was subject to enzymatic treatment in order to remove unwanted nucleic acid contaminants which may interfere with the clear interpretation of GC-MS chromatogram spectra. Unfortunately, after this purification both a GC-MS and DOC PAGE analysis proved that no LPS could be identified: no signals seen on the chromatogram spectra and no laddering seen after silver staining. Another reason in which the Kdo may not have been identified could be due to the chemical treatments of the sample involved to derivatise the sample for the GC-MS analysis may have modified Kdo sugars. If the Kdo monosaccharides had become chemically modified during the AMG reactions, then the mass spectrometry analysis could fail to give an accurate analysis.

Subsequent acid hydrolysis was performed to cleave the acid-labile bond between Kdo and GlcN from the lipid A of an LPS (if present). The analysis by ^{1}H NMR analysis of the water-soluble phase did not provide any further insight into the carbohydrate composition or structure of the extracted LPS, whereas it confirmed the hypothesis of the occurrence of an acidic exopolysaccharide material likely composed of a variety of sugars with glucose being the main species since this has been found as the main peak in all the GC-MS analyses executed on both *V. parvula* ATCC 10790 batches. Unfortunately, acid hydrolysis of the extracted LPS can create unwanted products such as reducing kdo sugars which make the analysis through NMR practically difficult therefore if more sample were available, a further analysis using a de-acylation reaction could be useful in obtaining the oligosaccharide component of the LPS (Di Lorenzo, 2014).

The conclusion to be drawn from this extraction and analysis would be to state that possibly the extraction method used caused a co-extraction of the LPS material and an exopolysaccharide whose presence impaired chemical analyses interpretation.

Comparison of the Two Strains

During this research two batches of cells were provided from separate laboratories with different growth conditions. The results from *V. parvula* DSM 2008 could provide an insight into the differences between a strain of bacteria isolated from a hospitalised patient which would have an active immune system at the time of extraction. LPS from ITCC 10790 strain contained deoxy-hexose, pentose, deoxy-hexosamine, hexose, hexosamine and heptoses residues; (Figure 21), compared to the *Veillonella* strain extracted from the hospitalised patient (DSM 2008) which lacked deoxy-hexoses but Kdo residues were identified (Table 2). Although the strains of *Veillonella parvula* are the same (DSM 2008 and ATCC 10790) the growth medium was different. The bacteria ATCC 10790 was grown in medium “anaerobose basal broth (OXOID)” which is commonly used for anaerobic bacteria (Gibbons and MacDonalnds, 1960). The growth media can have a direct influence on the structure of the LPS and therefore may play a role in the compositional difference seen in this research.

As stated in *Chapter 4* in the analysis of the ITCC 10790 strain, the chemical analysis of the extracted LPS could be hindered by the impurity through the co-extraction exopolysaccharide whose presence may impair the chemical analyses interpretation. Therefore, only speculations can be made of this analysis.

Overall Conclusion and Discussion

The intestinal tract is colonised by 10^{13}-10^{14} organisms composing the gut microbiota which play a vital role in the health of the human host (Sender, Fuchs and Milo, 2016). There is very little understanding of the biological mechanisms which allow the human body to host a complex microbial ecosystem without the constant activation of the immune system. A better understanding of the interactions occurring between the gut microbiota and human immune system could influence our current views on gut pathologies and human nutrition (Waldor *et al*., 2015).

The elucidation of the structure of LPS from bacteria found within the gut microbiota is of particular importance in expanding our understanding of the role which LPS plays in gut bacteria. The structure of the LPS, specifically the lipid A, activates the host immune system through the TLR4/MD-2 receptor complex in a structure dependent manner (Silipo and Molinaro, 2011). Therefore, this study of the LPS from the bacteria species *Veillonella*, a key coloniser of the intestinal tract and oral cavity, furthers our knowledge in the growing biomedical field of the gut microbiota. Bettering our understanding of how bacteria inhabit within the human host, play a role in the activation and or suppression of the immune system response will lead to greater improvements in medical care in the future.

This research focused on two batches of *Veillonella parvula* cells, enabling full compositional analysis of the saccharide composition of the LPS from the bacterium. The compositional analysis through gas chromatography- mass spectrometry was the main technique used throughout and

provided a key foundation for the ^{1}H NMR experiments and the prospective 2D NMR analysis. Furthermore, the composition of the monosaccharides within the separate domains (the core and O-antigen) were successfully obtained through liquid chromatography and analysed using GC-MS and NMR analytical methods.

Within this study the core region of the LPS was found to contain a mixture of glucose, galactose, glucosamine, galactosamine, heptoses and Kdo residues. The core composition is of particular interest due to its interaction with the immune system through binding to C-type lectins (Zhang *et al*., 2006). The binding to C-type lectins leads to the activation of phagocytosis however resistance to this phagocytosis has been observed in bacteria which possess LPS with an O-antigen saccharide component (Burns *et al.,* 1998) (Cortes *et al.* 2002). This protective method against phagocytosis may also be employed by *Veillonella* as during this study it was discovered that these Veillonella species possess the *S*-type LPS, containing an O-antigen polysaccharide. This could be further investigated by performance of immunological assay of the LPS from *Veillonella* with and without the O-antigen region.

The composition of the O-antigen was found in the research to be composed of repeating units of pentose and hexose residues. Although separation was enabled through liquid chromatography, further purification would enable a more detailed analysis of the polysaccharide.

A compositional analysis of the fatty acids within the lipid A, the glycolipid part of the LPS, was performed which was supported by the successful elucidation of the full lipid A structure through MALDI-TOF and MS^{2} mass spectrometry. The main species composing of two ester linked C13:0 (3-OH) and two amide linked C15:0 (3-OH) acyl chains and further substitutions on both the primary acyl chains of the non-reducing GlcN by tridecanoic acids (C13:0) (seen in Figure 16). It is important to highlight the heterogeneity of the lipid A species identified within the analysis performed, finding variation in the level of phosphorylation, acyl chain lengths and substitutions. The structure of the lipid A species elutes varied effects on the immune system response: activation and/or suppression. *Veillonella Parvula* has been identified as an

opportunist coloniser therefore it could be hypothesized that the ratio of these lipid A structure could vary dependent on the strain, environment or where they are isolated from (Mashima and Nakazawa, 2015) (Marchandin *et al.,* 2001). A further investigation and comparison into the lipid A structure of LPS from *Veillonella* grown in different growth conditions would be a useful support for this hypothesis.

The findings within this research are novel as it is first structural elucidation of the lipid A component from the bacteria species *Veillonella Parvula*. Secondly, the elucidation of the sugar composition of the individual components of the LPS from *Veillonella Parvula* will lead to an increased knowledge of how the chemical structure of LPS from the microbiota relates to their interaction with the host. The successful separation of the O-antigen and the core enables the project to be continued into a deeper investigation with an aim to complete the full structure of the LPS from *Veillonella parvula.* For this more detailed investigation into the structure of the LPS, a continuation of the full structure of the core and O-chain region needs to be performed, which would identify the order of the residue linkages, the O-antigen repeat unit and the link between the core and O-antigen.

To understand how alterations within the growth environment affects directly the composition of the core region in *Veillonella* LPS, a comparative study between mutant core and/or O-chain knock-out strains and wild-type strains would be ideal (Wang *et al.* 2015). To confirm the importance and role of the O-antigen and/or core region of the LPS from *Veillonella* species research could be applied using mutated bacteria with knock-out of the genes coding for proteins involved in the production of the O-chain and/or core sugar linkages. It is known that *Veillonella* can be an opportunist pathogen as well as commensal (Mashima and Nakazawa, 2015) (Marchandin *et al.,* 2001) so a mutation study could identify if the LPS are the causative agents within infections.

Summary

- Successful extraction and purification of the LPS from the gut microbiota bacteria *Veillonella parvula* has been achieved followed by separation of the two diverse saccharide components: O-antigen and core oligosaccharide.
- Structure of the lipid A of the LPS from *Veillonella parvula* has been found using a combination of gas chromatography/mass spectrometry, MALDI-TOF mass spectrometry and tandem mass spectrometry (MS^2)
- Compositional analysis of the monosaccharide residues composing the Core region and O-antigen region of the LPS from *Veillonella parvula*, using gas chromatography mass spectrometry and NMR techniques.

Statement of collaboration

All spectra obtained by MALDI-TOF were prepared and performed by collaboration with Dr. Flaviana Di Lorenzo within years of 2017-2018, at the University of Federico II, Naples.

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