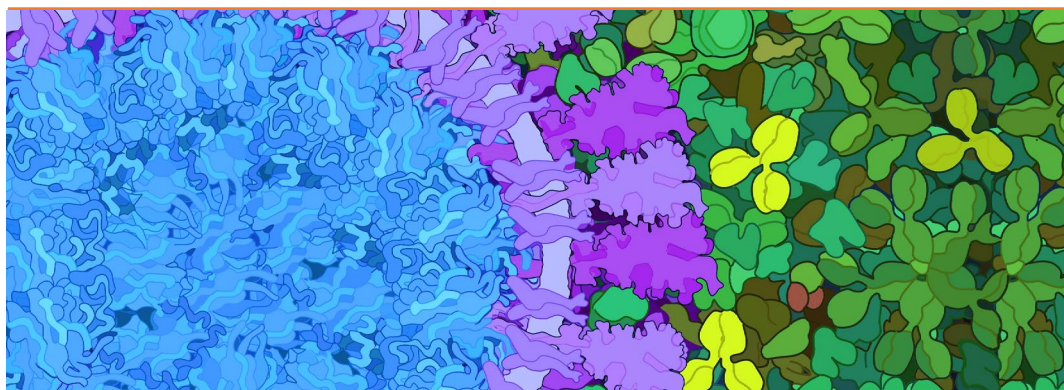


University of Naples Federico II
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Ph.D. in Chemical Sciences

Full characterization of bacterial
envelope components



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*A mi yaya,
luz de mi vida.*

ABSTRACT

Gram-negative bacteria possess an outer membrane primarily composed of glycans. There are still many open questions on how bacterial surfaces of pathogens and commensals interact with host receptors to avoid immune recognition and produce harmful or beneficial effects.

This thesis project shed some light into the study of bacterial glycans and their immunomodulatory properties, offering insights into their chemical structures and their implications for modulating the immune response. The research is structured into chapters, each of which focuses on the isolation and characterization of bacterial lipopolysaccharides (LPS), lipooligosaccharides (LOS), and capsular polysaccharides (CPS), key components of the outer layer of the outer membrane in Gram-negative bacteria. Each chapter contributes to a deeper understanding of how bacterial glycans influence the immune response, providing crucial insights to differentiate between pathogenic and beneficial bacteria, thus paving the way for potential therapeutic applications.

To this aim, the structures, composition and immunostimulatory properties of glycans from commensal bacteria such as *Bacteroides eggerthii*, *Akkermansia muciniphila*, *Pseudoruminococcus massiliensis*, and pathogens like *Fusobacterium gonidiaformans* are characterized in the following chapters.

In essence, the composition of the bacterial envelope is crucial for its interaction with the host, highlighting the need to understand the structure of its components for further functional exploration and comprehension of their specific roles. In this framework, gaining insights into the molecular characteristics of both bacteria and pathogens in the microbiota is an essential starting point. By delving deeper into the characterization of glycans, my PhD thesis highlights the unexpected structures and activities of these compounds, shedding light on previously unrecognized facets of their biological importance.

Keywords: Gram-negative bacteria, Glycans, Immunomodulation, Host receptors, Microbiota

The results presented in this PhD thesis were adapted from the following articles co-written by the author of the present thesis:

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ABBREVIATIONS

AA	Acetylated Alditols
AMG	Acetylated Methyl Glycosides
COSY	Correlation Spectroscopy
CPS	Capsular Polysaccharide
DC	Dendritic Cells
DMSO	Dimethyl Sulphoxide
DNase	Deoxyribonuclease I From Bovine Pancreas
DOSY	Diffusion Ordered Spectroscopy
EPS	Exopolysaccharide
GC-MS	Gas Chromatography Mass Spectrometry
GM	Gut Microbiota
HEK	Human Embryonic Kidney (Cells)
HMBC	Heteronuclear Multiple Bond Correlation Spectroscopy
HSQC	Heteronuclear Single-Quantum Correlation Spectroscopy
IBD	Inflammatory Bowel Disease
IL	Interleukin
LBP	LPS Binding Protein
LipA	Lipid A
LPS	Lipopolysaccharides
LTA	Lipoteichoic Acid
MALDI -TOF	Matrix-Assisted Laser Desorption/Ionisation Mass Spectrometry
MAMP	Microbe Associated Molecular Pattern
MAPK	Mitogen-Activate Protein Kinase
MD-2	Myeloid Differentiation Factor 2

MoDC	Monocyte-derived Dendritic Cell
MS	Mass Spectrometry
MWCO	Molecular Weight Cut Off
NABD₄	Sodium borodeuteride
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Spectroscopy
OM	Outer Membrane
OS	Oligosaccharide
PAGE	Polyacrylamide Gel Electrophoresis
PCP	Petroleum/Chloroform/Phenol
PDB	Protein Data Bank
PGN	Peptidoglycan
PMAA	Partially Methylated Acetylated Alditols
PS	Polysaccharides
RNase	Ribonuclease A
ROESY	Rotating Frame Nuclear Overhauser Effect
SDS	Sodium Dodecyl Sulphate
SEC	Size-Exclusion Chromatography
TFA	Trifluoroacetic Acid
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor α
TOCSY	Total Correlation Spectroscopy
WT	Wild Type

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CHAPTER

I

Gram-negative bacteria and
their cell surface glycans

1.1. Gram-negative and Gram-positive bacteria

One fundamental truth has been known with certainty since the late 1830s: all living organisms are composed of elementary entities known as cells, finite units with a clear boundary, the plasma membrane.

Yet beneath the surface lies a complex tapestry that goes far beyond a simple membrane. Unlike their counterparts in higher organisms, bacteria thrive in an environment as capricious as challenging, characterized by unpredictability, dilution, and sometimes hostility. In response to these demanding conditions, bacteria have meticulously designed a sophisticated and evolved cellular envelope that protects and sustains them.¹

Over a century ago, Christian Gram devised a staining method that allowed almost all bacteria to be classified into two distinct groups, a technique that bears his name today and is still widely used.² Essentially, one group of bacteria retains the stain, known as Gram-positive, while the other group, known as Gram-negative, does not. The rationale for the Gram staining method lies in the profound structural disparities within the cell envelopes of these two bacterial groups.^{1,3} The cell envelope of Gram-positive bacteria exhibits several notable differences from its Gram-negative counterpart (**Figure 1.1**). Chief among these disparities is the absence of the outer membrane, a crucial feature for protecting Gram-negative organisms from environmental hazards and providing additional stabilization around the cell. This absence leaves Gram-positive bacteria, including those living in challenging environments such as the gut, without this protective barrier. In compensation, Gram-positive microorganisms rely on remarkably thick layers of peptidoglycan to withstand the pressure exerted on the plasma membrane. These layers are spanned by long anionic polymers known as teichoic acids, composed primarily of repeats of glycerol phosphate, glucosyl phosphate, or ribitol phosphate. While the teichoic acids of the wall are covalently linked to

peptidoglycan, lipoteichoic acids are anchored to the head groups of membrane lipids.⁴

There are three principal layers in the envelope of Gram-negative bacteria (**Figure 1.1**): the outer membrane (OM), the peptidoglycan (PG) cell wall, and the inner membrane (IM). These layers define a distinctive arrangement where the outer and inner membranes enclose an essential aqueous compartment termed the periplasm. The periplasm serves as a dynamic space that facilitates various crucial processes within the bacterial cell.

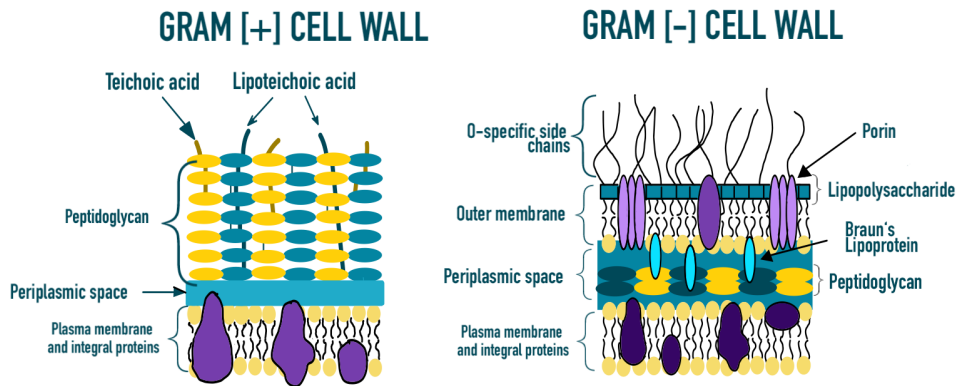


Figure 1.1. The difference between the cell envelopes of Gram-positive and Gram-negative bacteria. Gram-positive bacteria are characterized by a dense peptidoglycan (PG) layer that protects the inner membrane (IM), coupled with the unique existence of teichoic acids and lipoteichoic acids within the PG. In contrast, Gram-negative bacteria possess an outer membrane (OM) alongside a thinner peptidoglycan layer and the IM. The region between the IM and the OM is recognized as the periplasm, a distinctive feature of Gram-negative bacterial structure.

1.2. The Gram-negative bacteria cell envelope

From the innermost layer outward, the initial stratum is the inner membrane (IM). Composed of a phospholipid bilayer, the IM is made up of phospholipids, integral transmembrane (TM) proteins with α -helical TM domains spanning the IM, and lipoproteins. Bacteria, devoid of intracellular organelles, carry out all

membrane-associated functions within the IM, encompassing vital processes such as energy production, lipid biosynthesis, protein secretion, and transport. Notably, while the membrane proteins responsible for these functions are conserved in bacteria, their cellular localization differs from that of eukaryotic organelles; in bacteria, these proteins are located within the IM. Bordered by the IM and the OM, the periplasm is a crucial aqueous cellular compartment. Within the periplasm, a variety of proteins can be found, including periplasmic binding proteins involved in sugar and amino acid transport, chemotaxis, and chaperone-like molecules crucial for envelope biogenesis.^{5,6}

Bacteria do not undergo lysis in distilled water due to their robust exoskeleton. The peptidoglycan, consisting of polysaccharide repeating units of N-acetylglucosamine and N-acetylmuramic acid, is cross-linked by peptide side chains of three to five amino acids.⁷ The OM is connected to the underlying peptidoglycan by a lipoprotein, known as Lpp, murein lipoprotein, or Braun lipoprotein, with lipids attached to its amino terminus allowing it to embed itself in the OM (**Figure 1.2**).

The OM is a distinctive feature of Gram-negative bacteria, it is absent in Gram-positive bacteria. Composed of an asymmetric lipid bilayer, the OM is composed primarily of lipopolysaccharides (LPS) in the outer layer and phospholipids in the inner layer, making the OM permeable to small hydrophilic compounds.^{8,9} This selective permeability allows interaction with the medium and exchange of materials. The different permeability properties of the inner and outer membranes play a key role in protecting the cell from external agents, which complicates the development of antibiotics against Gram-negative bacteria. The external surface of the outer membrane is composed of glycolipids, mainly LPS (**Figure 1.2**).

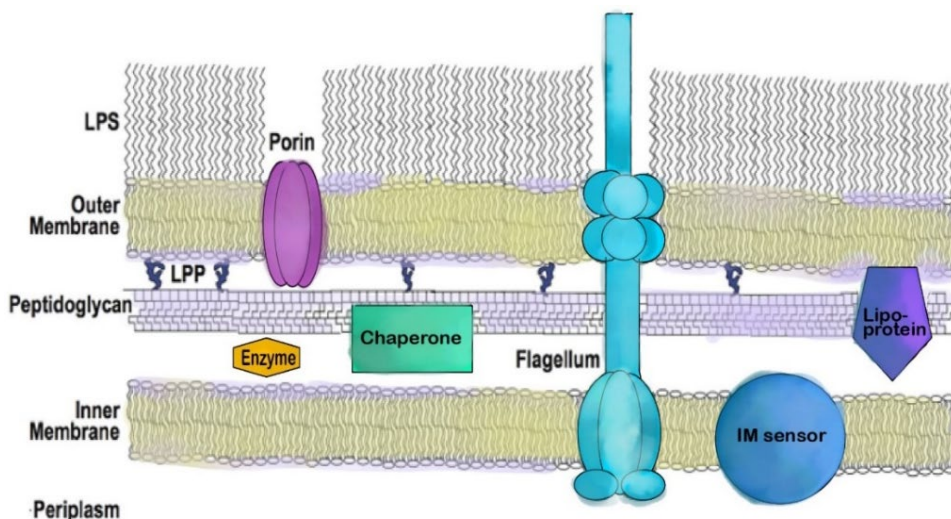


Figure 1.2. Cell envelope of gram-negative bacteria. The inner membrane is composed by a glycerolphospholipid bilayer, between the IM and the OM there is a region called periplasm, where there are a wide variety of enzymes and have different functions. Also present within the periplasmic space is a layer of interconnected sugars and amino acids known as peptidoglycan layer. The peptidoglycan is linked to the OM through covalent transpeptidase connections involving the abundant outer membrane lipoprotein Lpp. Additionally, a variety of sensors are located in the IM with periplasmic domains that detect changes in the environment. Multi-component protein complexes like the flagella machine extend across both membranes (IM, inner membrane; Lpp, Braun's lipoprotein, LPS, lipopolysaccharide).

With few exceptions, the proteins present in this the OM can be divided into two subtypes, lipoproteins and β -barrel proteins.⁸ In the case of lipoproteins, a lipid fraction can be found bound to an amino-terminal cysteine residue. There are about 100 OM lipoproteins in *E. coli* and the functions of the vast majority are still unknown. Almost all transmembrane proteins of the OM assume a β -barrel conformation, referred to as outer membrane proteins (OMPs)¹⁰. These proteins enable Gram-negative bacteria to selectively assimilate nutrients and secrete compounds into the environment.

All components of the Gram-negative cell envelope are synthesized either in the cytoplasm or at the inner surface of the IM. Accordingly, all of these components must be translocated from the cytoplasm or flipped across the IM.

Periplasmic components must be released from the IM, peptidoglycan components must be released and polymerized, and OM components must be transported across the aqueous, viscous periplasm and assembled into an asymmetric lipid bilayer (**Figure 1.3**).

The proteins destined to the periplasm or the OM are initially made in precursor form with a signal sequence at the amino terminus. The signal sequence targets them for translocation from the cytoplasm. This translocation reaction is catalysed by an essential, heterotrimeric IM protein complex called SecYEG.¹¹ The essential ATPase SecA, together with the proton motive force, drives this translocation reaction. The translocation of periplasmic and OMPs is generally achieved in a posttranslational manner, where synthesis and translocation are not directly coupled.¹² Once released, periplasmic proteins are home, but it seems likely that chaperones function to prevent misfolding and aggregation. The periplasmic and OMPs are delivered to a recently identified assembly site in the OM, called the Bam complex, where they are integrated into the membrane (**Figure 1.3**)^{13,14} This process involves a sequence of different steps and requires the coordinated effort of several protein complexes and chaperones to ensure proper insertion and folding within the cell envelope.^{15,16}

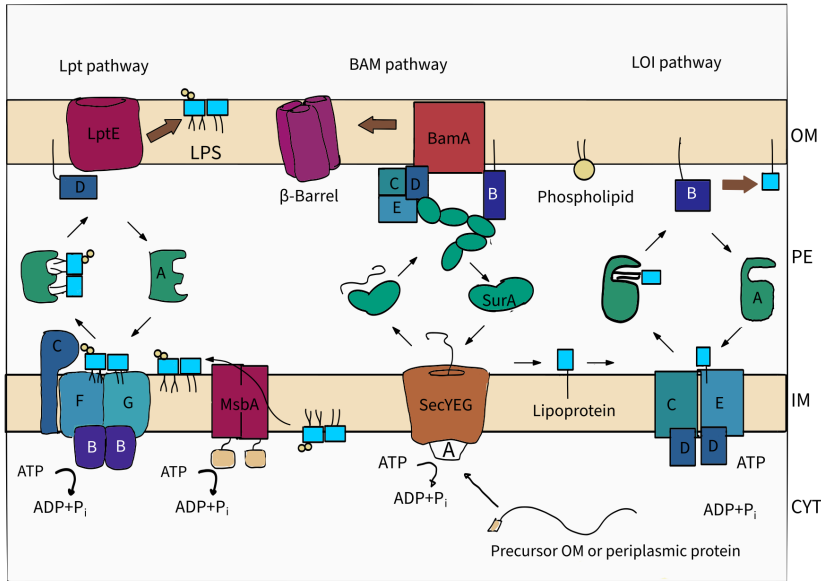


Figure 1.3. Cellular machinery for the biogenesis of the OM. The Lpt pathway, working together with MsbA, transports LPS from their production site to the surface of the cell. Alongside this, β-barrel proteins and lipoproteins are initially present in the cytoplasm in their preliminary forms, which include a signal sequence found at the amino terminus. This signal sequence guides the β-barrel proteins to the Bam machinery for assembly within the inner membrane. When it comes to outer membrane lipoproteins, the removal of the signal sequence and the attachment of lipids to the amino-terminal cysteine residue are followed by the action of the Lol machinery, which is responsible for their transportation to the OM.

Lipoproteins are initially made with an amino-terminal signal sequence and are translocated by the Sec machinery. These proteins are then transported to the outer membrane through a series of steps involving the Lol system. The Lol system consists of an ATP-binding cassette (ABC) transporter called LolCDE in the inner membrane, which utilizes ATP hydrolysis to extract the lipoproteins from the inner membrane. The extracted lipoproteins are then passed to a soluble periplasmic carrier called LolA, which delivers the lipoproteins to the outer membrane assembly site, known as the lipoprotein LolB (**Figure 1.3**).^{17–19}

LPS, including the core polysaccharide, and the O-antigen (**Figure 1.4**) are both synthesized on the inner leaflet of the IM. Once synthesized, LPS is

translocated from the inner leaflet of the inner membrane to the outer leaflet of the inner membrane by the ABC transporter MsbA. Following this, LPS is transported to the cell surface through a series of steps facilitated by essential proteins collectively known as Lpt (lipopolysaccharide transport). The Lpt system involves a complex machinery of proteins such as LptA, LptB, LptC, LptD, LptE, LptF, and LptG, which work together to transport LPS across the periplasm to the outer membrane assembly site.

1.3. Lipopolysaccharides

As mentioned, the outer membrane is peculiar due to its high asymmetry derived from the distribution of glycolipid lipopolysaccharides on the external face. LPS is an amphiphilic molecule with considerable chemical variability between species. These particular molecules participate in the stabilization of the outer membrane, are involved in host-microbe interactions, elicit immune responses in a structural-dependent manner and contribute to antibiotic resistance.²⁰

1.3.1. Structure of LPS

As a general overview, LPS primarily comprises three components: a phosphorylated glycolipid known as lipid A, an oligosaccharide core, and an O-antigen sugar chain or O-polysaccharide (**Figure 1.4**).

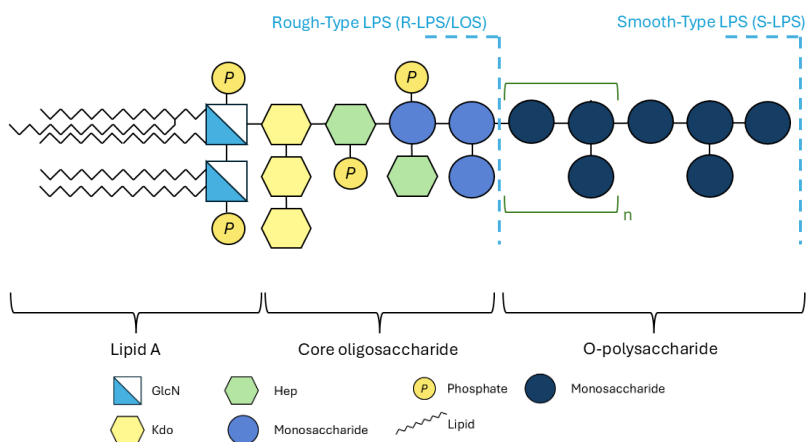


Figure 1.4. Schematic representation of a lipopolysaccharide. They are divided into three moieties: the lipid A, the core oligosaccharide and O-polysaccharide (or O-antigen). Indicating the two types: smooth-type LPS (S-LPS or LOS) and rough-type LPS (R-LPS). “n” represents the undefined number of repeating units.

Moreover, LPS can be classified based on their appearance into smooth or rough types. The smooth form encompasses all three fractions, while in the rough type, also known as lipooligosaccharides (LOS), the O-antigen fraction is notably absent.

The composition and structure of lipid A show intricate variations among bacterial species. Lipid A, typically characterized by a bisphosphorylated diglucosamine backbone connected via a β -(1 $\rightarrow$ 6) bond, is typically adorned with 4–7 acyl chains. The primary acyl chains form direct ester bonds with the sugar moiety, whereas the secondary acyl chains establish ester bonds with the hydroxyl groups of the primary acyl chains. The fundamental backbone of lipid A is [P $\rightarrow$ 4- β -D-GlcpN-(1 $\rightarrow$ 6)- α -D-GlcpN-1 $\rightarrow$ P] (**Figure 1.5**).²¹ Notably, the saccharide skeleton predominantly features 2-amino-2-deoxy-D-glucopyranoses (D-GlcpN). However, other variations include 2,3-diamino-2,3-dideoxy-D-glucopyranose (D-GlcpN3N) which contains an additional amino group,²² often present in *Rhizobiaceae* and in *Pseudomonas carboxydovorans*.²³

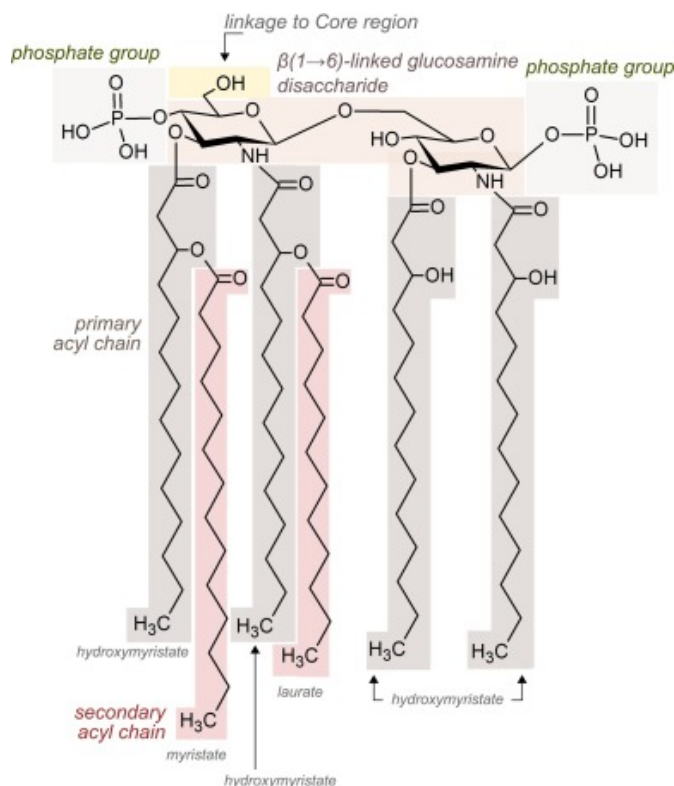


Figure 1.5. Representation of the *E. coli* lipid A. It consists of a glucosamine disaccharide backbone linked through $\beta(1 \rightarrow 6)$ bonds. This backbone, shaded in light brown, serves as the foundation. The connection between lipid A and the Core region is facilitated by the hydroxyl group (light yellow), present on the distal glucosamine. Furthermore, the figure showcases two phosphate groups in light green. The primary acyl chains, shaded in light grey, are directly attached to the sugar components, while the secondary acyl chains, represented in light red, are esterified with the hydroxyl groups of the primary acyl chains. Notably, all primary acyl chains in *E. coli* lipid A are hydroxymyristates. Among the two secondary acyl chains, one is myristate, with the other being laurate.⁴

Regarding acylation patterns, fatty acids can be attached symmetrically (3+3) or asymmetrically (4+2) on the disaccharide backbone, as observed in bacteria such as *Neisseria meningitidis* and *E. coli*, respectively (**Figure 1.6**). The number of acyls varies among the lipid A species. The prototype hexa-acylated lipid A from *E. coli* contains six acyl chains asymmetrically distributed on the sugar

skeleton. However, certain bacterial species can provide “underacylated” lipid A structures, like the tetra-acylated *Helicobacter pylori* lipid A,^{24,25} or even “overacylated” structures like the hepta-acylated *Acinetobacter* spp. lipid A or *Campylobacter jejuni* (**Figure 1.6**).^{26,27}

Disaccharide backbone phosphorylation patterns show significant diversity among bacterial species. The phosphate groups can be absent or further substituted. For example, phosphate groups can be replaced by mannose²⁸ to neutralize the negative charges of the lipid A or D-galacturonic acid²⁹, features that reduce LPS affinity for cationic peptides. Phosphate (P), Etanolamine (EtN), P-methyl, 4-amino-4-deoxy-L-arabinose (Ara4N)³⁰, amino-ethyl-phosphate (PEtN) or further phosphate groups generating a pyrophosphoryl moiety³¹, P-D-GalpN, P-D-Manp, and P-heptopyranose (P-Hepp) can be present. The presence of phosphate groups allows bacteria to modulate the net charge of their surface, which aids in responses to environmental factors such as temperature, pH, and exposure to antibiotics and antimicrobial peptides (AMPs). These interactions and modifications of phosphate groups contribute to the stability of the bacterial outer membrane and the regulation of cell wall permeability.

In some strains such as *Neisseria meningitides*, the introduction of PPEtN groups influences the structure and function of lipid A (**Figure 1.6**).³² Notably, Lipid 1-Arap4N modifications hold a significant role in *Pseudomonas aeruginosa*³³ and the *Burkholderia cepacia* complex, prominent Gram-negative opportunistic pathogens that target the respiratory tract in cystic fibrosis patients (**Figure 1.6**). In the case of *Burkholderia*, the lipid A structure consists of a GlcpN disaccharide backbone with two phosphate groups non-stoichiometrically substituted with 1-Arap4N and adorned with four or five acyl chains.³⁴ Furthermore, additional substitutions and decorations can alter the behaviour and interactions of lipid A, such as neutralizing negative charges, reducing the affinity of LPS for cationic peptides, and modulating immune recognition of lipid A.

These modifications show the adaptive strategies employed by bacteria to ensure survival and adaptability under varying environmental conditions.^{4,35}

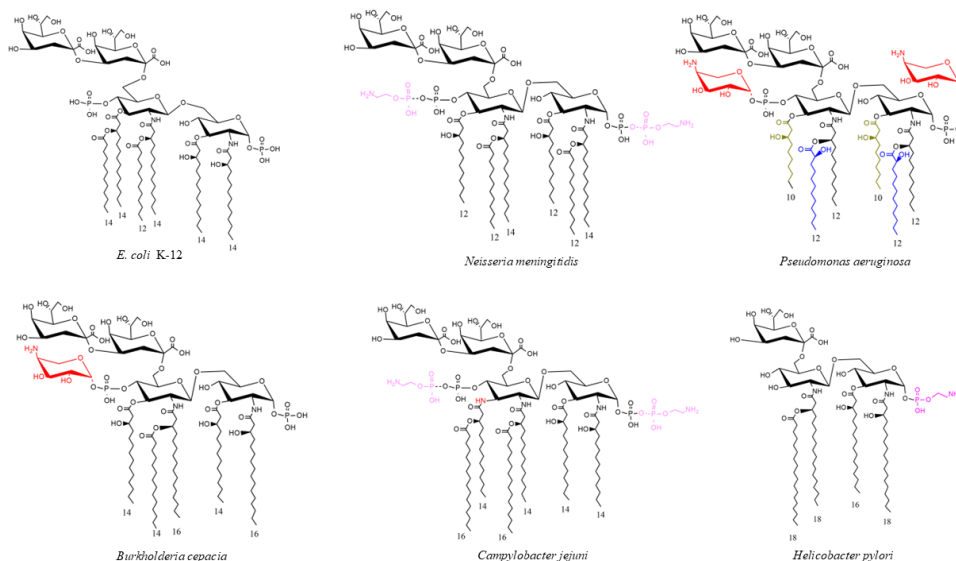


Figure 1.6. Representation of different bacterial lipid A species. The structural heterogeneity of the lipid A can be seen in the different length of the fatty acid chains, the substitution pattern and in the phosphate groups or its substituents. In *Neisseria meningitidis* the phosphates are substituted by PPEtN groups (in pink), also in *Campylobacter* and non-stoichiometrically in *Helicobacter*. In *Pseudomonas* and *Burkholderia* the phosphates are decorated with l-Arap4N (in red). Also, in *Pseudomonas* there are particular fatty acids (C10:0 (3-OH) in green and C12:0 (2-OH) in blue).

The oligosaccharide core is composed of an inner and an outer core, with the sugar Kdo (3-deoxy- α -D-manno-octulosonic acid) serving as a hallmark for Gram-negative bacteria within the inner core. The outer core generally contains additional heptose sugars, some of which may be phosphorylated or modified with phosphoethanolamine or derivatives. Some species contain D-glycero D-manno-heptose as well³⁶ or D-glycero-D-talo-oct-2 ulosonic acid (Ko).³⁷ The Kdo is commonly found linked to an heptose (L,D- Hepp and D,D-Hepp), but it also can be replaced by other sugars, as in the case of *B. vulgatus* where the residue directly linked at position 5' of the Kdo is a rhamnose.³⁸ It is important to note, that

structural variations within the core can alter the interactions between the LPS and the TLR4/MD-2 receptor. The presence of phosphate groups and aminosugars can modify the overall charge of the molecule, thus altering the recognition process. The structural variability of the inner core is limited within each genus or family whereas the outer core region has higher structural heterogeneity. In fact, the outer core is the most exposed portion for a rough-type LPS and is the bridge between the core and the O-antigen in the smooth-types.

The O-antigen, covalently linked to the core oligosaccharide, consists of a highly diverse polymer with repeating sugar units. The size and composition of O-antigens (OPS) can be highly variable; structures consist of repeating oligosaccharide units (O-units), typically containing two to eight residues from a wide range of common and rare sugars and their derivatives.^{39,40} The O-antigen exhibits variation in the types of sugars present, their arrangement within the O-unit, and the linkages within and between O-units, making it one of the most variable cellular constituents. It is exposed on the cell surface, is highly immunogenic and is also used as a receptor by bacteriophages, which may contribute to maintaining diversity by intermittent selection against specific forms of O-antigen. Variability can be observed both in the sugar units, position and stereochemistry of the O-glycosidic linkages, and the presence of noncarbohydrate substituents. O-repeat units from different structures may comprise varying numbers of monosaccharides, they may be linear or branched, and they can form homopolymers or, more frequently, heteropolymers.

Most of the sugars present in the O-antigen are hexoses, other sugars such as pentoses, deoxyhexoses, lactyl substituted hexoses, heptoses and nonuloses can also be found and, in particular, the most unusual sugar is an acidic diamino sugar, a derivative of 5,7-diamino-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulosonic (pseudaminic) acid, found in *Shigella boydii* type 7.⁴¹ Also, sugars that rarely occur in nature such as yersinirose A (3,6-dideoxy-4-C-[(S)-1-

hydroxyethyl]-D-xylo-hexose) and yersiniose B (3,6-dideoxy-4-C-[(R)-1-hydroxyethyl]-D-xylo-hexose) can be found have been reported in *Legionella* and *Burkholderia* O antigens.^{42,43} Two sugars, 4-deoxy-D-arabino-hexose and 3,6-dideoxy-D-xylo-hexose (abequose), are also uncommon in nature but are present in the *Citrobacter* genus.⁴⁴ Some unique monosaccharides have been described in OPS like abequose, colitose, paratose or tyvelose among many others (**Figure 1.7**).^{45–47} The most common arrangement of sugar residues is the cyclic form, however, residues in the open form have been described as well.⁴⁸ In addition, non-carbohydrate substituents are often present such as phosphate, amino acids, sulphate, methyl, acetyl, or formamide groups.⁴⁹ Each small difference in composition has great consequences on the chemical properties of the sugar chain.⁵⁰ Therefore, the detailed characterization of nature of sugar units; stereochemistry; absolute configuration (D or L); anomeric center (α or β); cyclic or open form; neutral, acidic and basic nature; decorations of the hydroxyl or other groups (acetylated, phosphate linked, amino acid linked, etc.), the specific attachment site, the linear or branched nature of the polysaccharide and the number of repeating units is fundamental in the study of its properties.

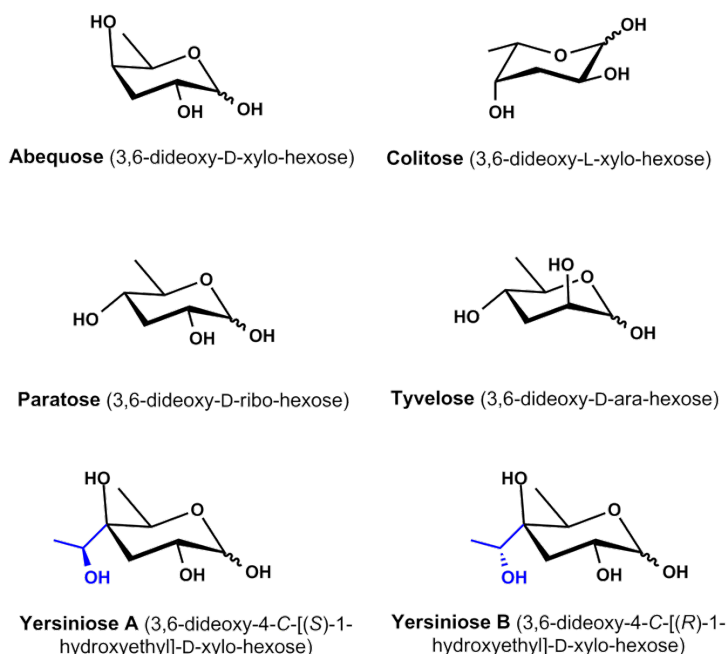


Figure 1.7. Some unique monosaccharides present in the OPS of different bacteria. Abequose present in the *Citrobacter* genus and its optical enantiomer colitose, present in *E. coli* O111.⁵¹ On the second row, the paratose sugar present in the O-polysaccharide unit of *Yersinia pseudotuberculosis*⁵² and tyvelose from *Trichinella spiralis*.⁵³ On the last row, yersiniose A and B, which differ in the configuration of the substituent in position 4 (in blue), and interestingly are present in different strains. Yersiniose A is present in the O-unit of *Y. pseudotuberculosis* while yersiniose B occurs in *Y. enterocolitica*.⁵⁴

1.3.2. Host-LPS interactions

As mentioned, in the bacterial world a critical element is LPS, a molecule with a multifaceted role in immune recognition. Historically known as an important virulence factor, the structure of LPS is determinant in its impact on the host. The lipid A portion of LPS is responsible for its endotoxicity, influencing immune responses and inflammation, while the oligosaccharide core and the O-antigen contribute to its antigenicity and serotype-specific immunogenicity, eliciting a response from the host immune system.

LPS acts as a microbe-associated molecular pattern (MAMP), recognized by several host's Pattern Recognition Receptors (PPRs) including Toll-like receptor

4 (TLR4), and TLR2, transient receptor potential (TRP) channels, caspases, C-type lectins receptor (CLR) and the complement, triggering a cascade of immune responses, inflammation and fever.⁵⁵ The diverse structures of LPS, particularly within the lipid A region, create biological diversity and a complex interaction with the host immune system. In this context, the structure of lipid A largely determines the immunogenicity of LPS and is therefore crucial for its function.^{56,57}

Referred to as "agonistic" lipopolysaccharides, some LPS trigger powerful pro-inflammatory reactions within host cells, while structurally distinct lipid A molecules may provoke milder host reactions, termed "weak agonistic," or even possess the ability to block pro-inflammatory responses by binding to specific host receptors, earning them the name of "antagonistic" lipopolysaccharides. Among Gram-negative bacteria, the expression of agonistic hexaacylated LPS, exemplified by *E. coli* LPS, stands out as a potent stimulator of the human innate immune system, showing the diverse effects of LPS.^{58,59} Typically, the number of acyl chains in the lipid A structure directly correlates with the molecule's ability to induce cytokine production, with hexaacylated forms being particularly potent immunostimulants.⁶⁰

The recognition of LPS by the host immune system involves a sophisticated molecular dance. TLR4, a transmembrane protein featuring 22 leucine-rich repeats (LRRs), a transmembrane domain, and a cytosolic Toll/IL-1 receptor (TIR) domain, rules in this complex system. While LPS resides in the bacterial outer membrane, its recognition by the host immune system necessitates its dissociation from the membrane structures, achieved either through bacterial cell death or via interactions with the soluble host lipopolysaccharide-binding protein (LBP).⁶¹ LBP, secreted into the intestinal lumen, acts as a vital protector, present in the bloodstream to detect the presence of bacteria. Furthermore, LBP extends its reach into the intestinal lumen, where it not only detects invading pathogens

but also responds to commensal-derived LPS.^{62,63} LBP also orchestrates the transport of monomeric lipid A molecules to CD14.

CD14 can be anchored to the membranes of TLR4-expressing cells or released into the extracellular environment as a soluble protein. Subsequently, CD14-associated monomeric LPS is transported to MD-2, forming a crucial interaction for downstream immune signalling pathways and the activation of numerous immune-related genes that regulate the release of proinflammatory cytokines, antimicrobial peptides, and other immune effectors.⁶⁴⁻⁶⁶ MD-2 may be a soluble protein secreted outside the cell or may be permanently linked to a TLR4 monomer via hydrogen bonds.^{67,68} Binding of LPS to MD-2 results in the formation of a heterooligomeric protein complex consisting of two MD-2 and two TLR4 molecules (**Figure 1.8**). This protein complex formation is initially induced through interaction at the extracellular domains. These protein-protein interactions result in dimerization of the intracellular cytosolic TIR domain-containing parts of TLR4 receptors, leading to myeloid differentiation primary response gene 88 (MyD88)- or TIR domain-containing adaptor-inducing interferon (TRIF)-dependent downstream signalling.⁶⁹ Binding to the TLR4 receptor complex activates more than 103 genes^{64,69} and results in secretion of proinflammatory cytokines such as TNF, IL-6, IL-1, as well as production of the superoxide anion O_2^- , hydroxyl radicals, NO, and antimicrobial peptides (AMP).^{65,66}

The first five acyl chains of lipid A (**Figure 1.8**) are known to be housed within a hydrophobic cavity of the TLR4 adaptor molecule MD-2, and the sixth chain facilitates binding to TLR4 to initiate TLR4-mediated intracellular signalling (**Figure 1.8**).^{70,71} Consequently, the absence of this sixth acyl chain significantly reduces TLR4-mediated signalling. In addition to acyl chains, the amount of phosphate groups also contributes to the immunogenic properties of lipid A. Removal of one phosphate group is associated with decreased endotoxic activity.

While most lipid A structures contain two phosphate residues, some bacterial lipid A lack one or both, modulating their immunogenic potential. From a structural point of view, Kdo and Hep sugars of LPS may facilitate the binding to TLR4.⁷² Monosaccharides decorating the lipid A, such as Ara4N, can also be involved in the interaction.⁷³

Recent studies remark the capabilities of TLR2 to engage with a diverse array of self and non-self-molecules. Some LPS can activate the TLR2, exerting a synergistic effect alongside TLR4 in the induction of nuclear factor- κ B production. The activation of TLR2 is vital for maintaining homeostatic conditions, particularly in supporting the equilibrium of the gut microbiota.^{74–76} TLR2 signaling occurs through heterodimerization with either TLR1 or TLR6, with pre-formed heterodimers present on the cell surface.^{77,78} These distinct heterodimers elicit specific immune responses, including both pro-inflammatory and anti-inflammatory reactions. The variation in immune responses is believed to be influenced by the temporal structure-activity relationship between the TLR and the ligand. Studies have shown that LPS from *Bacteroides fragilis*, *Legionella pneumophila*, *Chlamydia trachomatis*, *Rhizobium* spp. can trigger TLR2-mediated inflammatory responses in immune cells.^{79–81} Additionally, LPS from *Ochromobactrum intermedium* has demonstrated weak activation of TLR4; however, in a preassembled receptor complex TLR4/TLR2/MD-2, a significant release of pro-inflammatory cytokines was observed.⁸² This implies that this LPS requires TLR4 alongside TLR2 to activate the innate immune response. This aspect is fascinating because research has demonstrated that the TLR2 pathway plays a crucial role in facilitating the establishment of commensals from the human intestinal microbiota, as seen with *B. vulgatus*³⁸ and *Akkermansia muciniphila* (see Chapter IV). Studying the immunological characteristics of these LPS using human *in vitro* models has uncovered the remarkable ability to generate

anti-inflammatory cytokines and trigger a cooperative effect between the MD-2/TLR4- and TLR2-mediated signaling pathways.

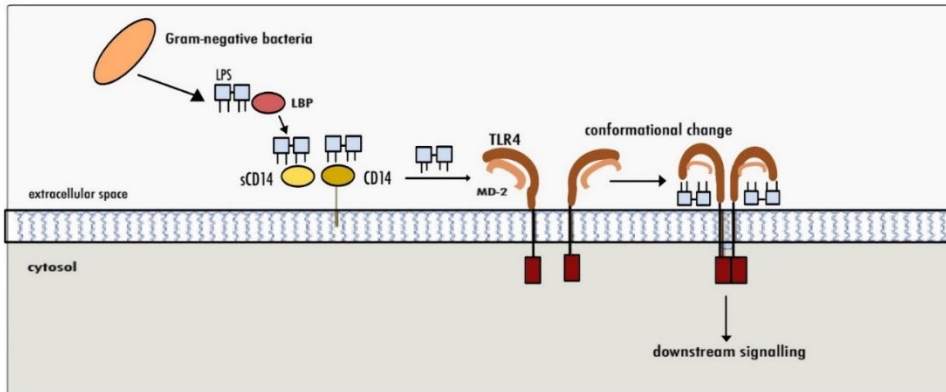


Figure 1.8. The extracellular sensing of bacterial LPS. Initially, the host lipid binding protein (LBP) interacts with LPS from the outer membrane of gram-negative bacteria and facilitates its transfer to host CD14. Notably, CD14 can exist in a soluble form (sCD14) or can be anchored in the membrane of TLR4 expressing cells. Following this, CD14 transports LPS to the TLR4 coreceptor protein MD-2, which triggers a conformational change in both MD-2 and TLR4. Subsequently, this conformational change leads to the dimerization of the extracellular domains and subsequently the intracellular TIR domains. The dimerization of the TIR domains is responsible for mediating downstream signalling, ultimately resulting in the enhanced expression of more than 1000 different genes.

Furthermore, the involvement of caspases, known for their roles in apoptosis and inflammation, introduces a layer of complexity to lipid A recognition and modulation, suggesting the deeply interconnected nature of cellular signalling pathways and immune responses. The amino-terminal domain of variable length precedes the formation of the large and small catalytic components, approximately 20 kDa and 10 kDa in size, respectively, which collectively create the protease domain in caspases. The subset of inflammatory caspases, crucial for combating microbial infections and initiating the immune defense against pathogens, includes murine caspases 1, 4, 5, and 11, as well as human caspases 4 and 5.^{83,84} These enzymes are initially produced as proteolytically inert zymogens, requiring proteolytic cleavage for enzymatic activation. Additionally, they share a common

N-terminal domain known as the caspase activation and recruitment domain (CARD), responsible for mediating essential protein-protein interactions. Notably, caspase-4, caspase-5, and caspase-11 display a distinct mode of activation by directly detecting cytoplasmic LPS, triggering their oligomerization and subsequent activation. Upon activation, inflammatory caspases drive the maturation of key pro-inflammatory cytokines such as IL-1 and IL-18, while also orchestrating a unique form of cell death called pyroptosis.⁸⁵

C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) encompass a diverse family of proteins, including both soluble and transmembrane extracellular entities (**Figure 1.9**).⁸⁶ Within the domain of tissue-resident antigen-presenting cells (APCs), activation of PRRs such as CLRs plays a pivotal role in triggering local cytokine production. This response serves to attract additional immune cells, particularly neutrophils, to the focal point of infection, facilitating clearance of invading microbes.^{87,88} Furthermore, CLR-induced activation of APCs plays a crucial role in shaping adaptive immunity.

For example, macrophage-inducible C-type lectin (Mincle) exhibits robust LPS-binding capacity, triggering cytokine production and influencing the downregulation of TLR4 signalling, while sensing alterations in the microbiota.⁸⁹ Similarly, the C-type lectin DC-SIGN on human dendritic cells plays a vital role in sensing the polysaccharide component of LPS. This recognition event leads to dendritic cell maturation, cytokine production, and T cell polarization.⁹⁰ Furthermore, the C-type lectin SIGNR1 on macrophages stimulates cytokine production and promotes TLR4 oligomerization, further emphasizing the diverse and critical roles of CLRs in immune orchestration.⁹¹

The multifaceted functions of CLRs encompass interactions with a wide range of bacterial pathogens such as *Mycobacterium leprae*, *Helicobacter pylori*, and *Lactobacillus species*. These interactions show the versatility of CLRs in shaping

immune responses and host-microbe dynamics.⁹² Notably, less explored CLR, such as DEC-205, have been shown to recognize specific bacterial components, shedding light on new facets of host-pathogen interactions and immune modulation.⁹³

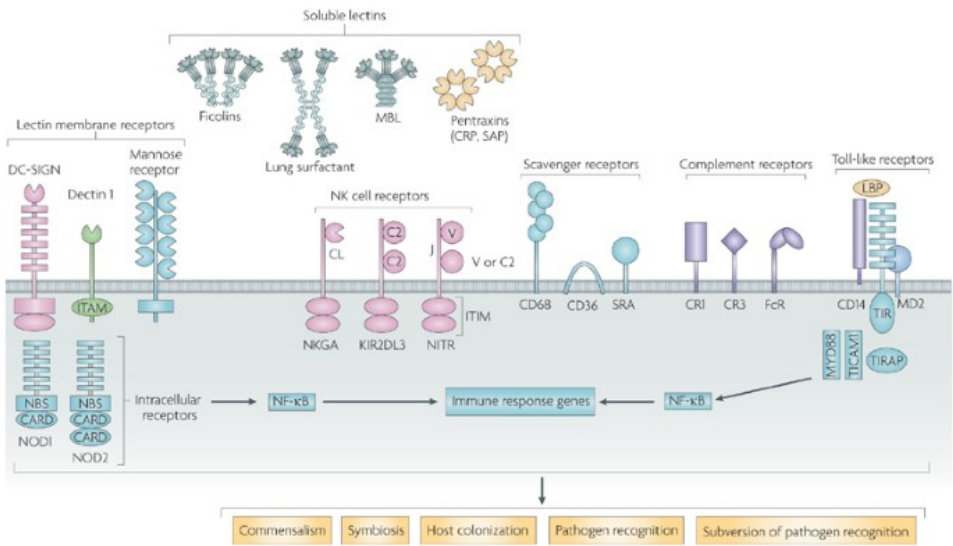


Figure 1.9. Schematic representation of different pattern-recognition receptors.⁹⁴

Siglecs

Finally, the family of sialic acid-binding immunoglobulin lectins (siglecs) encompasses a diverse array of human and murine cell surface receptors expressed on various white blood cells within the immune system (**Figure 1.10**).^{95–98} Siglecs play a pivotal role in recognizing ubiquitous sialic acid (Sia) epitopes present on various glycoconjugates in the cellular glycocalyx, and through signal transduction, they regulate the immunological and inflammatory activities of these cells.

Particularly, the subset known as CD33-related Siglecs serves as primarily inhibitory receptors, responsible for suppressing leukocyte activation. Intriguingly, recent research has unveiled that several bacterial pathogens utilize

sialic acid mimicry to activate these Siglecs as an immune evasion strategy. Pathogens such as *Campylobacter jejuni* have been implicated in human infections causing foodborne enteritis and triggering cases of Guillain-Barré syndrome, an acute peripheral neuropathy.^{99–101} The interaction of *C. jejuni* LOS expressing terminal α 2,8-linked Sia or α 2,3-linked Sia with Siglec-7 and sialoadhesin is notable and contributes to the immune modulation.^{102,103} Moreover, *Pseudomonas aeruginosa* is involved in the acquisition of Sias, ultimately leading to the interaction with multiple human CD33r Siglecs, which negatively regulates neutrophil activation, resulting in impaired innate bactericidal responses.^{104,105}

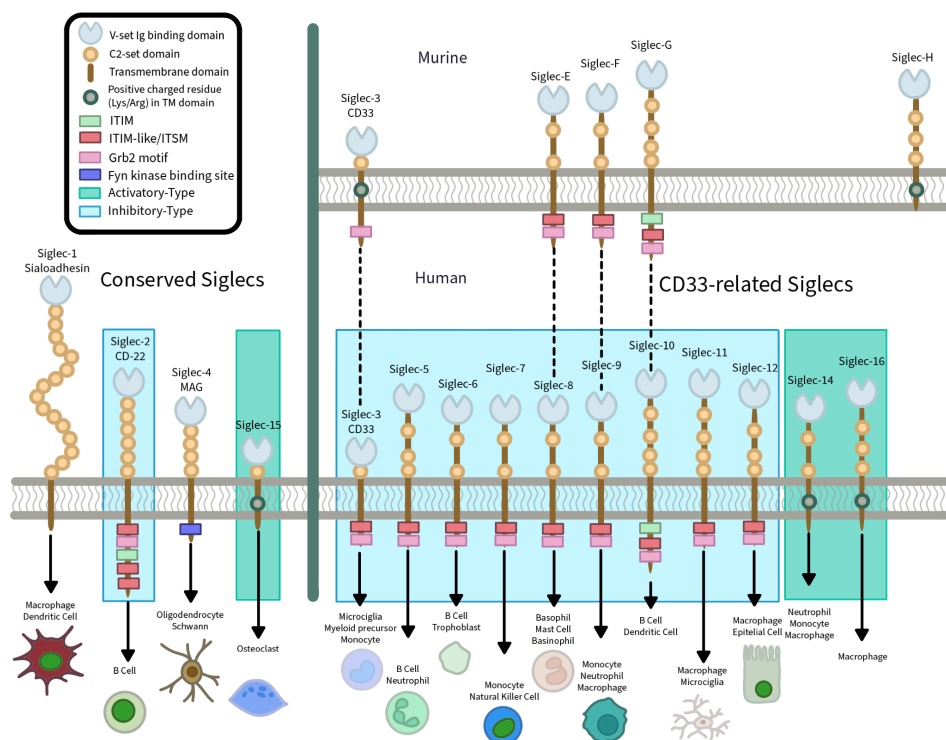


Figure 1.10. Human and murine Siglecs schematic representation. On the left the conserved Siglecs family whereas on the right the CD33-related Siglecs. Highlighted in red and green the inhibitory and activator Siglecs respectively. Grey dash lines connect Murine Siglecs with their human homologs.

Interestingly, bacteria have developed a fascinating ability to modify the structure of their LPS, avoiding detection by the host immune system. Through this process, they can evade defense mechanisms such as cell damage and opsonization by expressing glycan epitopes that closely resemble the host's own components, effectively mimicking self-associated molecular patterns (SAMPs). These structural modifications in O-polysaccharides (OPS) not only increase the potential virulence of bacteria, but also help evade immune surveillance.¹⁰⁶ Surprisingly, the same OPS that enhance bacterial virulence are harnessed for vaccine production, taking advantage of their ability to stimulate specific and memory immunity.¹⁰⁷ While it may seem paradoxical, the main function of O-polysaccharides appears to be the protection of bacteria.¹⁰⁸ The last decade has demonstrated an increase in interest for O-antigen polysaccharide vaccines with encouraging preclinical studies. For example, the OPS from *Klebsiella pneumoniae* has been used to develop glycoconjugate vaccines^{109,110} against a broad range of clinical isolates to date. In another study, showed that the conjugate of oligopolysaccharide of *Brucella melitensis* has the potential to stimulate the humoral and cellular immune systems against *B. abortus* 544 infections.¹¹¹

In animal pathogens, these polysaccharides play a pivotal role in contributing to bacterial evasion of host immune responses.¹¹² Notably, the LPS of certain bacteria, such as *Helicobacter pylori*, *Neisseria gonorrhoeae*, *N. meningitidis*, and *Haemophilus influenzae*, have been found to contain O-antigen structures that bear a striking resemblance to human glycosphingolipids. This similarity arises from the presence of common host carbohydrate residues, such as N-acetylneuraminic acid or L-fucose.¹¹³

For example, the predominant human gastroduodenal pathogen, *H. pylori*, produces LPS O-antigen units that can be uniquely embellished by the addition of fucose residues, creating Lewis's antigens.¹¹⁴ These carbohydrates are also

expressed in human gastric epithelium, further underscoring the tactics that bacteria employ to subtly impersonate host elements.

1.3.3. LPS biosynthesis

The process of LPS biosynthesis holds the key to understanding and combating Gram-negative pathogens and potentially life-threatening conditions like sepsis shock.^{115,116} While LPS adorns the surface of bacterial cells like a shield, its journey begins deep within the bacterial cytoplasm. For LPS biosynthesis, export and assembly a large number of enzymes and structural proteins are required.¹¹⁷ Lipid A from *E. coli* has been widely studied and is composed of a phosphorylated and glycosylated glucosamine disaccharide at the 6' position with two Kdo residues. Both the core and lipid A are assembled on the cytoplasmic side of the inner membrane by the action of the ABC transporter MsbA (**Figure 1.11**).

Lipid A biosynthesis was characterized by Raetz and his group for *E. coli*, culminating in lipid A-Kdo₂.^{733,35} It requires nine conserved enzymatic reactions that occur in the cytoplasm, with the participation of soluble and membrane-associated enzymes. The reaction begins with an ester linkage of an acyl chain to the 3-OH group of a uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) group, the reaction catalyzed by LpxA.^{118,119} This is followed by a deacetylation reaction of UDP-3-O-(acyl)-GlcNAc performed by LpxC.¹²⁰ The Raetz pathway continues with the transfer of another acyl chain to the newly generated amino group to form UDP-2,3-diacylglucosamine, a reaction catalyzed by LpxD (**Figure 1.11**).¹²¹ The phosphoanhydride bond of UDP-diacylglucosamine is then hydrolysed by the action of the pyrophosphatase LpxH, resulting in UMP and 2,3-diacylglucosamine-1-phosphate (lipid X).¹²² The next step in the synthesis is the phosphorylation at the 4' position of the intermediate to form lipid IV_A. This reaction is catalyzed by the LpxK kinase which requires the hydrolysis of ATP for its function.²⁰ The addition of the two 3-deoxy-D-manno-octulosonic acid (Kdo)

residues at the 6'-position of the distal glucosamine of the newly formed lipid IV_A requires a step prior to the formation of the lipid A-Kdo₂ molecule. This step is catalyzed by the bifunctional enzyme WaaA (also known as KdtA),²⁰ using the sugar nucleotide CMP-Kdo as donor. The resulting Kdo₂-lipid IV_A undergoes further reactions catalysed by LpxL and LpxM to form Kdo₂-lipid A (**Figures 1.-12**).

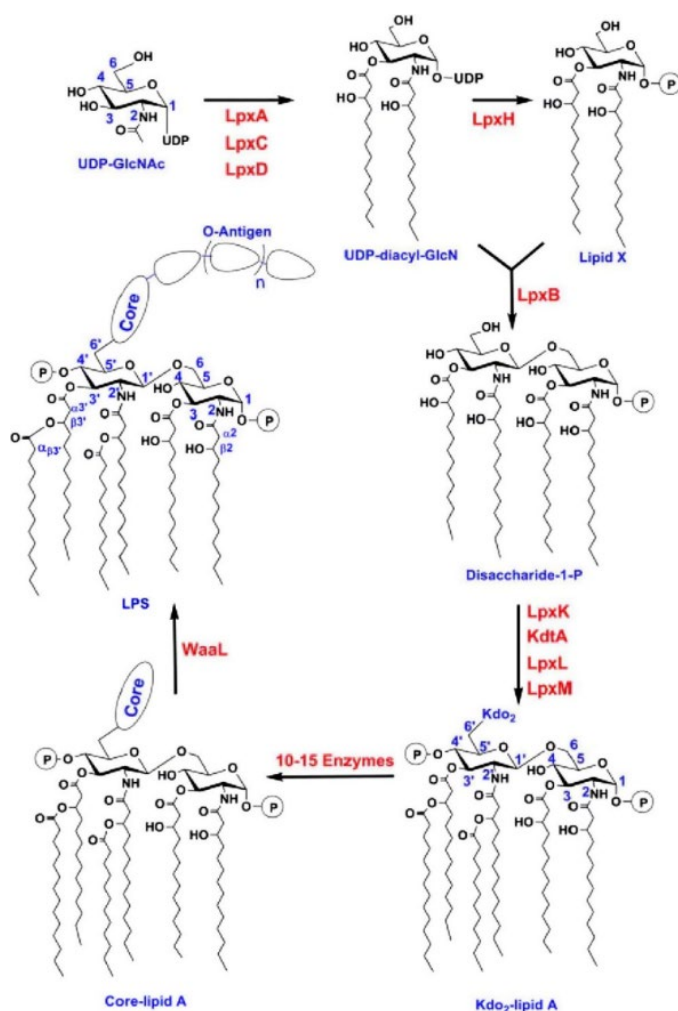


Figure 1.11. Structure and biosynthesis of the LPS in *E. coli*. The reactions are catalysed by different enzymes (in red) (from X. Wang, P.J. Quinn / Progress in Lipid Research 2010).¹¹⁶

LpxL adds a secondary lauroyl (C12:0) residue to the 2'-position of Kdo₂-lipid IV_A and is followed by LpxM-addition of a myristoyl (C14:0) residue to the 3'-position, allowing the formation of lipid A-Kdo₂.

The structure of lipid A is highly conserved, while the structure of the core oligosaccharides shows some variations. The core oligosaccharides are sequentially assembled on lipid A at the cytoplasmic surface of the inner membrane in a process that involves several membrane-associated glycosyltransferases, using nucleotide sugars as donors (**Figure 1.12**). The biosynthesis of core oligosaccharides is rapid and efficient, suggesting that the glycosyltransferases function as a coordinated complex.^{35,123,124}

The oligosaccharide core typically includes hexoses, heptoses and two forms of the octulosonic acid Kdo or D-glycero-D-talo-oct-2-ulopyranosonic acid (Ko). The structure of the "inner" core is generally well conserved among bacteria. In most Gram-negative bacteria, the synthesis of this inner core is completed by heptosyltransferases-I and -II, also known as WaaC and WaaF, respectively. The substrate they use is ADP-L-glycero-D-manno-heptose. The b-configuration of this substrate is essential since the reaction occurs through an inversion mechanism resulting in the formation of alpha-glycosidic bonds. For the assembly of the "external" core a long variety of glycosyltransferases that use activated nucleotides as precursors to transfer hexose units to the glycosylated lipid A are critical in this process.¹²⁵ After all, the core region can be modified by the enzymes WaaP (kinase), WaaY (phosphorylase), WaaQ (adds branch to the heptose) and WaaZ (adds third Kdo residue). Finally, WaaG and other glycosyltransferases finish the outer core.¹²⁶

The O-Antigen biosynthesis also occurs at the cytoplasmic surface of the inner membrane (**Figure 1.12**). The initiator enzymes are membrane proteins that catalyse the formation of a phosphoanhydride bond between undecaprenyl phosphate (Und-P) and a 1-phosphate sugar transferred from UDP, resulting in the

release of uridine monophosphate. Two enzyme superfamilies catalyse the initiation of the reaction: polyisoprenyl-phosphate N-acetylaminosulfonate-1-phosphate transferase (PNPT) and polyisoprenyl-phosphate hexose-1-phosphate transferases (PHPT). The synthesis in most cases requires a lipid acceptor on which the polysaccharide is built by the action of glycosyl transferases (**Figure 1.12**).¹²⁷

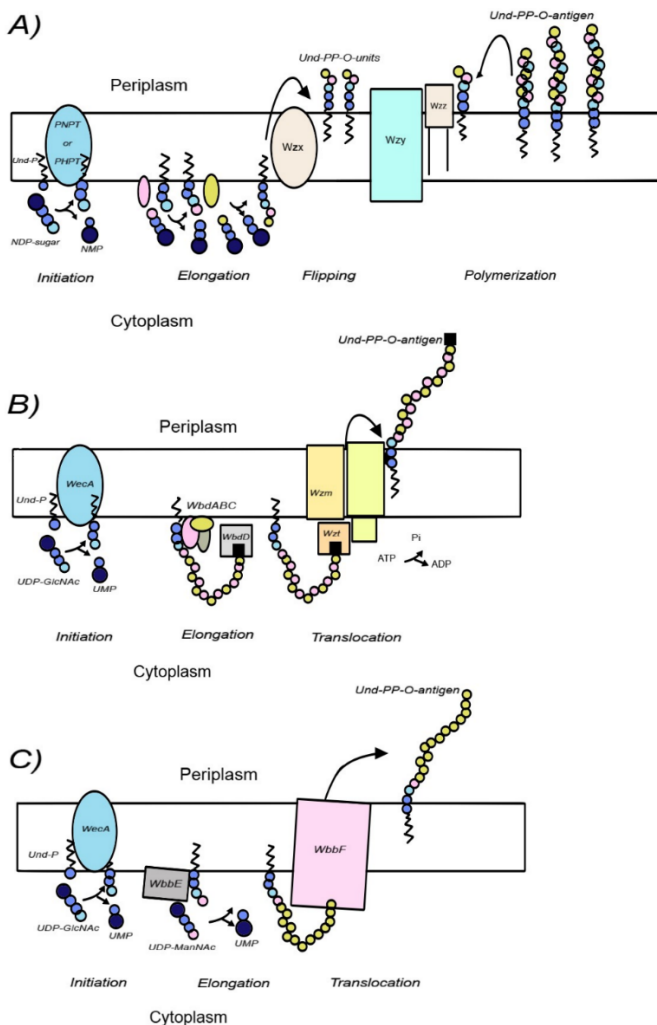


Figure 1.12. Oag assembly pathways. (A) The Wzy-dependent pathway. **(B)** ABC-dependent pathway. **(C)** Synthase-dependent pathway.

The Und-P molecule is also used for synthesis of peptidoglycan and capsular polysaccharides. LPS is assembled in the periplasm but precursors of LPS, the core-lipid A and the O-antigen, are synthesized at the cytoplasmic surface of the inner membrane. Therefore, the core-lipid A and the O-antigen must be transported across the inner membrane. The transport of core-lipid A is carried out by the ABC transporter MsbA. Translocation occurs by a flip-flop mechanism with ATP hydrolysis.¹²⁸ For the O-antigen the lipid carrier linked to the polysaccharide is flipped from the inner leaflet to the outer leaflet of the IM. The three pathways are: the Wzx/Wzy dependent pathway, the ABC-transporter dependent pathway and the Synthase dependent pathway. The Wzx/Wzy and the ABC-transporter dependent pathways have been described in *E. coli* groups 1 and 2 respectively (**Figure 1.13**).²⁰

The Wzx/Wzy dependent pathway

The Wzy protein is required for the polymerization of Und-PP-linked-O-units on the periplasmic face of the cytoplasmic membrane. The reaction involves the transfer of the nascent polymer from its Und-PP carrier to the reducing end of the new Und-PP-linked-O-unit with the formation of a novel glycosidic bond joining the O-antigen units. This step is repeated for each repeating unit present in the OPS, recycling the und-PP. Finally, the Wzz processing protein stops the elongation of the OPS.¹²⁹

The ABC-transporter dependent pathway

In contrast, within the ABC-transporter dependent pathway, also known as the adenosine triphosphate (ATP)-binding cassette, the complete O-specific polysaccharide (OPS) is assembled on the lipid acceptor before undergoing transport across the IM. The specific composition of the lipid acceptor is dictated by the polysaccharide being synthesized. This pathway is tailored for OPS structures that follow a linear arrangement, with an OPS unit consisting of

approximately eight to ten sugar units tightly integrated between the carbohydrate-binding domain and the nucleotide-binding domain.¹³⁰

The synthase-dependent pathway

Conversely, within the synthase-dependent pathway, the secretion of the OPS can occur either with or without the involvement of distinct lipid acceptors. Both the synthesis and transportation processes are facilitated by a membrane glycosyl transferase.¹³¹

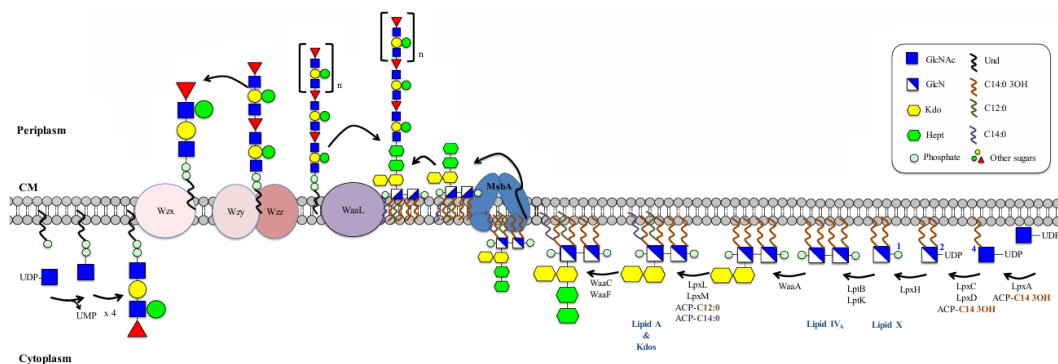


Figure 1.13. Lipid A and O-polysaccharide biosynthesis.

The next question is how LPS crosses the periplasmic space and reaches the outer face of the outer membrane. The lipid A-core is transported across the periplasmic layer of the inner membrane by the ABC transport MsbA.¹²⁸ LPS is then transported across the periplasm and assembled on the outer face of the outer membrane. This process requires a complex protein machinery known as the Lpt machinery, consisting of seven essential proteins, LptABCDEFG.^{132–135} LPS is extracted from the periplasmic side of the IM by an ATP-binding cassette (ABC) transporter LptB2FG and transported to another IM protein LptC. LptB is a cytoplasmic protein that has a nucleotide-binding domain. LptF and LptG are the transmembrane subunits, whereas LptC is a bitopic membrane protein with a single transmembrane domain and a long soluble periplasmic portion. LptA functions as the periplasmic LPS-binding component and is a key protein for this

pathway since it is speculated to connect the inner and outer membranes. At the outer membrane, the LptDE complex is required for anchoring of LPS to the external face of the OM. LptD is an essential β -barrel protein. Recognition of LPS by LptE may trigger a conformational change in the protein that could be transmitted to LptD to allow translocation of LPS and complete its assembly at the OM (**Figure 1.14**).

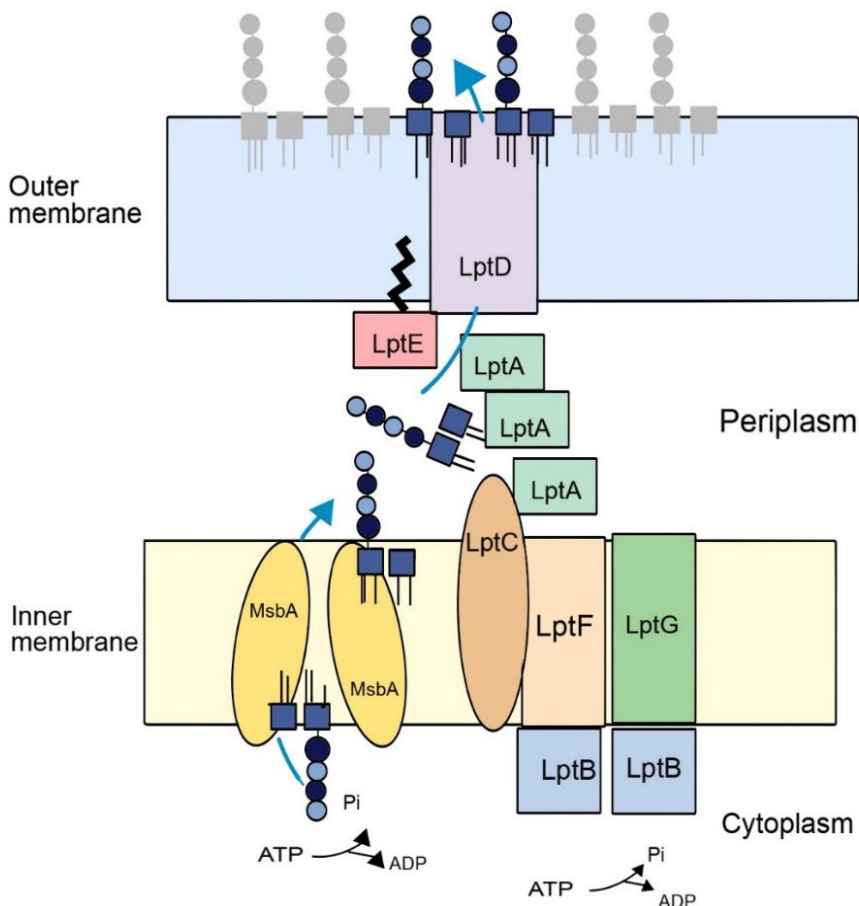


Figure 1.14. LPS export to the outer membrane via the action of Lpt machinery. The ABC transporter MsbA flips the core-lipid A from the inner surface to the outer surface of the inner membrane. The Lpt machinery (LptABCFG) help to assemble the LPS in the outer membrane through the peripasm. LptE and LptD are required for the assembly in the outer surface of the outer membrane-

1.4. Capsular polysaccharides

In the world of microbiology, capsules are significant outer structures of bacteria tightly linked to the cell surface, making them difficult to separate. These capsules play a crucial role in the appearance and morphology of bacterial colonies when grown on agar medium. While capsulated bacteria form "smooth" colonies with distinct features, non-encapsulated counterparts exhibit "rough" morphology, characterized by smaller size and a drier appearance.

A high percentage of Gram-negative bacteria produce capsules, primarily composed of negatively charged capsular polysaccharides (CPS) that form a gel-like structure around the cell, containing up to 90% water.

Historical observations shed light on the importance of bacterial capsules, notably in *Streptococcus pneumoniae*, where capsules have long been recognized as key virulence factors.¹³⁶ Early microbiologists, like Kruse and Pansini in 1891, noted that the absence of the capsule in *S. pneumoniae* resulted in a decrease in virulence.¹³⁷ This discovery marked the beginning of a deeper exploration into the role of CPS in stimulating the immune system, paving the way for the development of modern CPS-based vaccines.^{138–140}

1.4.1. CPS structure

CPSs are polysaccharide acidic polymers formed by intricate structures composed of repeated oligosaccharide units that can exhibit both linear and branched configurations (**Figure 1.15**).¹⁴¹ The negative charges inherent in these structures stem from acidic sugar components or substitutions with charged non-carbohydrate entities. Extensive studies have delved into the diverse compositions of CPSs across various bacteria, including notable examples such as *Streptococcus pneumoniae*, *Escherichia coli* or *Bacillus anthracis*, the CPS of the latter having a particular poly- γ -glutamate composition (**Figure 1.15**).^{142–144}

Substitutions on CPSs may include O-formyl or O-acetyl groups, pyruvates, or even amino acids linked to sugar rings or hexuronic acids.¹⁴⁵ Notably, in many Gram-negative bacteria, the reducing end of CPSs is terminated by a lipid moiety, serving as a cell anchor and fostering association with the cell surface through hydrophobic interactions.¹⁴⁶ In select bacterial strains like the CPSs of *E. coli* group 2, *Neisseria meningitidis* group B and *Haemophilus influenzae* type b, reducing sugars on CPSs can be replaced with phosphatidic acid in a phosphodiester linkage, while the CPS of *E. coli* K5 predominantly features phosphatidyl-Kdo substituents.¹⁴⁷

Despite the wealth of knowledge surrounding CPS compositions, secondary structures of bacterial capsules remain largely uncharacterized. Studies suggest that certain CPSs, like the K1 antigen of *E. coli* and CPS of *N. meningitidis* group B, exhibit helical traits, either along the polysaccharide chain or within specific regions.^{148–150}

In the realm of Gram-negative bacteria, CPSs typically surpass O antigen polysaccharides (O-PS) in length.¹⁴¹ Notably, the same polysaccharide structures may be displayed either as CPSs (K antigen) or O-PSs (O antigen). For instance, the K87 CPS of *E. coli* O8:K87 and the O32 LPS of *E. coli* O32 share an identical structure showcasing the multifaceted nature of polysaccharide structures in bacterial biology.¹⁵¹

of type-specific CPSs, and their number depends on the complexity of the respective CPS.^{141,145}

Escherichia coli serves as a quintessential model for investigating the genetics of capsular biosynthesis in Gram-negative bacteria. In *E. coli*, capsule gene *loci* are designated *cps* in cluster 1, *kps* in clusters 2 and 3, and *rfp* in cluster 4. Each cluster encompasses both common and CPS-specific genes, with the latter dictating the biosynthesis of the specific CPS within the cluster and varying in size according to the complexity of the CPS (**Figure 1.16**).^{146,153}

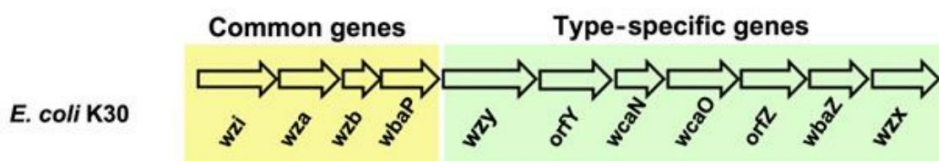


Figure 1.16. Organization of capsule biosynthesis genes in *Escherichia coli* K30.

1.4.2. Host-CPS interactions

For decades, dating back to the pioneering experiments of F. Griffith on the transformation of avirulent non-encapsulated pneumococci into encapsulated pneumococci and their virulence, it has been well-established that the expression of a capsule serves as an essential virulence factor. In cases of invasive bacterial infections, the interactions between the capsule and the host immune system play a critical role in determining the outcome of an infection. This principle is illustrated by the observation that numerous pathogens increase their encapsulation during systemic infections; for instance, group B *streptococci* can produce high levels of CPS, masking cell wall complement-activating components, showing greater virulence than isolates with lower levels of this CPS.¹⁵⁴

Certain CPS structures may be particularly effective in shielding bacteria from host immunity, aiding in adhesion/colonization, or facilitating dissemination

during infections. Notably, in a mouse peritonitis model, capsule type K1 and K2 isolates of *K. pneumoniae* strains exhibit high virulence, whereas strains expressing other CPS types demonstrate limited or no virulence.^{155,156}

Despite the diverse structural and immunological characteristics of capsules, they all primarily serve a common recognition function of protecting bacteria from immune and phagocytic eradication.¹⁵⁷ Capsules play a crucial role in evading complement-mediated opsonophagocytosis.¹⁵⁸ The complement system, an important component of the innate immune system, plays a key role in combating microbial infections.¹⁵⁹ Activation of the complement system through the classical, lectin, and alternative pathways can lead to bacterial lysis via the formation of the membrane attack complex (in the case of Gram-negatives) or by phagocytosis (against both Gram-negative and -positive bacteria). Without specific anticapsular antibodies, the activation of the complement system and its attachment to the bacterial surface depend on the alternative complement pathway mediated by the binding interactions between complement protein C3 and the microbial cell wall, or the lectin pathway involving the soluble mannose-binding lectin and C-type lectin receptors.^{157,160} The CPS avoid the activation of the complement.^{161,162}

The CPSs of certain bacteria possess structural characteristics that imitate host molecules, a phenomenon known as molecular mimicry.¹⁶³ This mimicry serves as a crucial evasion mechanism. For instance, capsules made of polysialic acid (PSA), consisting of N-acetylneuraminic acid (Neu5Ac) monomers linked with multiple glycosidic bonds, are produced by strains of *E. coli* K1, *N. meningitidis* serotype B, *Moraxella nonliquefaciens*.^{164–167} This PSA capsules share the same structure with the terminal polySia region of the embryonic form of the neuronal cell adhesion molecule (NCAM). Other examples are *E. coli* K5 strains and *P. multocida* type D strains are composed of heparosan.^{168,169} Heparosan, composed

of $[\rightarrow 4)\text{-}\beta\text{-D-GlcA-(1}\rightarrow 4)\text{-}\alpha\text{-D-GlcNAc-(1}\rightarrow]_n$ repeating disaccharide units, is identical to the mammalian precursor of heparin and heparan sulfate.¹⁷⁰

Early investigations into pneumococcal capsules provided initial evidence that CPS possess ample immunogenicity to elicit protective immunity against similar bacterial infections.¹⁷¹ CPS are T-cell independent antigens, they can stimulate IgM antibody responses independently of T-cells, without the potential for booster responses upon repeat exposure.^{172,173} Nevertheless, when bound to a carrier protein or presented within complete bacteria, CPS can provoke a T-cell dependent immune reaction, resulting in the production of IgG antibodies that can be effectively reinforced through subsequent immunogen administrations.

The successful application of purified CPS as vaccines against *H. influenzae*, *S. pneumoniae* and *S. typhi*, and *N. meningitidis* has been noted.^{174–176} Additionally, the chemical conjugation of CPS with carrier proteins has been shown to elicit T-cell dependent immune responses and notably enhance the immunogenicity of CPS, particularly in young children.¹⁷⁷ These advancements have led to extensive research and the formulation of conjugate vaccines in recent years.

At present, conjugate vaccines have obtained approval for various encapsulated pathogens. Notably, the first conjugate vaccine was developed for *H. influenzae* type b.¹⁷⁸ Recently, the pneumococcal conjugate vaccine PCV7, originally introduced for pediatric use in the United States and subsequently adopted in multiple countries, incorporates conjugates of diphtheria toxoid with CPS from seven serotypes.¹⁷⁹ Also, available vaccines exist for preventing other life-threatening diseases such as the *Neisseria meningitidis*, the CPS-conjugated vaccines are being used for serogroup MenA, MenC, MenW and MenY infections.¹⁸⁰

1.5. Study of bacterial glycans host perception

Knowing the importance of LPS and CPS for virulence and evasion of host immune recognition for Gram-negative bacteria, the study of LPS/CPS structures and their interactions with host receptors becomes indispensable. Understanding the basis of these interactions is essential for the development of innovative therapies and diagnostics. NMR-based methods are widely used for the characterization of both the structures and the interactions between the protein and the ligand.^{181–184} Here is a wide collection of NMR methodologies that can be used to investigate these dynamics, and access to high-field magnets such as 1.2 GHz has provided new advances in sensitivity and resolution.¹⁸⁵

The study of complex saccharides faces challenges like low sensitivity and resolution issues, which can make it difficult to analyze their structures. To address these obstacles, researchers have developed labeling strategies to enhance the accuracy of their investigations.

The signals obtained from the nuclei of different sugar rings in ^1H NMR spectra can overlap due to their similarities, making it tricky to identify key parameters clearly.¹⁸⁶ Advances in molecular biology strategies such as stable isotope labeling of receptors like ^2H , ^{13}C , ^{15}N , and ^{19}F , as well as paramagnetic lanthanide atoms that binds to sugars through lanthanide-binding-tags (LBTs), have allowed a deeper study of these interactions.^{187–189}

Automated glycan assembly (AGA) synthesis has enabled the creation of well-defined hexasaccharides with specific ^{13}C -labeled glucose units, making it easier to study individual glycosidic linkages and residues in detail, including the crucial $^1\text{J}_{\text{CH}}$ values. This advancement allows for a more precise investigation of molecular structures and.¹⁹⁰

Fluorinated sugars are also widely used in chemistry and biology due to the unique behaviour of fluorine nuclei in NMR spectra, providing valuable insights into molecular interactions and the involvement of specific amino acids. In this context, Patrick *et al.* through a study utilizing ^{19}F and ^{31}P NMR spectroscopy, investigated the conformational and dynamic changes in a glycosyltransferase (WaaG) involved in *E. coli* LPS synthesis (**Figure 1.17**).¹⁹¹

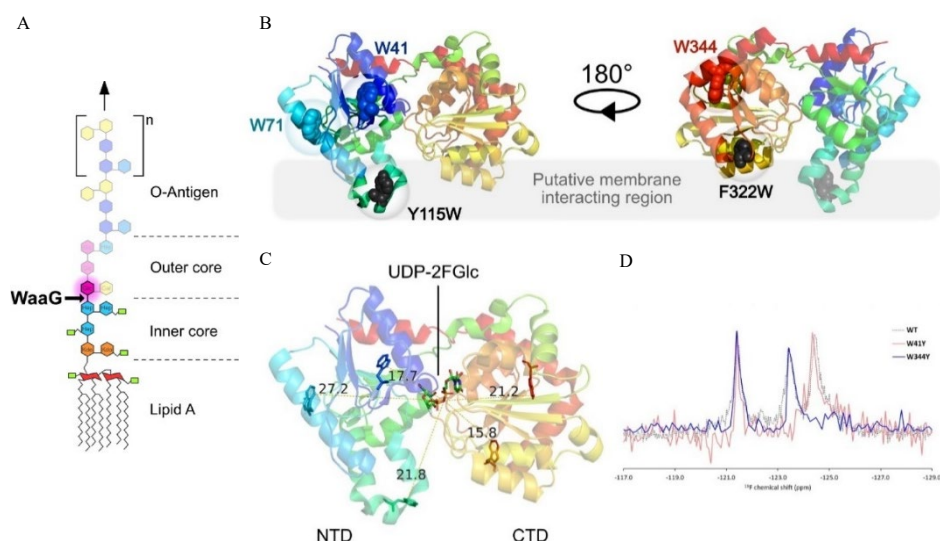


Figure 1.17. (A) Structure of LPS molecule from *E. coli* K12 indicates the essential role of WaaG in adding glucose to the core. (B) Schematic of WaaG depicts intrinsic Trp residues (W41, W71, W344) in cyan, blue and red; and the inserted Trp residues (in point mutants WaaG^{Y115W} and WaaG^{F322W}) in black. (C) Highlighted crystal structure of WaaG features bound UDP-2FGlc (UDP-2-deoxy-2-fluoro glucose), between the N- and C-terminal domains. Distance measurements (in Å) between UDP-2FGlc and five tryptophan residues. (D) Overlaid 1D ^{19}F NMR spectra of wtWaaG (black dotted), WaaG^{W41Y} (pink) and WaaG^{W344Y} (blue) comparing how ^{19}F peak assignments were made, facilitating characterization of WT WaaG peaks as W71, W41, and W322 (from Patrick *et al.* 2023).

1.6. Gut microbiota

Humans are colonized by microbes; the set of these microbial colonies is commonly known as human microbiota. These microbes inhabit different areas of our body and perform an important function in fighting infectious diseases. The microbiota is complex, performs metabolic functions and complements the biology of the host. It protects the host by acting as an important barrier that combats the invasion of external pathogens, which have developed sophisticated mechanisms to avoid the microbiota.

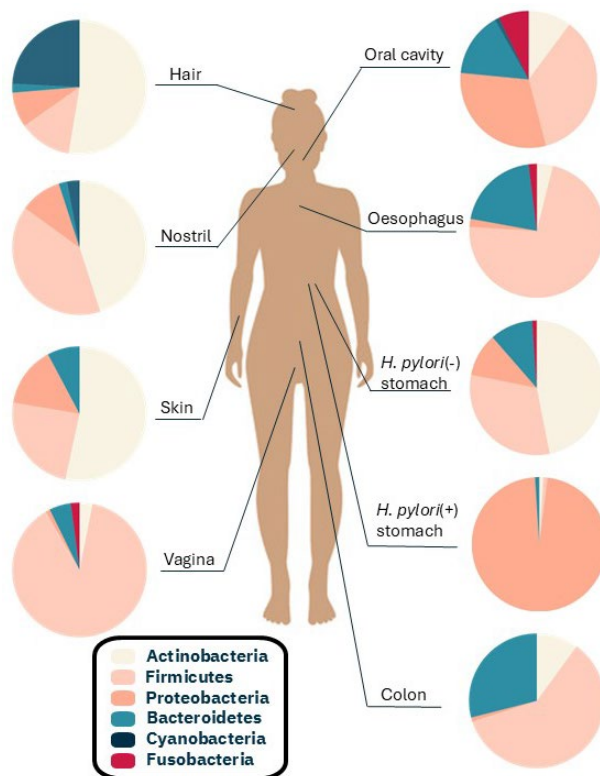


Figure 1.18. Composition of the human microbiota. The four dominant phyla are: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*

We are home to around 10^4 bacteria, a number greater than our own cells,¹⁹² but the microbiota is also composed of archaea, eukaryotes and viruses. The vast

majority of bacterial species in the human microbiota are derived from four major phyla – the Gram-positive Firmicutes and Actinobacteria, and the Gram-negative Bacteroidetes and Proteobacteria (**Figure 1.18**).¹⁹³

While diversity in the microbiota is high, each individual has a particular, much smaller, subset of species, which become established during the first few years of life.¹⁹⁴ In adulthood, the composition generally remains stable over time, although species abundance can be affected by environmental factors such as changes in diet, antibiotic use or disease processes.^{195–198}

Despite these variations, the presence of a diverse and stable microbiota seems to be related to the health of the host. It breaks down and differentiates dietary fiber that is not usable by the human host, through a fermentation process that results in short-chain fatty acids, which will subsequently be metabolized by the intestinal epithelium for cell growth.^{199,200} In addition, several essential vitamins are synthesized, such as vitamin K, riboflavin (B2), biotin (B7), folate (B9), and cobalamin (B12).^{199,200}

Furthermore, the microbiome plays a key role in the development of major components of the host innate and adaptive immune system.²⁰¹ The production of interleukin-10 (IL-10), the best studied and described anti-inflammatory cytokine, has the function of limiting the secretion of pro-inflammatory cytokines and controlling the proliferation of T, B lymphocytes and macrophages.²⁰² For example, the commensal bacteria *Bifidobacterium longum* 5^{1A} was used for treatment on mice infected with *Klebsiella pneumoniae*, inducing an increase of the IL-10 secretion in the lung and helping to the inflammation, the decreased of the lung damage and reduction of bacterial infection.²⁰³ The microbiota also contributes to host protection by acting as a barrier against external microbes, in a phenomenon called "colonization resistance".

But alterations in the microbiota can produce a phenomenon known as "dysbiosis", in which the composition of the microbiota changes and some species

can outgrow. This phenomenon has been linked to different events including invading pathogens, diet, toxins or drugs, and consequently the host becomes more susceptible to infections, chronic inflammation, metabolic disorders, allergies, diabetes, obesity...²⁰⁴

Therefore, and given the importance of the microbiota in health and disease, the study and understanding of this complex community can be of great help in treating some disorders.

1.6.1. Microbiota Pathogen Interactions

The indigenous microbiota has been increasingly studied as a key player in the study of infectious diseases. Growing evidence points to its significant role, particularly in the intestines, where extensive research has focused on pathogen biology and host immune responses.

Commensal bacterial species and microbial products protect against infections indirectly by activating immune responses that in turn attack pathogenic bacteria; for example, *Bacteroides thetaiotaomicron* stimulates the C-type lectin-secreting protein III γ that will bind to peptidoglycan (REGIII γ), which is an antimicrobial peptide used primarily to attack Gram-positive bacteria.²⁰⁵ Other bacteria compete for nutrients or induce the secretion of inhibitory substances that directly inhibit intestinal pathogens. For example, *Bacillus thuringiensis* secretes a bacteriocin that directly attacks spore-forming bacilli and clostridia, including *Clostridium difficile* (renamed as *Clostridioides difficile*).^{206,207} Also, some *Bifidobacterium* spp. produce organic acids and peptides that affect the growth and adhesion of pathogenic *E. coli* to enterocytes. Thus, the microbiota acts as an additional layer of defense against these invaders.²⁰⁸

Resistance to colonization can be due to both microbe-microbe and host-microbe interactions (**Figure 1.19**). For example, microbes will directly compete against invading pathogens for available resources, sources, and mucosal

adhesion, and will produce various antimicrobial compounds, such as bacteriocins, that inhibit the growth of these pathogens.²⁰⁹ In the gut, commensals and their secreted products, such as short-chain fatty acids (SCFAs), also contribute to the modulation of the host immune response.²¹⁰ Indirectly, SCFAs provide additional benefits through their role as a primary energy source for the gut lining, promoting cell regeneration, and mucus production. This strengthens the integrity of the gut barrier, making it more resistant to pathogen infiltration.²¹¹

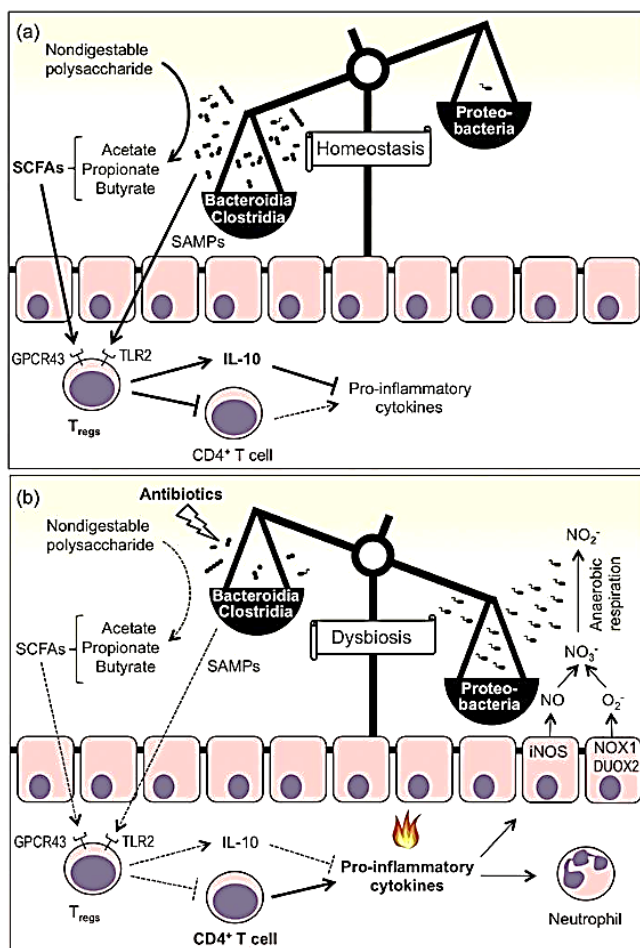


Figure 1.19. Factors mediating colonization resistance and dysbiosis in the colon. **(a)** Under healthy conditions the microbiota is dominated by obligate anaerobic lineages such as *Clostridia* and *Bacteroidia*, which feed on host dietary substrates and produce short-

chain fatty acids (SCFAs). SCFAs and symbiont-associated molecular patterns (SAMPs) induce regulatory T cells (Tregs), which dampen inflammatory response from the host. The intact microbiota effectively outcompetes invading pathogens. **(b)** In dysbiosis the microbiota is disturbed by an external factor such as antibiotic use, diarrhea or pathogenic infiltration. This leads to a reduction in SCFAs and SAMPs, triggering an inflammatory response from the host. Inflammation-associated compounds such as reactive oxygen and nitrogen species (NO/O_2^-) can be utilized as growth substrates by *Proteobacteria* species, which can then outcompete and inhibit the re-growth of a healthy microbiota.²⁰⁴

All of these processes contribute to preventing colonization of the digestive tract by invading pathogens. But it should be emphasized that these processes also appear to suppress the expansion of potentially harmful species (also called pathobionts) that may be members of the microbiota. Examples of this are *Clostridioides difficile* and vancomycin-resistant *Enterococcus* (VRE) that exist as part of the microbiota, but their imbalances are major causes of infectious diarrhea and healthcare-associated colitis. Isolation and characterization of intestinal commensals that prevent the growth of these bacteria may aid the use of particular species or specific combinations of protective commensals to treat infections.²¹²

But pathogens have evolved and developed sophisticated tactics to overcome resistance to colonization. One of the most studied examples is *C. difficile*, which is able to overgrow in the colon after treatment with broad-spectrum antibiotics, thus generating dysbiosis.²¹³ This is due in part to the loss of native species such as *Bacteroides* due to antibiotics, which weakens resistance to colonization and allows the growth of these pathogenic species. This is the case of *Salmonella Typhimurium*, which has developed mechanisms to benefit from the abundant hydrogen sulfide (H_2S) produced by commensals, but if it is not converted to thiosulfate ($\text{S}_2\text{O}_3^{2-}$) it can produce intestinal inflammation.²¹⁴ Just like *Salmonella*, pathogenic *E. coli* and *Citrobacter rodentium* also seem to benefit from this inflammation and overgrow in the intestine.^{215,216} This indicates that inflammatory situations may be beneficial for invading pathogens, causing damage to the

microbiota by reducing their population and the resources they can use, and allowing pathogens to persist if balance in the gut is not restored. This damage fuels the progression of harmful pathogens like vancomycin-resistant *Enterococcus* (VRE). Both animal and human studies have highlighted the microbiota as an indicator of VRE expansion, leading to increased vulnerability to infections and the potential development of sepsis among hosts.²¹⁷ The complexity of these diseases stems from the intricate interplay of diverse microbes within the microbiota. In situations of microbiota imbalance, often characterized by reduced diversity and domination by anaerobic bacteria like Proteobacteria, an inflammatory environment sets the stage for disease progression. Restoring the health of the microbiota emerges as a crucial factor in combating these menacing pathogens.^{218–220}

Another further concern is horizontal gene transfer between microbiota and pathogens, which raises concerns about the transmission of virulence factors, antibiotic resistance determinants, and other deleterious traits. Recent metagenomic research has revealed high levels of antibiotic resistance genes in the microbiome, particularly in regions with extensive antibiotic use.²²¹ Furthermore, studies have shown the persistence of resistance genes in the microbiota long after cessation of antibiotic treatment, with densely populated bacterial communities such as the colon creating favourable conditions for horizontal gene transfer, potentially leading to the spread of antimicrobial resistance determinants to pathogens.²²²

1.6.2. Human microbiota as a Therapeutic Target

Given the connection between microbiota and infections, one goal of emerging therapies is to recruit microbial communities. It is also hoped to limit the use of antibiotics or use those with narrower spectra to avoid damaging the microbiota as much as possible. A recently developed macrocyclic antibiotic known as

fidaxomicin has demonstrated efficacy in combating *C. difficile* infections. While *C. difficile* clearance rates are similar to those of vancomycin, it markedly reduces relapse rates after removal of antibiotic treatment.²²³

An alternative strategy is to reinforce the microbiota by promoting species beneficial to the host through probiotics or by providing useful substrates to beneficial species (prebiotics) that support their growth. Another approach has been to introduce a health-associated microbiota into a sick individual through fecal transplantation, but the risk of introducing new pathogens limits the use of this procedure.^{224,225}

Some probiotic strains have been found in different bacterial genera, including *Bifidobacteria* and *Lactobacilli*. For example, administration of *Lactobacillus casei* in diabetic KK-Ay mice significantly reduces plasma glucose levels and inhibits the production of B-cell-specific CD4⁺ T cells and cytokines such as interferon- γ (IFN γ) and interleukin 2 (IL2), which are major factors involved in the development of autoimmune diabetes.²²⁶

1.6.3. LPS from gut microbiota

Research has uncovered the ability of glycoconjugates, such as lipopolysaccharides from gut bacteria, to initiate widespread pro-inflammatory and immunomodulatory responses throughout the body.²²⁷ This finding is particularly intriguing given the challenge faced by the immune system in distinguishing beneficial microbes from harmful pathogens, even when commensal bacteria share molecular patterns with their pathogenic counterparts. Thus, the importance of the chemical composition of these macromolecules is unquestionable.

The natural heterogeneity observed in the lipid portion of LPS, lipid A, can produce modulatory effects on immune responses. This immunomodulation has been observed to be closely related to the acylation state of lipid A. Anti-

inflammatory LPS (A-LPS) are often hypoacylated, with the identification of tetra- or penta-acylation of A-LPS, as opposed to the well-established hexa-acyl pro-inflammatory lipid A of *E. coli*, expected to be a characteristic of hypoacylation structure in various Bacteroidetes bacteria, including *Bacteroides dorei*, *Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, and *Bacteroides uniformis*, among others.²²⁸ Another example is *Campylobacter jejuni*, a food-related pathogen, whose hypoacylated LPS is capable of inducing a moderate TLR4-dependent immune response.²²⁹ Furthermore, certain examples of LPS from microbiota bacteria have been identified to instigate immune responses. For instance, the Bacteroidetes phylum has been found to achieve resistance to cationic antimicrobial peptides associated with inflammation by dephosphorylating lipid A.²³⁰

Klebsiella escapes from the prompt and prolonged host immune response by modifying its lipid A through the addition of Ara4N and switching from a potent agonist hexa-acylated form to a weaker agonist hepta-acylated species.²³¹ Furthermore, it is important to note that even similar structures can elicit significantly different immune responses, as is the case of the structurally similar penta-acylated LPS from *Porphyromonas gingivalis* and *B. thetaiotaomicron*, highlighting the importance of the exquisite structural variations of LPS lipid A in immune regulation.²³²

Furthermore, the reduction of main diglucosamine chain hypophosphorylation greatly diminishes LPS toxicity. Monophosphorylated lipid A is present in A-LPS obtained from mucosal-associated gut bacteria such as *B. thetaiotaomicron* and *Prevotella intermedia*, resulting in moderate immune-stimulating properties and potential use as immunological adjuvants to elicit antigen-specific immune responses.²³³

1.7. Aims of the thesis

The complex nature of bacterial cell envelopes renders them dynamic structures that play a variety of protective and adaptive roles. In particular, I focused on the isolation and characterization of bacterial glycoconjugates like LPS, EPS and CPS as key components of the outer membrane external leaflet. Since the structure of these glycans is closely related to the immune response they trigger, their structural and functional properties in harmful and beneficial bacteria must be different. Therefore, this thesis project focuses on i) the comprehensive characterization of the chemical structures of bacterial glycans, both from microbiota members (**Chapter III, IV, V, VI**) and from pathogens (**Chapter VII**) and ii) on the investigation of their immunomodulatory capacities, through *in vitro* studies with different human cell lines. The final aim is to obtain information on the molecular patterns that are recognized by the human host as "harmless" or as "dangerous". This work has been divided into the following chapters:

Chapter III: *Bacteroides* species maintain a complex and generally beneficial relationship with the host as part of the gut microbiota. They coexist by adapting to nutrient availability and influencing the host immune system. Due to the importance of *Bacteroides* species as members of the gut microbiota, given their ability to establish beneficial associations with the human host or influence the immune system, this chapter focuses on *Bacteroides eggerthii* 1_2_48FAA. This bacterium has been reported to be abundant in people with type 2 diabetes and to promote colitis in mice. Like other members of the *Bacteroides* genus, its role as a pathogen or commensal is under discussion. Therefore, we here studied the structure of its LOS and its immunological properties *in vitro*.

Chapter IV: Excess weight and metabolic disorders are prevalent among many adults; an essential aspect that disrupts metabolism and affects overall health is the gut microbiome. *Akkermansia muciniphila* stands out as a promising next-generation beneficial microbe. The levels of this microbe in the gut are negatively

correlated with obesity, diabetes, cardiometabolic issues, and chronic inflammation. Moreover, research has shown that pasteurizing *A. muciniphila* enhances its stability and, more significantly, boosts its effectiveness. This places *A. muciniphila* at the forefront of new candidates for creating innovative pharmaceutical supplements that offer health benefits. In summary, similar to how plants and pharmacognosy have historically informed drug development, we posit that understanding microbes could provide a foundation for future therapeutic strategies. Consequently, this chapter will explore the LOS of *A. muciniphila* and its interaction with TLR4 and TLR2 signalling pathways.

Chapter V: Dietary and host-produced glycans influence the composition of the human gut microbiota, and key carbohydrate-degrading species are essential for the integrity and functionality of gut microbial communities. Recently, *Pseudoruminococcus massiliensis* E10-96H has been characterized as a strict anaerobe within the family *Ruminococcaceae*. The closest recognized relative of this species is *Ruminococcus bromii*, recognized as a critical component of the human gut ecosystem due to its ability to degrade resistant starch and its important role in colonic fermentation. Resistant starch fermentation by these bacteria plays a vital role in cross-feeding interactions with butyrate-producing species, a crucial bacterial metabolite involved in maintaining gut homeostasis, improving epithelial barrier function, and reducing inflammation. Furthermore, *P. massiliensis* E10-96H does not trigger proinflammatory reactions in enterocytes, indicating a potentially beneficial relationship with the host epithelium. This research explores the newly identified species *P. massiliensis* by examining the components of its bacterial envelope and provides initial insights into its interactions with the human host.

Chapter VI: *Fusobacteria* are Gram-negative, non-spore-forming, rod-shaped, anaerobic bacteria that produce butyric acid as the main fermentation product. Of note is the distinctive ability of *Fusobacterium* to invade as a pathogen. A poorly

studied species, *Fusobacterium gonidiaformans*, is commonly isolated from the urogenital and intestinal tracts in humans. It is pathogenic and often associated with conditions such as sporadic cases of peritonsillar infections complicated by bacteremia, meningitis, abscesses, intra-abdominal infections, and endometritis. As a potential pathobiont, the specific role of *F. gonidiaformans* in inflammation-related diseases requires further investigation. In this chapter, the focus is on the major component of its outer membrane, LPS, and its impact on the innate immune system.

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CHAPTER II

The study of LPS/CPS
and their interactions
with host receptors

2.1. Introduction

To pave the way for innovative therapies and diagnostics, a profound understanding of the elements composing the bacterial envelope and their interactions with host receptors is necessary. This knowledge helps the design of pharmaceuticals with diagnostic and therapeutic purposes, unlocking the potential to target biologically significant markers with precision and novelty.

Therefore, a diverse array of methodologies is required, including biophysical, spectroscopic and computational methods, serving as robust instruments to reveal the complex structures and interactions existing at the molecular level. In this thesis, the structure of different bacterial cell envelope components and their interactions with host receptors have been studied by nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) in combination with biophysical approaches and computational studies.

2.2. Bacterial glycoconjugates (LPS/CPS) extraction and purification

To study bacterial glycans such as LPS, LOS or CPS from Gram-negative bacteria, the first step is the extraction of the material of interest from the cells without affecting their chemical structure. The quest for bacterial components unfolds through the utilization of two distinct yet synergistic approaches: the phenol-chloroform-petroleum ether (PCP) method and the hot phenol-water extraction technique. The LOS structure, characterized by a shorter saccharide chain with higher hydrophobicity, is effectively extracted using the PCP method, while the presence of the O-polysaccharide antigen in LPS, enhancing hydrophilicity, necessitates the hot phenol-water extraction method. The latter process not only liberates LPS, CPS, and various glycoconjugates but also releases nucleic acids and proteins from the cellular matrix.¹

Initiating the PCP extraction entails the treatment of dry cells with a blend of petroleum ether, chloroform, and 96% aqueous phenol in an 8:5:2 volume ratio,

supplemented with solid phenol until achieving a clear solution. Gradually add water into the phenol solution until the LOS precipitates. Conversely, the hot phenol-water extraction procedure entails treating cells with a 1:1 phenol-water mixture at 65–70 °C, followed by dialyzing the distinct water and phenol phases until the phenolic aroma dissipates, finishing in a freeze-drying process for the extraction of crude extracts. The extracted phases are subjected to enzymatic hydrolysis with DNase, Rnase and protease to remove nucleic acids and proteins. Furthermore, contaminating polysaccharides such as glycogen are attacked with the amylase and pullulanase enzymes, administered together with the nucleases before proceeding to the protease phase. Subsequently, the purified samples are scrutinized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained by silver nitrate.

Observations with SDS-PAGE reveal distinctive patterns: the presence of LPS is indicated by a ladder-like pattern, resulting from the varying lengths of the O-antigen, while LOS leaves its mark as a distinctive band located at the lower end of the gel due to its comparatively lower molecular weight. To achieve a refined sample for further characterization, additional purification techniques such as ultracentrifugation and column chromatography can be employed. These steps allow obtaining a pure sample, preparing the ground for subsequent derivatization procedures essential for comprehensive characterization.

2.3. Structural characterization of LPS/LOS/CPS

Through structural characterization of glycoconjugates, a wide range of valuable information can be obtained, including the qualitative and quantitative composition of the sugar residues, offering insight into the specific types and proportions of sugars comprising these molecules. Also, determining the absolute configuration of each monosaccharide provides detailed knowledge of the three-dimensional arrangements of the sugar residues. Furthermore, comprehensive

analysis allows for the identification of ring sizes and bonding patterns between monosaccharides, shedding light on the structural nuances and conformational complexities of LOS and LPS. Characterization efforts also contribute to unravelling the monosaccharide sequences within these molecules, revealing the specific arrangements and order of the sugar moieties. These structural studies aid in the determination of non-carbohydrate appendages, revealing additional components or modifications that may affect the functional properties and interactions of LOS, CPS and LPS. Together, these in-depth analyses yield an understanding of the structural diversity, composition, and functional characteristics of glycoconjugates.

2.3.1. Monosaccharide analysis

The analysis of oligo- and/or polysaccharide components by gas chromatography-mass spectrometry (GC-MS) requires their transformation into volatile derivatives, which is achieved by replacing polar groups with nonpolar alternatives. This process allows for the subsequent separation of compounds by gas chromatography, where the stationary phase facilitates their elution at different retention times. The subsequent use of a mass spectrometer detector generates various fragments that reflect the chemical characteristics of each component. Depending on the specific derivatization techniques employed, the integrated evaluation of retention time and fragmentation patterns serves to discern the identity, binding patterns, or absolute configuration of individual components. Thus, the sugar composition can be obtained by acetylated alditols (AA) or acetylated methyl glycosides (AMG), while the absolute configuration of these monosaccharides is provided by acetylated octyl- (or butyl-) glycosides (AOG) and the monosaccharide bonds by partially methylated and acetylated alditols (PMAA).

2.3.1.1. Acetylated alditols (AA)

The process of generating these derivatives proceeds according to the following protocol (**Figure 2.1**). Initially, a primary hydrolysis step, often involving trifluoroacetic acid (TFA) to cleave the glycosidic bond, precedes a subsequent reduction step targeting the unbound carbonyl group, ending in the final acetylation of the released hydroxyl moieties. While this approach effectively detects aldose and ketose monosaccharides, its applicability is restricted to neutral or basic species, precluding the detection of acidic monosaccharides unless the carboxylic group undergoes reduction.

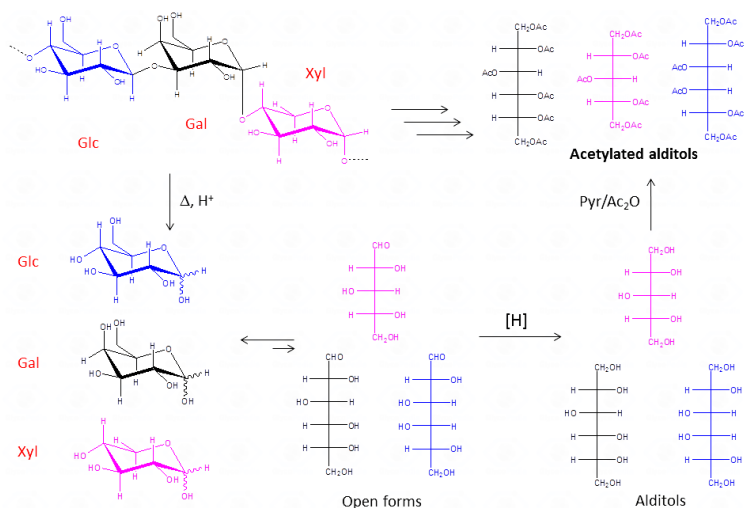


Figure 2.1. Reaction scheme of the acetylated alditols reaction. The glycan is derived into the corresponding acetyl alditols. Image adapted from (De Castro, 2010).¹

2.3.1.2. Acetylated methyl glycosides (AMG)

The process for deriving these compounds involves a method outlined below (**Figure 2.2**). It begins with the methanolysis of the saccharide sample under controlled degradation conditions (MeOH/HCl 0.5-2M at 85°), followed by the acetylation of the resulting monosaccharides. The initial step, a methanolysis under anhydrous conditions, necessitates particular caution. Subsequently, the acetylated O-methyl glycosides undergo analysis via GC for comparison with

standards, or through GC-MS, where their identity is established via fragmentation patterns and retention times. Although adept at detecting most monosaccharide types (including neutral or basic aldoses, uronic and ulosonic acids), this method falls short in identifying. Notably, this approach differs markedly from the use of acetylated alditols due to the non-exposure of the reducing end of monosaccharides, preventing the occurrence of unwanted aldehyde group reactions. Consequently, it results in a heightened recovery yield for each component. Moreover, this reaction mechanism yields a mixture of diverse glycosides for each sugar, varying in configuration at the anomeric center (α and β) as well as in ring size (pyranic or furanic).

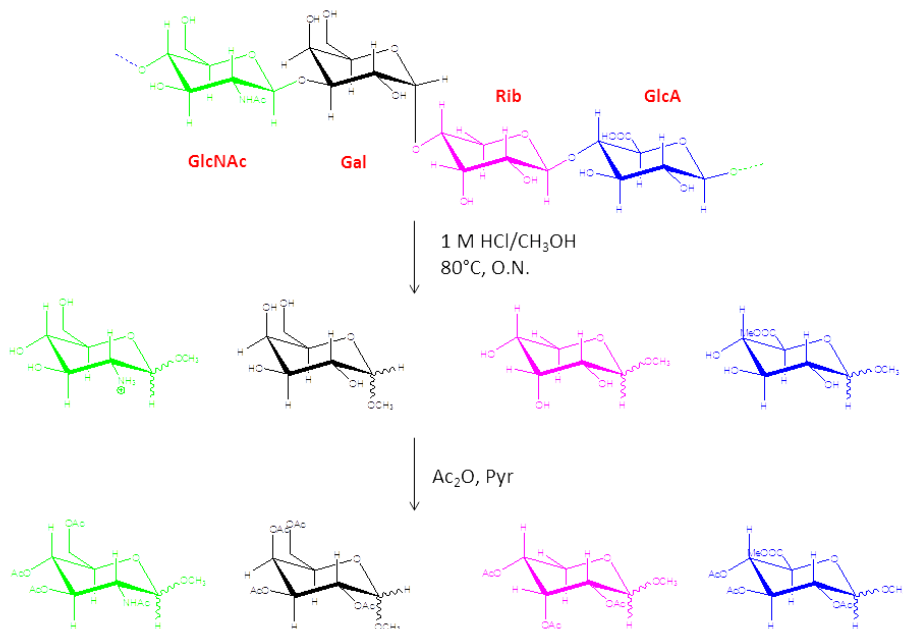


Figure 2.2. Reaction scheme of the AMG reaction. The polysaccharide will be derived into the corresponding acetyl alditols. Image adapted from (De Castro, 2010).¹

2.3.1.3. Partially methylated and acetylated alditols (PMAA).

Similar to the method employed for acetylated alditols derivatives, this technique is applicable for aldose and ketose monosaccharides. However, its use

is confined to neutral or basic species. In the case of acidic monosaccharides, an additional step is necessary to reduce the carboxymethyl group of the fully methylated polysaccharide. The process involves methylation of the sample at its free hydroxy groups, followed by hydrolysis, reduction with NaBD₄, and acetylation. The reduction of the carbonylic function with sodium borodeuteride enables the differentiation between fragments originating from the reduced position (even masses) and those from the last position (odd masses, refer to **Figure 2.3**). Known as linkage analysis, this approach predicts the substitution pattern of monosaccharides. It offers a means to identify the hydroxyl function of each monosaccharide involved in a glycosidic linkage. Moreover, it can sometimes provide insights into the ring shape of the monosaccharide. This procedure converts the monosaccharide units into their corresponding partially methylated acetylated alditols. Each derivative consistently contains two acetyl functions, typically at positions 1 and 5 (in the case of an aldose in the pyranose form, see **Figure 2.4**).

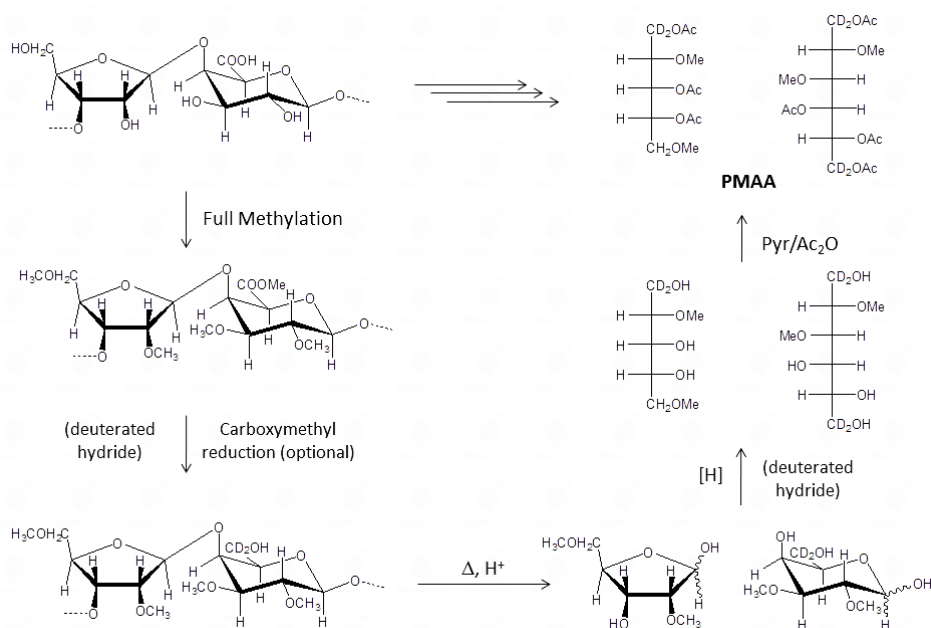


Figure 2.3. Scheme of the reactions occurring during transformation of a sugar in the corresponding Partially Methylated Acetylated Alditols. Image adapted from (De Castro, 2010).¹

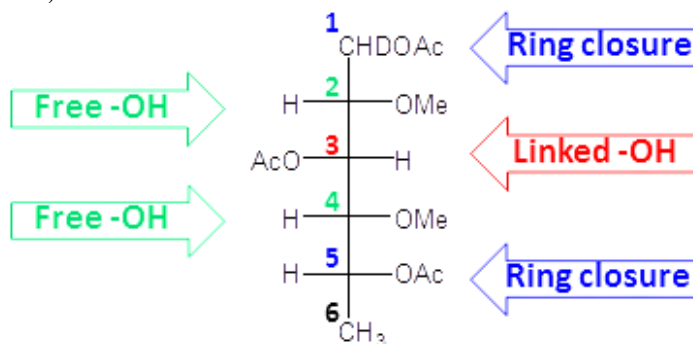
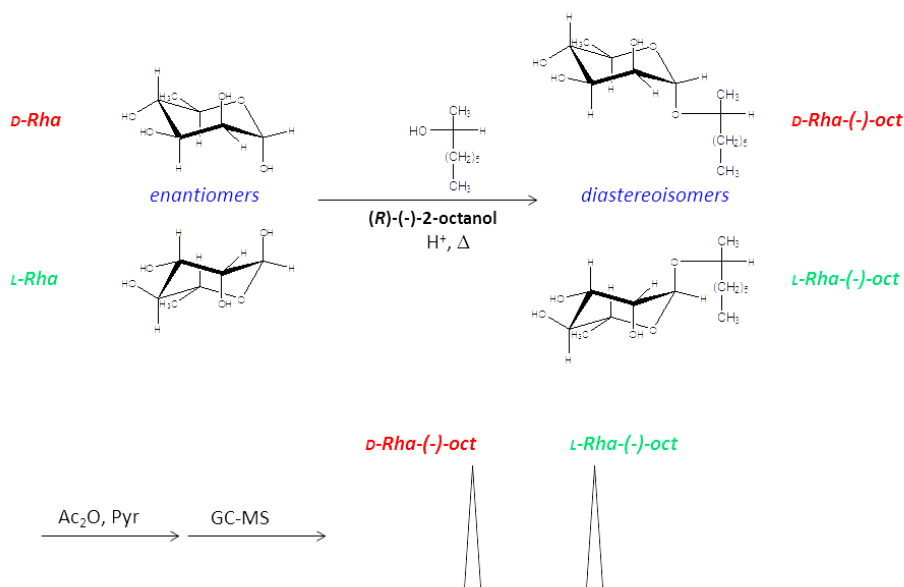


Figure 2.4. PMAA depicting a 3-linked 6-deoxyhexose: methyl groups are introduced on free hydroxyl functions present in the original polysaccharide. It is anticipated that acetyl groups will be present at positions 1 and 5 due to the cyclic (pyranosidic) structure of the residue, while the acetyl at position 3 indicates the involvement of this hydroxyl in a glycosidic linkage. Image adapted from (De Castro, 2010).¹

2.3.1.4. Acetylated octyl- (or butyl-) glycosides.

In nature, monosaccharides such as rhamnose, fucose, or arabinose, exist in both D and L configurations. None of the previously discussed methods are capable of assessing the absolute configuration. The analysis of these derivatives provides a method for determining the absolute configuration of a monosaccharide unit. To resolve the enantiomeric mixture, it is common to convert it into a mixture of diastereoisomers by employing an enantiomerically pure alcohol, such as 2-(+)-octanol or 2-(+)-butanol (**Figure 2.5**). The utilization of enantiomerically pure alcohol enables the formation of a diastereomeric mixture, which can then be separated in the chromatographic column of the GC-MS. Discrimination between the two diastereoisomers is achieved by comparing their retention time with that of the reference compounds. In terms of standard preparation, only one of the two potential configurations of the monosaccharide is utilized, while it is essential to use the optically active alcohol in both its racemic and enantiomerically pure forms (**Figure 2.5**).



b)

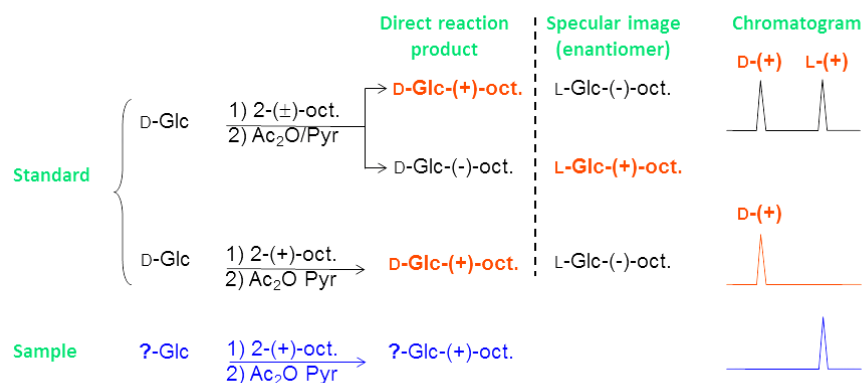


Figure 2.5. a) Scheme of the reaction of Acetylated Octyl Glycosides for the enantiomeric mixture of rhamnose using chiral *(R)*-(-)-2-octanol. B) Strategy used to prepare 2-octylglucoside standards.

2.3.2. Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) is a powerful tool for exploring compounds in solution, providing detailed information at the atomic and

molecular level by observing atomic nuclei within a magnetic field. Furthermore, the non-destructive nature of this technique allows for the complete recovery of samples that can be used for further investigations. Consequently, NMR stands out as one of the most widely used techniques for characterizing molecules, including oligosaccharides and polysaccharides.

In liquid-state NMR, a magnetic field is applied to molecules dissolved in a suitable deuterated solvent, which has been selected to stabilize the field during experiments. The most commonly used solvent is deuterium oxide (D_2O), but other solvents such as CD_3Cl can be used if necessary. The residual water signal can be intense and may cover signals that are nearby, with the result that some information may be lost or overlooked. It occurs at approximately 4.7 ppm at 300 K, i.e. in the same region as the anomeric signals of carbohydrates, which are among the most important signals for the characterization of these molecules. In the structural characterization of glycans, the initial step is usually one-dimensional spectroscopy (1D NMR), with the proton spectrum (^1H), although additionally other nuclei such as ^{13}C , ^{31}P or ^{15}N can be investigated. The proton spectrum can be divided into different regions, each of which provides valuable information on the composition of the sample (**Figure 2.6**). Thus, the anomeric region (5.6–4.4 ppm) comprises relevant information on the anomeric protons of the aldose units. The next region (4.4–3.0 ppm) is the most populated part of the spectrum, where the carbinolic protons of the monosaccharide residues are found. The information present in this region is so essential that it is considered the “fingerprint” of a glycan. The region in higher fields (3.0–1.0 ppm) is the aliphatic one, where we can find the key signals to identify deoxysugars such as fucose. The methyl group of 6-deoxyresidues can be found around 1.1–1.3 ppm, but other interesting protons can also be found in this zone, such as the two diastereotopic protons of the methylene group at C-3 of Kdo (3- deoxy-2-keto-D-manno-octulosonic acid), the hallmark of bacterial lipopolysaccharides.²

Finally, in the case of having aromatic residues as aglycone substituents, the spectrum area in the range of 8–7 ppm is the one that will provide the necessary information for their identification. It is important to note that these divisions should not be taken as immutable since different substituents such as phosphates or sulphates can shift the geminal proton.

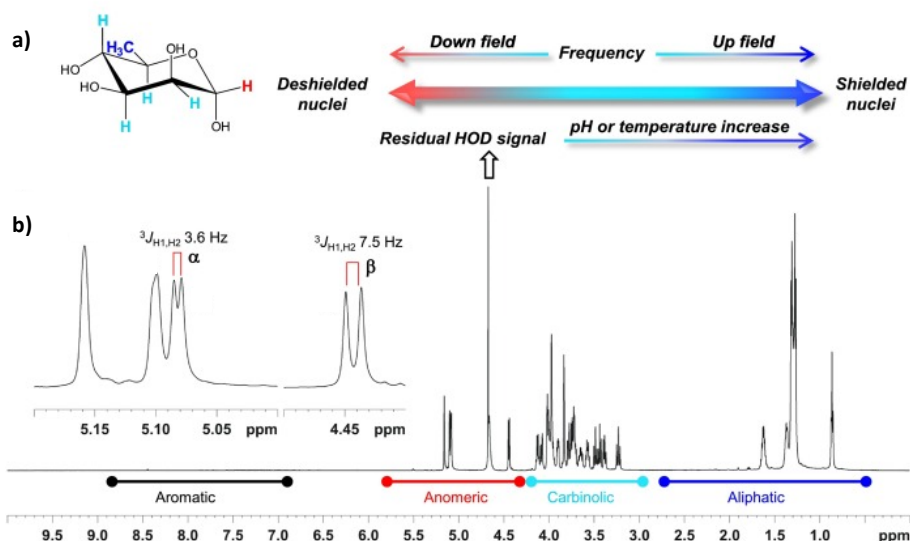


Figure 2.6. a) Visual representation in a cartoon format illustrates the significant downfield or upfield regions of the spectrum pertinent to monosaccharide residues. Using D-Rha (D-Rhamnose) as an exemplary, the coloration of its protons aligns with their classification and position within distinct spectrum regions: anomeric, carbinolic, and aliphatic. B) ¹H NMR spectrum with the regions of the spectrum divided by the same colors as in a), and with a zoom in the anomeric region to show α and β anomeric signals. Figure modified from.²

Generally, for sugars in their pyranose form anomeric protons downfield of 4.7 ppm indicate an α configuration, below them are the β configured (**Figure 2.6**). Each anomeric signal can be observed split into two due to the existence of the “coupling constant” with the neighbouring proton (i.e. H-2) and depends on the relative orientation of the protons. It is denoted as $^3J_{H1,H2}$ and is expressed in Hz, thus protons in β having a trans-diaxial orientation of H-1/H-2 will have doublets

with a $^3J_{H1,H2}$ value of $\sim 7\text{--}9$ Hz. While for those with the α configuration, the protons will form a dihedral angle of $\sim 60^\circ$ (equatorial–axial arrangement), giving a small $^3J_{H1,H2}$ value of $\sim 2\text{--}4$ Hz.

In the process of establishing the sugar sequence, a series of bidimensional (2D) NMR spectra provide different pieces of information depending on the phenomena studied: scalar coupling (through-chemical bond) or dipolar (through-space) interactions between the spins of two (or more) nuclei, and diffusion-based mobility of the molecule in solution. Initial experiments focus on nuclear Homo correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY), which correlate the chemical shifts of ^1H nuclei with geminal and vicinal couplings. These protons are either geminal or vicinal, so separated from two (^2J) or three (^3J) chemical bonds (**Figure 2.7**). In contrast to COSY, the TOCSY spectrum uncovers correlations among protons within a spin-spin coupled chain of protons, facilitating magnetization propagation through the spin-locking sequence. TOCSY offers a view of all nuclei within the same spin system, irrespective of direct coupling through ^2J or ^3J constants (**Figure 2.7**). The use of TOCSY is essential for solving the most populated regions of the spectra. By starting from distinctive signals like the anomeric signal, TOCSY delineates protons within the monosaccharide spin system, aiding in the selection of correlated protons in the COSY spectrum. Complementarily, TOCSY assists in the preliminary identification of the relative stereochemistry of the residue, like the monosaccharide has a *manno*, or a *gluco*, or a *galacto* (or another) relative configuration. Taking glucose as an example the TOCSY is expected to have six different correlations, one for each proton of the monosaccharide that appears thanks to the favorable proton-proton coupling constant values between all protons. However, when an epimer of glucose is studied, the TOCSY changes due to the presence of coupling constant values that interrupt the magnetization propagation.

Subsequent to TOCSY, the exploration of nuclear Overhauser effect spectroscopy (NOESY) enables the evaluation of cross-relaxation rates, which provides information on the spatial proximity of the protons (**Figure 2.7**). NOESY, a homonuclear experiment unlike COSY and TOCSY, capitalizes on the nuclear Overhauser effect to detect cross-relaxation phenomena between nuclei, emphasizing spatial interactions beyond bond numbers. The closer the nuclei are, the greater the signal intensity is, with 4 Å being the distance limit for this effect to be detected.^{3,4} When dealing with minimal NOE scenarios, as seen in small oligosaccharides such as trisaccharides, the utilization of ROESY (rotating frame Overhauser effect spectroscopy) under spin-locked conditions yields positive signals.

Moving on to carbon-proton correlations, HSQC (heteronuclear single quantum correlation), is used to directly correlate ^{13}C and ^1H nuclei attached utilizing the one-bond coupling $^1J_{\text{CH}}$, generally set to ≈ 145 Hz to detect both anomeric ($^1J_{\text{CH}} \approx 160\text{--}170$ Hz) and carbinolic ($^1J_{\text{CH}} \approx 140$ Hz) correlations (**Figure 2.7**). The carbon chemical shifts of carbohydrates can be divided in different regions: 10–25 ppm is the aliphatic region and contains the methyl groups of 6-deoxysugars and of the acetyl groups; 45–60 ppm is typical of carbon bearing an amino group; 60–70 ppm show the presence of the hydroxymethylene ($-\text{CH}_2\text{OH}$) carbons, either with the free hydroxyl function (60–63 ppm) or substituted (64–70 ppm); 70–85 ppm contains the carbinolic ($-\text{CHOH}-$) carbons; 90–110 ppm is the region of anomeric carbons. In general, in glycans the ^{13}C values of the α anomers are at about 98–103 ppm, while those of the β anomers are at 103–106 ppm.⁵

Table 1. Typical ^1H and ^{13}C chemical shift values of sugar compounds.

Δ (ppm)	^1H
8.5 – 7.5	Amide resonances
5.5 – 4.2	Anomeric protons
4.5 – 2.8	Sugar ring protons
2.6 – 1.8	α -methylene protons of deoxy sugars
2.0 – 1.0	Methyl protons of the 6-deoxy sugars and of the acetyl groups
δ (ppm)	^{13}C
160 – 180	Carbonyl carbons
90 – 110	Anomeric carbons
60 – 85	Sugar ring carbons
45 – 60	Nitrogen bearing carbon signals
~ 30	Aliphatic methylene carbons of deoxy sugars
10 – 25	Methyl carbons of deoxy sugars, acetyl groups

Furthermore, the HMBC (Heteronuclear Multiple Bond Correlation) experiment is used to determine the long-range ^1H - ^{13}C connectivity through multiple bond couplings over two or three bonds, typically characterized by a coupling constant (J) range of 2-15 Hz. Accordingly, this sequence detects proton/carbon pairs separated from two (^2J) or three (^3J) linkages, without the possibility to make a distinction between them because the size of the coupling constant (2–15 Hz) is the same in both cases (**Figure 2.7**). Despite this limitation, the HMBC sequence serves for the structure elucidation of polysaccharides, seamlessly interlocking diverse molecular fragments to form a complete structure.

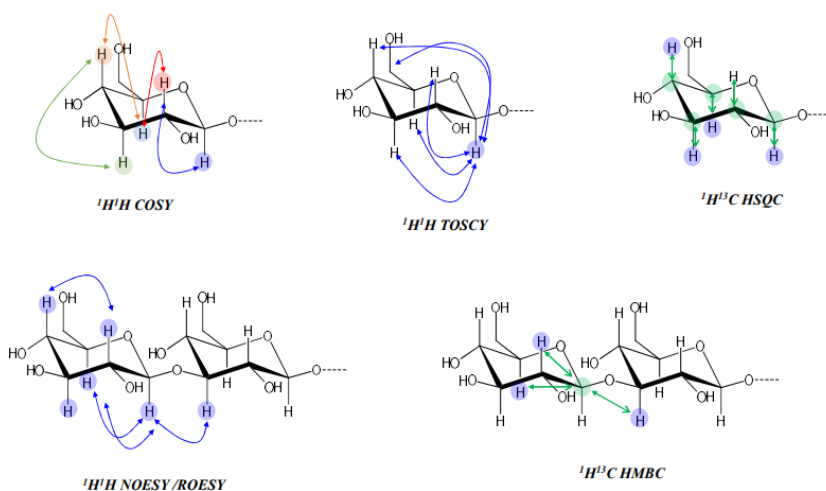


Figure 2.7. Representation of the different correlations obtained via 2D NMR experiments.

2.3.3. Mass Spectrometry analysis

In recent times, there has been a broad array of mass spectrometry applications utilized to uncover the structure of LPS. Enhancements in methodologies and separation techniques have shown the adaptability of mass spectrometry in examining LPS mixtures. Currently, mass spectrometry (MS) using a soft ionisation method such as matrix-assisted laser desorption/ionization (MALDI) and soft electrospray ionization (ESI) are important tools for the characterization of oligosaccharides and LPS. Additionally, the analyzers can be chosen in order to adapt the method to the requirements, for example Quadrupole (Q), Quadrupole Ion Trap (QIT), Fourier transform Ion Cyclotron Resonance (FTICR) and Time-of-Flight (TOF) can be found among the options.⁶

MALDI operates by directing pulsed laser light onto a metal target where a co-crystallization of an organic acidic matrix and the target structure has occurred. The matrix is essential in the ionisation process, but also absorbs the laser energy protecting the sample and assuring for a soft ionisation with formation of single charged ions.^{7,8} In addition, by using a MALDI-TOF/TOF system, tandem mass

spectrometry can be performed as is an efficient approach for elucidating the sequence of oligosaccharide chains and has proven to be invaluable for studying a variety of biomolecules. Through tandem MS (MS^n), specific ionic species are isolated and further fragmented to acquire structural information. This method swiftly screens large glycans, providing details on sequence, monosaccharide composition, branching, linkage types, connectivity, ring size, and compositional isomers. However, the fragmentation does not necessarily occur at each glycosidic linkage therefore other fragments can be produced complicating the analysis. The nomenclature of this fragments was implemented by Domon and Costello⁹ and divides these fragments in two types: the ones from the glycosidic cleavages ((B-, C-, Y-, Z-type), and the other ones from cross-ring fragmentations (A- and X-type) where A, B and C-type are related to fragments with a terminal sugar moiety (non-reducing end) X, Y and Z-type contain the reducing end of the sugar or the aglycon (**Figure 2.8**).

MALDI boasts the advantage of enabling the analysis of complex oligosaccharide mixtures, such as methylated lipid A in positive ion mode or underivatized lipid A in negative ion mode. Singly charged ions of intact LPS are predominantly formed during MALDI, simplifying the interpretation of mass spectrometry spectra. The produced fragments typically correspond to core oligosaccharide and lipid A derived from the cleavage of the labile ketosidic linkage of the Kdo (**Figure 2.8**).¹⁰ In the resulting MALDI-TOF spectra three regions can be distinguished: the ions originated by the LPS, the core OS and the lipid A. Interestingly, in the particular case of S-LPS, due to the different weights of the O-antigen repeating unit, information about the size can be obtained.⁶

MALDI-TOF MS/MS is also used to analyze the lipid A samples, which typically result in a complex array of peaks representing individual molecular species. Negative ion mass spectra are dominated by intense signals of deprotonated $[M - H]^-$ ions of *mono*- and *bis*-phosphorylated lipid A species (with

a mass difference of 80 Da), exhibiting varied degrees of acylation (ranging from tri- to hepta-acylation, depending on the bacterial strain), with satellite peaks of sodium and potassium adduct ions also potentially present $[M + Na - 2H]^-$ and $[M + K - 2H]^-$ (with differences of 22 and 38 Da from the main peaks, respectively), while differences of, for example, 131 or 123 Da indicate substitutions with groups like Ara4N or PEtN.

Recent developments with various techniques like IMS-MS (ion mobility mass spectrometry) and tandem mass spectrometry (IMS-MS/MS) have been achieved. These techniques offer two-dimensional data sets that provide m/z , drift time, and ion intensity information, aiding in the resolution of oligosaccharide and lipid A fragments based on size, shape, and charge.⁹

ESI MS is another valuable method for analyzing oligosaccharides, particularly if glycan acids are present due to their reduced fragmentation with this technique. When coupled with GC-MS, ESI MS facilitates the determination of monosaccharide composition and linkage analysis. Its attributes complement endotoxin analysis well, as it allows direct analysis of biological extracts, interfaces easily with other techniques like liquid chromatography, generates molecular ions, produces multiply charged ions, and offers high sensitivity and a broad dynamic range.

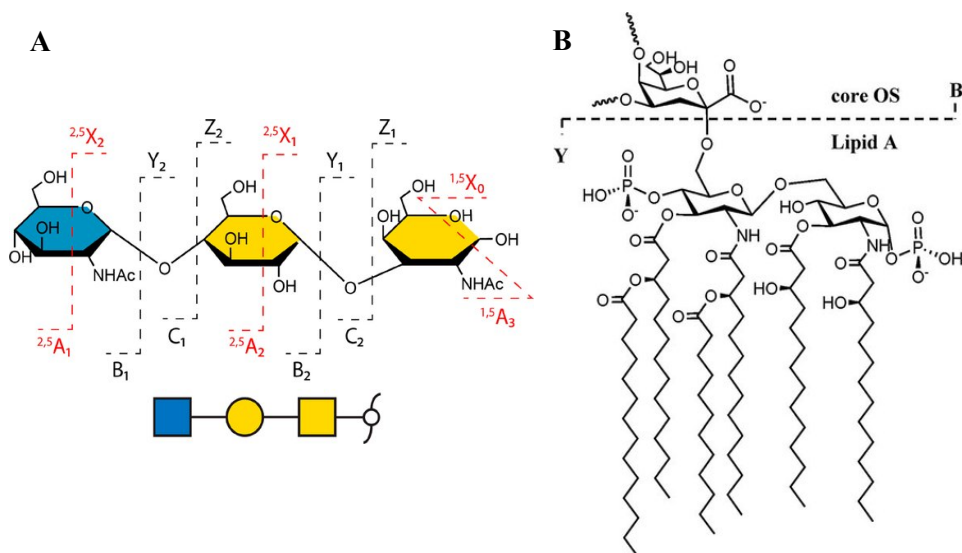


Figure 2.8. (A) A scheme showing the systematic nomenclature for carbohydrate product ions generated by tandem MS approach. The glycosidic cleavages labelled B-, C-, Y-, Z-type and the A- and X-type of the cross-ring cleavages. (B) A schematic representation indicating the cleavage occurring in MALDI-TOF MS analysis of a LPS. Indicated are the B-type (core OS) and Y-type (lipid A) ions which occur in the spectrum. Figure modified from ⁹.

2.4. Immunological test

Recognition of microbial pathogens is what allows the immune system to distinguish between non-infectious self and non-self-infectious. Toll-like receptors play an important role in pathogen recognition and the initiation of inflammatory and immune responses. Stimulation of Toll-like receptors by microbial products results in the activation of signalling pathways that result in the induction of antimicrobial genes and inflammatory cytokines. Furthermore, stimulation of Toll-like receptors triggers maturation of dendritic cells and results in the induction of costimulatory molecules and enhanced antigen-presenting capacity. Thus, microbial recognition by Toll-like receptors helps direct adaptive immune responses to antigens derived from microbial pathogens (see Chapter 1. Host-LPS interactions).¹¹

2.4.1. HEK-Blue TLR4/MD-2 and TLR2 cells

Toll-like receptors (TLRs), have a cytoplasmic domain for proinflammatory signaling, and recognition by TLRs is directly linked to inflammatory responses. Consequently, it is important to understand how TLRs recognize microbial products.¹² Human Embryonic Kidney (HEK) 293 is a widely used cell line for *in vitro* models as it is simple to grow in culture and easy to introduce genetic modifications in these cells.¹³ In particular, HEK-Blue TLR cells are designed to provide a fast and sensitive method to screen TLR agonist and antagonist. These cells co-express a human TLR gene and an NF- κ B inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Upon TLR engagement SEAP will be released producing a purple/blue color and can be measured using a spectrophotometer (**Figure 2.9**).¹⁴

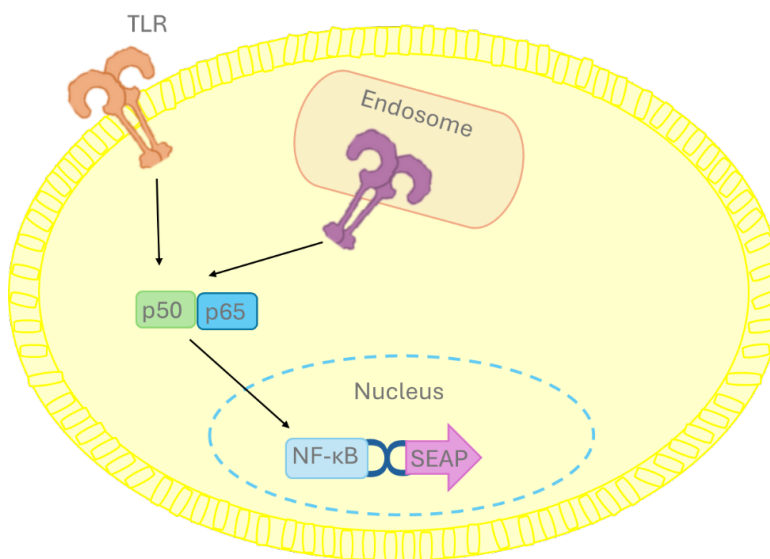


Figure 2.9. Signalling pathways in HEK-Blue TLR cells

2.4.2. Monocyte-derived dendritic cells (moDCs)

Dendritic cells (DCs) are the most important antigen-presenting cells of the immune system, allowing proper T cell responses upon maturation induced by

different stimuli. The generation of DCs from human peripheral blood monocytes is widely used as an *in vitro* model for the study of DC maturation and activation. After isolation of monocytes from blood, they are cultured with IL4 and GM-SCF, which induce their differentiation into immature monocyte-derived DCs (moDCs) after 6-7 days. Upon triggering PRRs on moDCs by different stimuli, these cells increase the expression of co-stimulatory molecules, such as CD40, CD80 and CD86, which facilitate the activation of T cells and the generation of an adaptive immune response. Finally, the analysis will be performed by flow cytometry to evaluate the cellular viability.¹⁵

2.5. Flow cytometry

Flow cytometry is an essential technique for the study of immune cells and their responses both *in vitro* and *in vivo*. It is based on a rapid multi-parametric analysis of single cells in suspension, allowing us to study multiple cell surface and intracellular markers simultaneously in any cell of interest. A traditional flow cytometer consists of a fluidics system, in which cells are focused into a liquid stream and intercepted by laser beams at the so called “interrogation point”. Then, in the optical system of the cytometer, which includes not only the lasers, but also the detectors where both visible light and fluorescence signals are captured, and the sample is analyzed. Finally, the electronics system converts these signals into digital information.

In order to perform a multiparametric analysis, cells are first labelled with antibodies against the markers of interest coupled with fluorochromes. When a labelled cell reaches the interrogation point, it scatters light and the fluorochromes present on that cell will be excited and fluoresce. Scattered light is measured in the forward direction (Forward Scatter or FSC) and at 90 degrees (Side Scatter or SSC), giving information about the size and complexity of the cells, respectively. A series of filters allow the fluorescent light to reach specific detectors depending

on the emission wavelength of each fluorochrome, giving information about the binding of labelled antibodies to the cells, ultimately showing the expression of the markers of interest in each cell.

2.6. Computational methods

Molecular modelling is a potent tool used to model or mimic the behaviour of molecules. The combination of the *in silico* methods with NMR and other biophysical techniques helps the elucidation of the conformational and dynamic properties of these saccharide structures.^{16,17} Determining the possible shapes of glycans, and understanding their implications in drug development, presents a complex challenge stemming from the vast array of monosaccharides, diverse glycosidic bonds, and the inherent flexibility of the rings. Furanose rings exhibit considerable natural flexibility, while pyranose rings tend to adopt energetically favourable conformations. The spatial arrangements of saccharide units are commonly described using the torsion angles of the Φ ($H_1-C_1-O_1-C_x$) and Ψ ($C_1-O_1-C_x-H_x$) glycosidic bonds (see **Figure 2.10**). However, when the glycosidic bond does not involve an endocyclic carbon (atom not located in the ring), such as for 1,6 linkages, the ω ($O_5-C_5-C_6-O_6$) angle is considered. The ω torsion angle was described by means of the populations of the gauche–gauche (*gg*), gauche–trans (*gt*), and trans–gauche (*tg*) rotamers.¹⁸

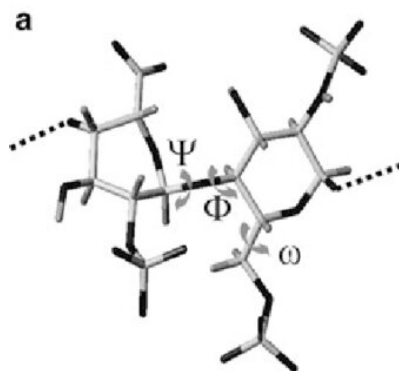


Figure 2.10. Graphical representation of the disaccharide repeating unit of heparin (2-OS)- α -LIdoA-1-4-(2-NS,6-OS)GlcNAc with indication of the torsion angles of interest. The dotted lines represent the extension of heparin sulfate chain.

The additional flexibility of α -(1 $\rightarrow$ 6) bonds complicates the determination of the preferred solution conformation of oligosaccharides containing these bonds. Newman projections for these cases are as follows (**Figure 2.11**).

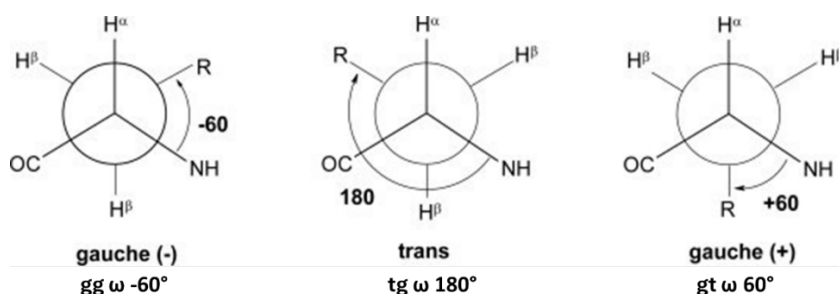


Figure 2.11. Newman projections of staggered rotameric states of the omega torsion angle along the C6-C5 bond: *gg* (gauche-gauche), *tg* (trans-gauche) and *gt* (gauche-trans).

Oligosaccharides are recognized as flexible molecules due to the presence of glycosidic bonds that confer a degree of conformational flexibility. Their intricate modelling is further complicated by specific stereoelectronic effects like anomeric and exoanomeric phenomena, associated with configurational and conformational shifts. Key force fields employed for carbohydrate structures include Amber/GLYCAM¹⁹ and CHARMM,²⁰ both encompassing a wide range of common monosaccharides and diverse glycosidic linkages. Notably, GLYCAM06 is well-suited for D and L enantiomers, mono- and oligosaccharides, and all types of glycosidic linkages, including N-glycosylation.²¹

Various methods exist for the calculation of Φ and Ψ to define the energy map, generally applied to each disaccharide unit in a glycan chain. MM3*²² is the main force field used to calculate local minima and flexibility of glycosidic torsions,²³ but free carbohydrate databases (such as <http://glycosciences.de>) are also

available. Thus, the potential energy surface showing conformational energy for the Φ and Ψ dihedral angles can be plotted on “adiabatic” maps, representing the energetically favourable conformations of a carbohydrate dimer. Molecular dynamics (MD) simulation stands out as a key tool in the computational exploration of biological molecules, offering detailed insights into the dynamics and structural alterations of molecules and their assemblies. During an MD simulation, the initial velocities and positions of all atoms within the biomolecular system are defined. By calculating the forces acting on each atom and applying Newton's second law of motion, the evolving spatial positions of the atoms over time are predicted. For an atom "i" with mass " m_i " and Cartesian position " x_i ", Fx_i denotes the force exerted on the atom at time "t".

$$\frac{d^2x_i}{dt^2} = \frac{Fx_i}{m_i} \quad [2.1]$$

The simulation is repeated at specific time intervals, evaluating the position and velocity of each atom at each step to generate a trajectory that captures the atomic-level configuration of the system over the entire duration. The initiation of a molecular dynamics (MD) simulation involves the initial phase of energy minimization, commonly achieved through the steepest gradient method to optimize the geometry of the system until a local minimum is reached. Incorporating the environment into the simulation, such as employing MD with explicit water molecules or other surrounding molecules, is crucial during this phase.²⁴ Carbohydrates, due to the presence of hydroxyl groups that form multiple hydrogen bonds, exhibit a strong affinity towards water molecules. The use of explicit water allows complex interactions to be simulated within a solvent box under periodic boundary conditions (PBC) to mitigate surface artifacts, with the widely used TIP3P model taking water molecules into account. Electrostatic interactions, including infinite ones, are calculated using the particle mesh Ewald (PME) summation, which splits the sum into short- and long-range components. The heating phase is then carried out to eliminate adverse contacts between

solvent and solute, such as steric clashes, by increasing the velocity of the atoms calculated according to the standard temperature-dependent Maxwell-Boltzmann distribution. The equilibration phase follows, which involves relaxation of the system under controlled conditions of energy, temperature, pressure, and volume. Finally, the production of the final trajectories concludes the process. Molecular dynamics simulations offer a valuable approach for studying carbohydrates in both their unbound and bound forms, often complemented with NMR data like NOE and residual dipolar coupling-based experiments. This integration allows for a comprehensive exploration of the conformational dynamics of ligands and the development of validated 3D models of complexes.

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CHAPTER III

The human *Bacteroides eggerthii*
expresses a new galactofuranose-
containing lipooligosaccharide with weak
immunostimulatory properties

3.1. Introduction

I will here focus on the characterization of the LPS isolated from the human gut bacterium *Bacteroides eggerthii* 1_2_48FAA. Among the members of gut microbiota, the obligate anaerobic *Bacteroides* species have garnered significant attention due to their prevalence across diverse human populations and adaptations to the gut ecosystem. Indeed, *Bacteroides* ssp. forge enduring associations with their human hosts, conferring a multitude of health benefits.^{1,2} These species are also able to influence the gut environment itself by promoting production of fucosylated glycoproteins, or by releasing fucose and sialic acid residues from glycoproteins for consumption by other microorganisms.³ Recent evidence showed that LPS from commensal *Bacteroides* ssp, such as *B. thetaiotaomicron* and *B. vulgatus* mpk, are poor activators of pro-inflammatory cytokines production while they are inducers of anti-inflammatory cytokine released likely via the synergistic activation of MD-2/TLR4- and TLR2-mediated signaling pathways.^{4,5} As a matter of fact, TLR2 and TLR4 play vital roles in distinguishing specific molecular patterns on invading pathogens, both acting as PRRs that link the innate and adaptive immune responses.⁶ Nevertheless, how the molecular interactions between LPS and host receptors, as well as the subsequent signaling pathways, contribute to the establishment of disease, commensalism, or even mutualism, is still at a descriptive level.

Bacteroides eggerthii, a member of the human gut microbiota, has been reported to be abundant in individuals with type 2 diabetes and to enhance colitis in mice.⁷⁻⁹ As other members of the gut microbiota, its role as a commensal or pathobiont is still a matter of debate. Intrigued by the controversial nature of this gut microbe, here we report that *B. eggerthii* 1_2_48FAA produces an R-type LPS (or lipooligosaccharide, LOS) composed of a heterogeneous mixture of tetra- and penta-acylated lipid A species with different degree of phosphorylation, and a compact galactofuranose-containing core oligosaccharide. Using *in vitro* human

cell lines, we showed that *B. eggerthii* 1_2_48FAA LOS acts as a weak activator of TLR4-mediated signaling. Moreover, we observed that expression of maturation markers CD40, CD80 and CD86 on monocytes-derived dendritic cells upon *B. eggerthii* 1_2_48FAA LOS exposure was significantly lower compared to pro-inflammatory *Escherichia coli* LPS. Taken together, these data provide new structural and biological insights into LPS from gut bacteria, underscoring the importance of structural features in modulating host immunity.

3.2. *B. eggerthii* 1_2_48FAA LOS isolation and chemical analyses

The LOS from *B. eggerthii* 1_2_48FAA was isolated following the hot phenol-water extraction and further purified by enzymatic treatments and gel-filtration chromatography. Monosaccharide analysis indicated the presence of L-rhamnose (L-Rha), L-fucose (L-Fuc), D-galactose (D-Gal), D-glucose (D-Glc), D-glucosamine (D-GlcN), 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) Methylation analysis showed the presence, in pyranose form, of 6-substituted and 3-substituted GlcN (6-GlcNp and 3-GlcNp), terminal Fuc (*t*-Fucp), 3-substituted Gal (3-Galp), 6-substituted Glc (6-Glcp), 2,6-substituted Glc (2,6-Glcp), 2,3-substituted Rha (2,3-Rha), terminal Glc (*t*-Glc), and of a terminal galactofuranose residue (*t*-Galf) (**Table 3.1**). Fatty acid compositional analysis revealed the presence of *anteiso*-pentadecanoic acid (*a*C15:0), *iso*-C15:0 (*i*C15:0) and C15:0, hydroxypentadecanoic acid [C15:0 (3-OH)], *anteiso*-heptadecanoic acid (*a*C17:0) and C17:0, hydroxyhexadecanoic acid [C16:0 (3-OH)], *anteiso*-hydroxyheptadecanoic acid [*a*C17:0 (3-OH)], *i*C-17:0 (3-OH), and C17:0 (3-OH) (**Table 3.1**), typical content of lipid A from other *Bacteroides* ssp.^{4,5,10}

Table 3.1. Monosaccharides and fatty acid components of *B. eggerthii* 1_2_48FAA LOS. Except for rhamnose and fucose, which are in L-configuration, all other monosaccharides are in D configuration. Relative abundances expressed in percentage of each partially methylated acetylated alditol derivative is reported in parentheses where inositol was used as internal standard and set as 100%. Hydroxylated fatty acids are in *R* configuration.

Monosaccharide composition	Fatty acid composition
3-substituted GlcNp (8.7%)	<i>anteiso</i> -C15:0
6-substituted GlcNp (25.3%)	<i>iso</i> -C15:0
Terminal Fucp (6.1%)	C15:0
3-substituted Galp (12.5%)	C15:0 (3-OH)
6-substituted Glcp (13.3%)	<i>anteiso</i> -C17:0
2,6-substituted Glcp (8.0%)	C17:0
2,3-substituted Rhap (13.1%)	C16:0 (3-OH)
Terminal Glcp (7.0%)	<i>anteiso</i> -C17:0 (3-OH)
Terminal Galf (6%)	<i>iso</i> -C17:0 (3-OH)
	C17:0 (3-OH)

3.3. Structure of *B. eggerthii* 1_2_48FAA fully de-acylated LOS (OS_{deAc})

To elucidate the structure of *B. eggerthii* 1_2_48FAA LOS, an aliquot was fully de-acylated and further purified by gel-filtration chromatography to get the pure saccharide fraction (OS_{deAc}), which then underwent extensive NMR investigation (Figures 3.1 & 3.2A). The anomeric configuration of the monosaccharide units was determined by evaluating $^3J_{H1,H2}$ and $^1J_{C1,H1}$ coupling constants, and further confirmed by *intra*-residue NOE contacts (Table 3.2). The relative configuration of sugar residues was elucidated by analyzing vicinal $^3J_{H1,H2}$ coupling constants and *intra*-residue NOE contacts. ^{31}P NMR experiments were pivotal to establishing the location of the phosphate groups. Nine spin systems were identified (A–K; Figure 3.1 & 3.2A, Table 3.2). Apart from C, all the monosaccharide residues were present as pyranose rings, as indicated by both ^{13}C

chemical shift values and the long-range correlations in the HMBC spectrum between positions 1 and 5 (for Kdo between C-2 and H-6).¹¹

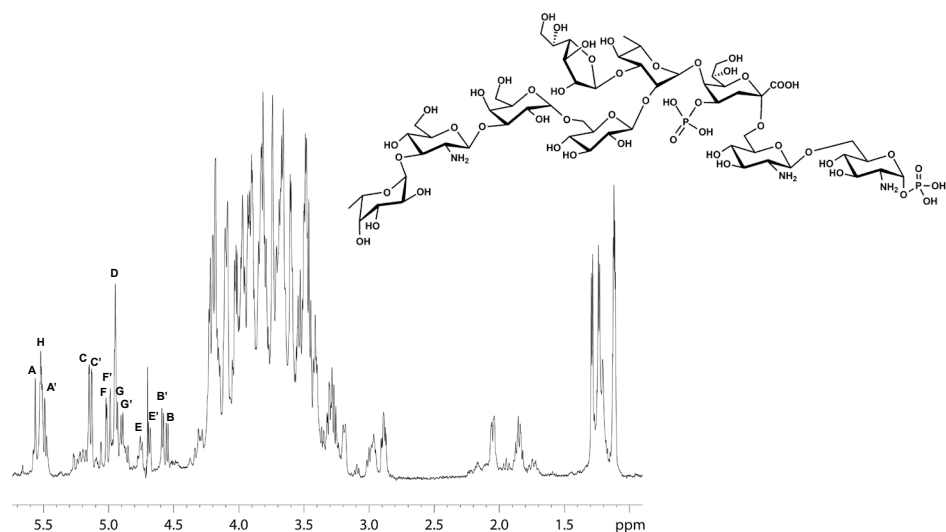


Figure 3.1. ^1H NMR of fully deacylated LOS (OS_{deAc}). Anomeric signals in the inset are as attributed in **Table 2**. Most of the NMR signals of sugar residues are split due to the non-stoichiometric phosphorylation of Kdo. Structure of OS_{deAc} deduced by NMR is also reported in the figure.

Table 3.2. ^1H , ^{13}C and ^{31}P chemical shifts of *B. eggerthii* 1 2 48FAA OS_{deAc}

	1	2	3	4	5	6	7	8
A/A' 2,3- α -Rha	5.56 / 5.48	4.20	4.01	3.49	3.91	1.23		
	99.2 / 99.0	80.4	77.9	71.1	69.3	16.9		
	$^3J_{\text{H1H2}} < 1\text{Hz}$							
B/B' 6- β -Glc	4.54/ 5.48	3.28	3.41	3.48	3.42	3.90 / 3.71 (3.65)		
	104.4/ 103.9	73.2	75.9	69.1	74.7	65.3		
	$^3J_{\text{H1H2}} = 8.2\text{ Hz}$							

C/C' t-β-Galf	5.14 / 5.2	4.09	3.97	4.02	3.73	3.50 / 3.73		
	109.2	81.5	82.0	76.0	70.4	61.5		
	³ J _{H1H2} = 2.4 Hz							
D 3-α-Gal	4.94	3.91	3.98	4.18	4.18	3.66		
	98.0	67.3	79.8	69.0	73.9	60.6		
	³ J _{H1H2} = 3.0 Hz							
E/E' 3-β-GlcN	4.69 / 4.76	3.00 / 2.97	3.62	3.46	3.77	3.84 / 3.70		
	102.43 / 102.0	56.3 / 56.6	83.9	69.7	70.3	60.2		
	³ J _{H1H2} = 7.6 Hz							
F/F' t-α-Fuc	4.98 / 5.01	3.82	3.74	3.74	4.22	1.12		
	101.3 / 101.1	69.4	70.4	71.8	67.2	15.4		
	³ J _{H1H2} = 3.4 Hz							
G/G' 6-β-GlcN	4.90 / 4.94	2.89/ 2.88	3.60	3.47	3.53	3.63 / 3.57		
	100.2 / 99.8	55.6 55.4	74.4	68.3	72.5	62.8		
	³ J _{H1H2} = 8.6 Hz							
H 6-α-GlcNIP	5.53	3.21 / 3.23	3.79	3.31	4.10	4.16 / 3.82		
	90.8	54.5 / 54.4	70.3	70.2	72.8	68.9		

	^{31}P 1.75 ppm; $^3J_{\text{P,H}}$ 4.1 Hz, $^3J_{\text{H1H2}} = 3.6$ Hz							
K 5- α -Kdo (4P)	-----	-----	2.06 / 1.84	4.18	4.18	3.79	3.84	3.99 / 3.85
	181.6	99.8	34.5	67.3	72.4	68.3	68.4	65.6
				^{31}P 1.77				

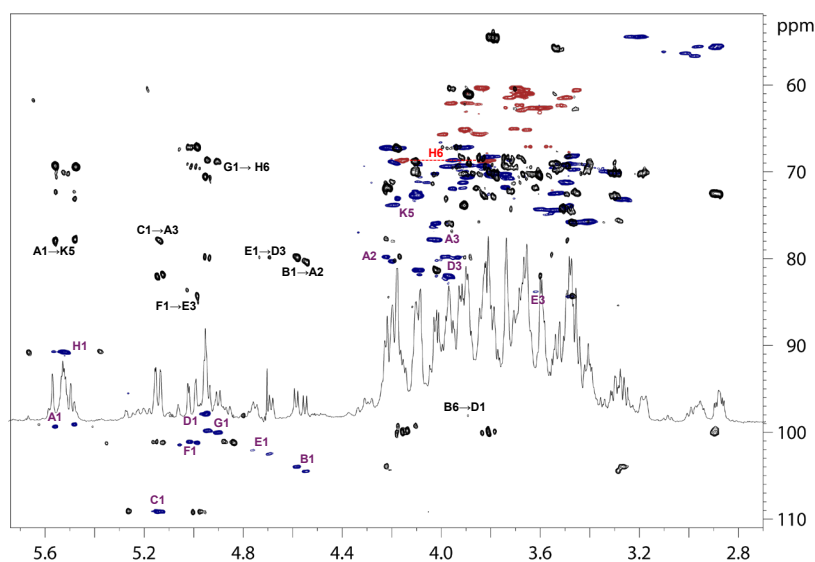


Figure 3.2. NMR structural assessment of **OS_{deAc}** that was obtained upon full de-acylation of an aliquot of purified *B. eggerthii* 1_2_48FAA LOS. Zoom of the HMBC (black) and HSQC (blue and red) and ^1H NMR spectra of the **OS_{deAc}** have been reported here. The key *inter-residue* long-range correlations involving sugar moieties (**A-K**) are indicated; letters are as in **Table 3.2**.

By contrast, residue C existed as a furanose ring, as evidenced by the low-field shifted carbon signals (**Table 3.2**) and corroborated by the intra-residue long-range correlation between positions 1 and 4. The non-stoichiometric

phosphorylation of Kdo splits most of the NMR signals of **OS**_{deAc} sugar residues (see **Table 3.2**); we here discuss the main species.

Spin systems **E**, **G**, **H** and **B** were all *gluco* configured sugar residues, based on the large $^3J_{\text{H,H}}$ ring coupling constants (8-10 Hz). Furthermore, **G**, **H** and **E** were identified as GlcpN residues based on the correlation of their H-2 resonance to nitrogen bearing carbon signals (**Figure 3.2A** and **Table 3.2**). The *intra*-residue NOE contact of H-1 with H-3 and H-5, $^3J_{\text{H1,H2}}$ and $^1J_{\text{CH}}$ values were indicative of β -anomeric configuration of **B**, **E** and **G**. Conversely **H** was identified as an α -Glc, whose anomeric configuration was determined according to $^3J_{\text{H1,H2}}$ and $^1J_{\text{CH}}$ values and the sole H1-H2 *intra*-residual NOE contact. Spin systems **D** and **F** were assigned to *galacto*-configured sugar units as observed in the $^3J_{\text{H3,H4}}$ and $^3J_{\text{H4,H5}}$ values (3 Hz and <1 Hz, respectively). Both $^3J_{\text{H1,H2}}$ and $^1J_{\text{CH}}$ coupling constants, together with *intra*-residual NOE contact of H-1 with H-2, were diagnostic for their α -configurations. Spin system **D** was identified as α -Gal, while the scalar correlations of ring proton signals with a methyl group at position 6 allowed to identify residue **F** as an α -Fuc. In contrast, spin system **C** was identified as a β -galactofuranose unit (β -Galf), according to compositional analysis, based on its chemical shifts, *intra* residue scalar correlations and the NOE contacts H1-H3 and H2-H4. Spin system **A** matched with an α -Rha, as attested by the scalar correlations of ring protons from H-2 till the methyl signal at position 6, visible in the TOCSY spectrum. The *manno* configuration was established by $^3J_{\text{H1,H2}}$ and $^3J_{\text{H2,H3}}$, both below 2 Hz and diagnostic of H-2 equatorial orientation; the α -anomeric configuration was assigned by the $^1J_{\text{H1,C1}}$ coupling constant. Spin system **K** was assigned to the Kdo residue based on the diastereotopic methylene proton signals resonating in the high-field region of the NMR spectrum; its α -anomeric configuration was assigned on the basis of the H-3 chemical shift values and the $^3J_{\text{H-7,H8a}}$ and $^3J_{\text{H-7,H-8b}}$ coupling constants (**Table 3.2**).¹¹⁻¹³ ^{31}P NMR spectra highlighted the presence of two phosphate groups, located on the reducing GlcN

of the lipid A backbone **H** and on O4 of the Kdo sugar unit **K**. The down-field shifted carbon signals were indicative of glycosylation at position O-6 of **B**, **G** and **H**, O-5 and O-2 of **K**, O-2 and O-3 of **A**, O-3 of **E**, whereas **C** and **F** were terminal sugar residues, in full accordance with the methylation data. The analysis of the *inter*-residual NOE contacts and the long-range correlations in the HMBC spectrum (**Figure 3.2**) allowed the definition of the complete sugar sequence of the LOS from *B. eggerthii* 1_2_48FAA. Residues β -(1 $\rightarrow$ 6) linked **G** and **H** composed the lipid A saccharide backbone (**Figure 3.2**). Unit **G** in turn was substituted at position O-6 by Kdo residue (**K**), as further confirmed by the weak downfield shift of signal of C-6 of residue **G** (δ 62.8 ppm, **Table 3.2**), which is expected for the α -(2 $\rightarrow$ 6) ketosidic linkage of Kdo with the β -GlcN of the lipid A.¹⁴ Kdo unit was, in turn, substituted at O-5 by the rhamnose residue **A**, as suggested by the corresponding *inter*-residue NOE contacts, together with the HMBC correlations **A1-K5** (**Figure 3.2**). Similarly, the following connections were identified between the sugar units: **C1-A3**, **B1-A2**, **D1-B6**, **E1-D3**, **F1-E3** (**Table 3.2**, **Figure 3.2**). The above information, in full agreement with the compositional analyses, allowed to determine the structure of *B. eggerthii* 1_2_48FAA OS_{deAc} as sketched in **Figure 3.3**.

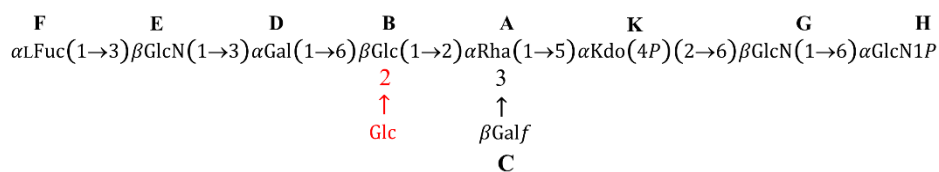


Figure 3.3. Structure of the OS_{deAc} from *B. eggerthii* 1_2_48FAA derived from the combination of chemical, NMR and MS investigations. In red the portion identified by merging data from chemical analyses and MS investigation.

This structural deduction was also confirmed and further integrated by a negative-ion MALDI-TOF MS analysis of OS_{deAc} (**Figure 3.4**), which detected a species at m/z 1640.9 ($[M - H_2O]^+$) matching with a core oligosaccharide built of

three HexN, two dHex, three Hex, one Kdo and two phosphate groups. Interestingly, it was possible to identify a second oligosaccharide species at m/z 1802.9 that carries an additional Hex unit, whose location was established by negative-ion MS/MS analysis (**Figure 3.4**). Indeed, the concomitant presence of several ion peaks among which those at m/z 503.3 ($Y_{6\alpha}C_{4\alpha}$) and m/z 931.3 ($Y_{5\alpha}B_6$) guided the positioning of this unit on the glucopyranose residue **B**, which was in line with the detection of 2,6-Glc in compositional analyses (**Table 3.1**). In addition, MS analysis of **OS**_{deAc} pointed out a certain degree of heterogeneity in terms of phosphorylation pattern, as other species carrying three phosphates, or two phosphates and one aminoethyl (EtN) group, or three phosphates and one EtN group were also detected (**Figure 3.4**). The third phosphate as well as the EtN group all belonged to the lipid A backbone as proven by the MALDI-TOF MS inspection carried out on the lipid A (see below).

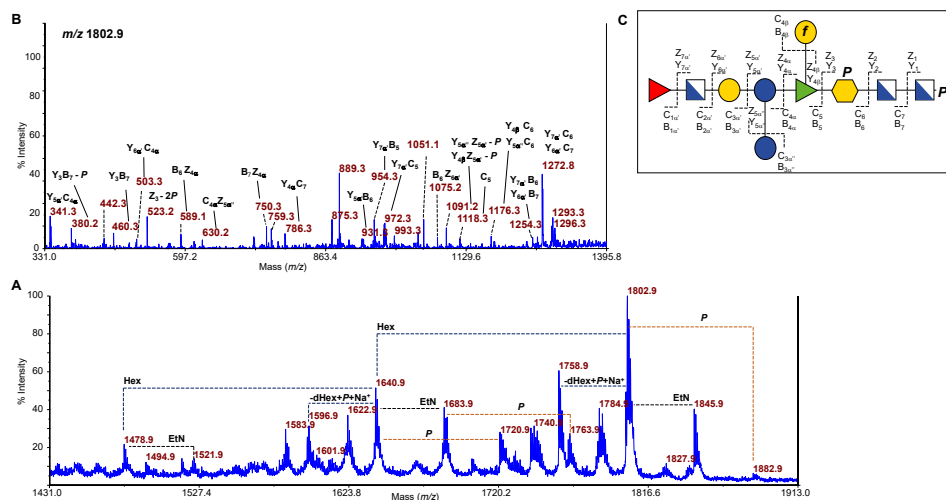


Figure 3.4. MS structural assessment of **OS**_{deAc}. (A) Negative-ion MALDI-TOF MS spectrum of **OS**_{deAc} recorded in reflectron mode. (B) Negative-ion MS/MS spectrum of the species at m/z 1802.9; fragmentations generated from glycosidic bond cleavage that were key for structural determination were indicated in the spectrum. (C) The proposed structure for the oligosaccharide species detected at m/z 1802.9 was drawn using the Symbol Nomenclature for Glycans (SNFG). The nomenclature of glycan fragment ions generated from the cleavage of a glycosidic bond used for the elucidated **OS**_{deAc} structure

is also reported. Symbols legend: red triangle: fucose; green triangle: rhamnose; blue circle: glucose; yellow circle: galactose; yellow circle with “f”: galactofuranose; blue/white square: glucosamine; yellow hexagon: Kdo; *P* stands for phosphate

3.4. Structure of *B. eggerthii* 1_2_48FAA core oligosaccharide (OS) and lipid A moieties

The LOS underwent mild acid hydrolysis that exploited the lability of the glycosidic linkage of Kdo and therefore split the lipid A and the core oligosaccharide (OS) (**Figure 3.5A**). NMR analysis of OS fully confirmed the above data, as well as the *N*-acetylation on the aminosugar residue **E**. In order to get a 3D model of *B. eggerthii* OS, the disaccharides constituting the glycan chain were built and the energetically accessible conformational regions were evaluated using standard protocols (see experimental section). The corresponding adiabatic energy maps for the glycosidic torsion angles (φ (H1-C1-O-CX'), ψ (C1-O-CX'-HX') and for 6-linked sugar units ω (O6'-C6'-C5'-O5')) were used to build the core OS and to run MD simulation in explicit solvent. Results showed that OS adopted a compact conformation and that, according to the multiplicity shown in NMR spectra (**Figure 3.5B**, zoom of the HSQC spectrum and extracted row corresponding to B6, proof of a *gg* conformation), the ω torsion angle of the Gal-Glc glycosidic linkage stably adopted the *gg* conformation, corresponding to the value of -60° (**Figure 3.5B**).

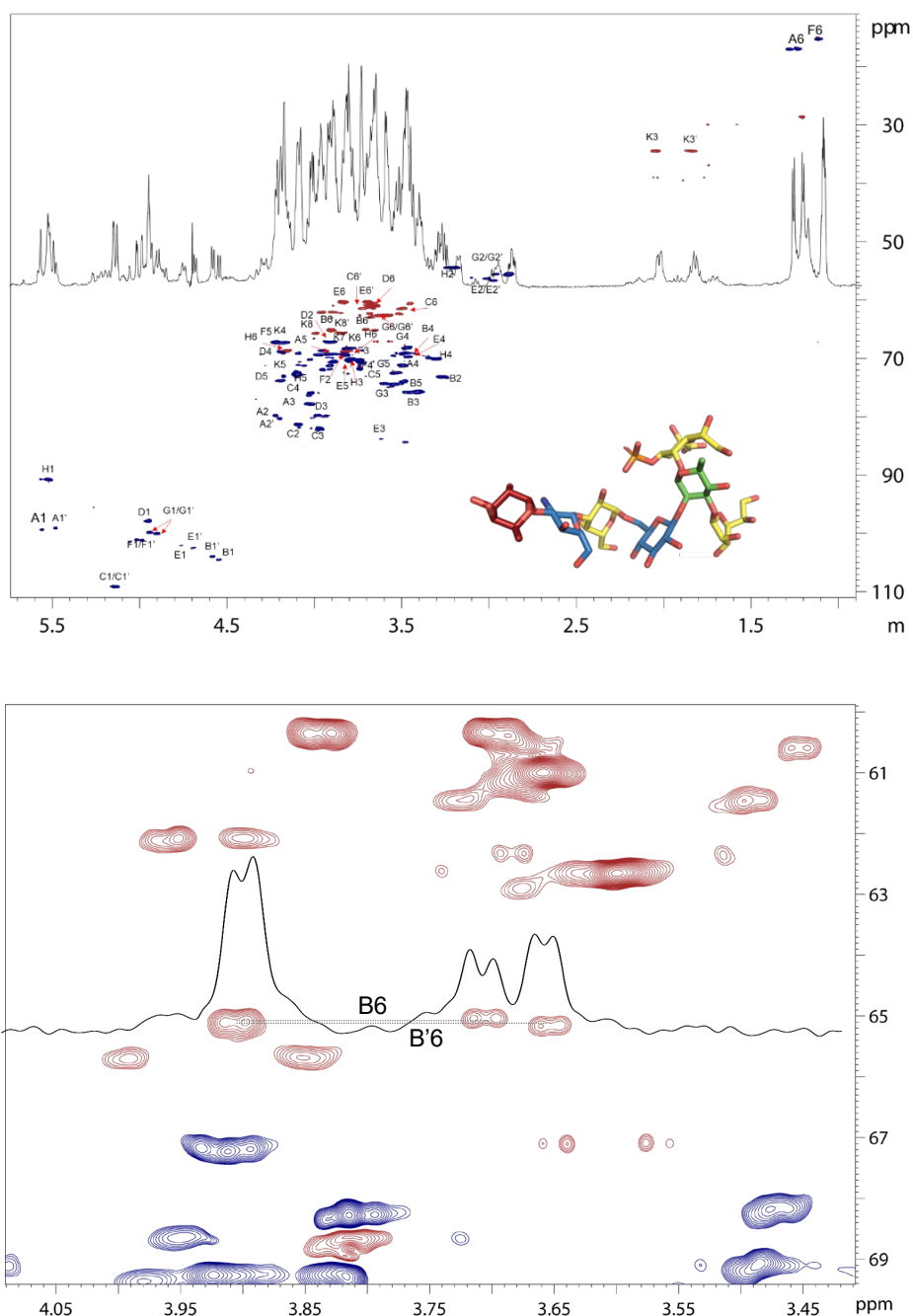


Figure 3.5. (A) NMR structural assessment of OS that was obtained after acid treatment of an aliquot of purified LOS. Multiplicity edited HSQC (blue and red) NMR spectrum,

assigned as attributed with the letters from the table 2, has been reported in (A), along with the structure of OS and a representative conformer. **(B)** Multiplicity edited HSQC (blue and red) NMR spectrum of **OS_{deAc}** from *B. eggerthii* 1_2_48FAA and zoom of the HSQC spectrum and extracted row corresponding to B6, proof of a *gg* conformation. A representative conformer of *B. eggerthii* 1_2_48FAA **OS**, obtained via computational studies, is shown.

The lipid A underwent compositional and MS investigations. The negative-ion MALDI-TOF MS spectrum showed a notable heterogeneity of lipid A species, with two main clusters of signals differing in the number of fatty acid chains. Each group of ions exhibited mass differences indicating variation in the fatty acid chain length within the lipid A species. In detail, the most intense cluster of ions in the mass range m/z 1600-1717 was consistent with *mono*-phosphorylated penta-acylated lipid A species whose fatty acid distribution and composition, as instance for ion peak at m/z 1688.1, was determined as two C17:(3-OH) as *N*-linked acyl chains, two C16:(3-OH) as primary *O*-linked fatty acids and C15:0 as a secondary *O*-acyl substitution, in accordance to fatty acid compositional analysis (**Table 1**) and literature data.⁵ The cluster of ions visible in the mass range m/z 1364-1463 was attributed to *mono*-phosphorylated tetra-acylated lipid A species with the main ion peak at m/z 1433.9 assigned to a lipid A species possessing the above-mentioned fatty acid distribution and composition but lacking one primary *O*-linked C16:(3-OH). Intriguingly, a very minor family of peaks in the mass range m/z 1754.0-1853.1 was also identified and assigned to penta-acylated lipid A species carrying two phosphates (m/z 1754.0-1796.0) and two phosphates and one EtN group. Finally, an aliquot of intact LOS was analyzed by negative-ion MALDI-TOF MS. The MS spectrum showed at higher molecular masses (m/z 2300-3300) a series of $[M - H]^-$ peaks attributed to the native LOS mixture, whose heterogeneity was immediately apparent. In fact, despite the low abundance due to the lability of the Kdo linkage, several LOS species were identified that confirmed the NMR and

MALDI MS structural determination, i.e. the occurrence of oligosaccharide species carrying three or four hexoses as well as heterogenous lipid A species which can be further decorated by an additional phosphate and by an EtN moiety (**Figure 3.6**).

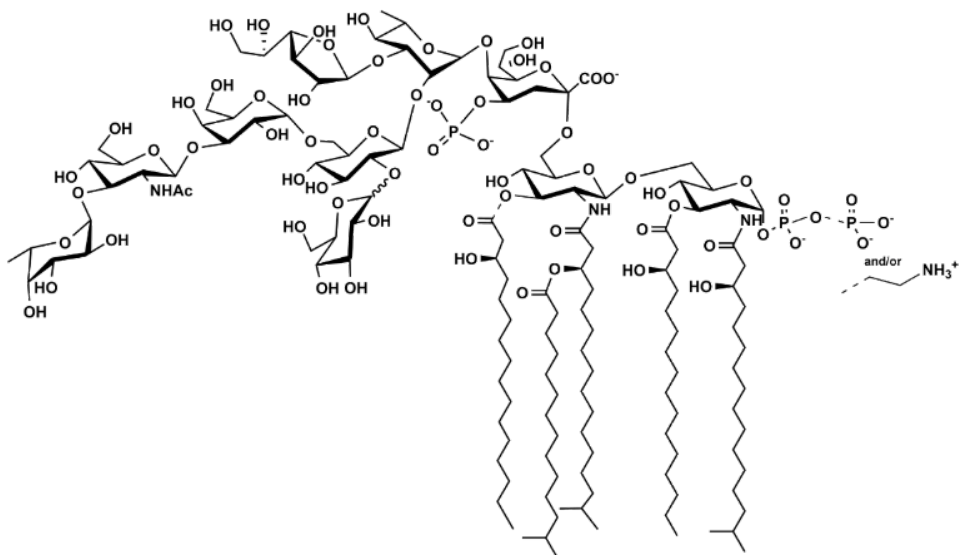


Figure 3.6. Full structure of the LOS from *B. eggerthii* 1_2_48FAA. The location of the EtN and of the second phosphate on the lipid A is tentative. Branched fatty acids are depicted in their *iso*-form, however they can also be found as linear or *anteiso* chains.

3.5. TLR4 and TLR2 activation by *B. eggerthii* 1_2_48FAA LOS and moDC maturation

To assess the immunological properties of *B. eggerthii* 1_2_48FAA LOS, different concentrations of purified LOS were tested in the HEK-Blue™ cells transfected with human TLR4, MD-2, and CD14 genes or HEK-Blue™ hTLR2 cells. *B. eggerthii* LOS exhibited a significantly weaker capability to activate TLR4 signaling compared to *E. coli* LPS used as a positive control (10 and 100 ng, *** $p < 0.001$) (**Figure 3.7A**). In parallel, no significant activation compared to untreated cells was observed in HEK-Blue™ hTLR2, indicating that *B. eggerthii* LOS is unable to activate TLR2 signaling (**Figure 3.7B**).

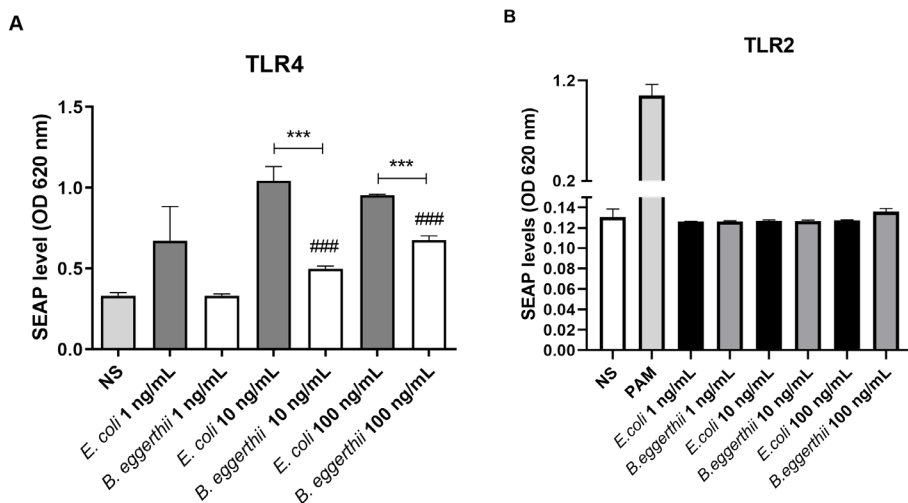


Figure 3.7. Analysis of TLR activation by *B. eggerthii* 1_2_48FAA LOS. **(A)** Stimulation of HEK Blue™ hTLR4 and **(B)** HEK Blue™ hTLR2 cells. Secreted embryonic alkaline phosphatase (SEAP) levels (OD) upon stimulation with LPS from *E. coli* and *B. eggerthii* 1_2_48FAA LOS at the indicated concentrations (1, 10, 100 ng/mL). In **(A)** Ultrapure LPS from *E. coli* 0111:B4, a potent TLR4 agonist, was used as a positive control, whereas in **(B)** it was used as a negative control, due its known inability to activate the TLR2 signaling. The positive control in **(B)** was Pam3CSK4 (500 ng/mL), a synthetic triacylated lipopeptide agonist for TLR2 signaling. **(A)** Significant difference between *B. eggerthii* 1_2_48FAA LOS generated values and the corresponding *E. coli* LPS (*B. eggerthii* vs. *E. coli*, *** $p < 0.001$) or untreated cells (*B. eggerthii* vs. untreated cells (NS), ### $p < 0.001$) values by the Student *t*-test are indicated. Data are expressed as mean $\pm$ SD of three independent experiments.

To further explore the immunological potential of *B. eggerthii* LOS, we evaluated its ability to induce maturation of monocyte derived dendritic cells (moDCs) by flow cytometry. Interestingly, *B. eggerthii* LOS stimulation showed significantly reduced expression of maturation markers CD40, CD86, and CD80 on monocyte derived dendritic cells (moDCs) (**** $p < 0.0001$) compared to *E. coli* LPS (10 ng/mL) (**Figure 3.8**). These findings revealed that *B. eggerthii* 1_2_48FAA does elicit a dose-response effect, but its ability to induce moDC maturation is consistently weaker than the LPS from *E. coli*. This indicates a distinctly less potent immunostimulatory profile for *B. eggerthii* LOS.

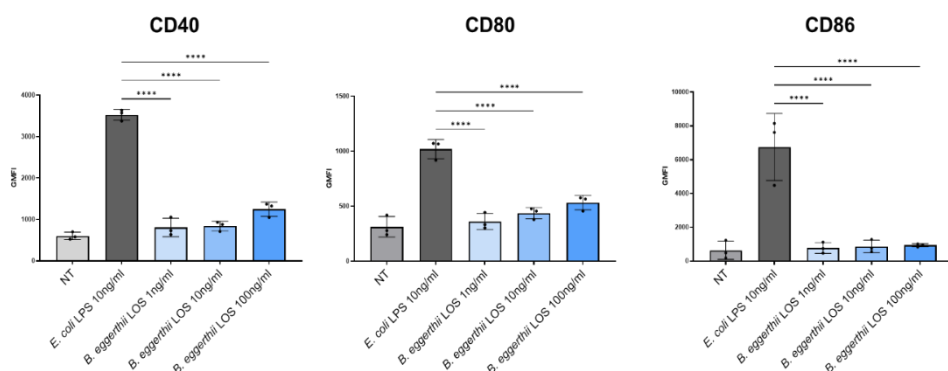


Figure 3.8. Analysis of moDC cell surface maker expression. The expression of cell surface markers CD40, CD80 and CD86 upon *B. eggerthii* LOS (1, 10, 100 ng/mL) and *E. coli* LPS (10 ng/mL) stimulation of moDCs was quantified by flow cytometry. Data is presented as geometric Mean Fluorescence Intensity (GMFI) and represents the results of three independent donors (*B. eggerthii* LOS vs. *E. coli* LPS) (**** $p < 0.0001$).

3.6. Discussion and conclusions

The increasing attention given to the role of LPS in mediating interactions between the host and the gut microbiota underscores the link between its intricate structure and immune potential.¹⁵ Here, we provided a comprehensive characterization of a novel LOS structure derived from *B. eggerthii* 1_2_48FAA. We showed that the lipid A consisted of a heterogeneous mixture of tetra- and penta-acylated species with a heterogeneous phosphorylation pattern. Therefore, *B. eggerthii* 1_2_48FAA lipid A shares features with other *Bacteroides* sp., such as *B. thetaiotaomicron* strain VPI 5482, *B. vulgatus* mpk, *B. fragilis*, and *B. gingivalis* in terms of the number, distribution and composition of the fatty acids.^{4,5,10,16} However, two phosphates and an EtN moiety were observed as additional substituents in minor species of *B. eggerthii* lipid A, in contrast to previously characterized *Bacteroides*-derived lipid A, which showed a sugar backbone decorated by only one phosphate group.^{16–19} The second phosphate and the EtN group might be speculated to be both on the reducing GlcN unit, as previously observed for lipid A of other Bacteroidetes.^{20,21}

The saccharide portion comprised a core region consisting of an octasaccharide with a phosphorylated Kdo unit, substituted at O-5 by a branched α -Rhap unit that carries a β -Gal f and a β -Glc p . This structure partially resembles that of *B. vulgatus* mpk, where connected to the Kdo is a branched α -Rhap. However, *B. eggerthii* 1_2_48FAA core OS carries a β -Glc p unit at position O-2 while in *B. vulgatus* mpk the β -Glc p carries a β -Gal p at O-4.⁵ Both *B. eggerthii* 1_2_48FAA and *B. vulgatus* mpk carry a β -Gal f at O-3 of the α -Rhap. In *B. eggerthii* 1_2_48FAA, the β -Glc p was further di-substituted at O-2 by a Glc residue and at O-6 by an α -Gal p in turn substituted at O-3 by a β -Glc p NAc, that carries at O-3 an α -L-Fuc p . Notably, no aminosugar residues have been reported in *B. vulgatus* mpk⁵ while Glc p NAc composes the core region of *B. thetaiotaomicron* VPI 5482.⁴

Gal f residues have been reported in the glycans of various human pathogens, such as the respiratory pathogens *Mycobacterium tuberculosis* and *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Vibrio cholerae* and *Helicobacter pylori*, as well as the pathogenic fungus *Aspergillus fumigatus*^{22,23} and in protozoan parasites such as *Trypanosoma cruzi* and *Leishmania*.²⁴ By contrast, Gal f is absent in mammals and its presence can trigger the immune recognition in humans, making them potential targets for drug development.^{22,23} The human intelectin-1 (ITLN-1), a host defense lectin secreted by intestinal epithelial cells and upregulated in some diseases, targets exocyclic 1,2-diols and therefore also Gal f residues present on microbial surface. As a result of this interaction, phagocytic clearance of microbes through their sequestration is fostered and targeting to neutrophils is enhanced.²⁵⁻²⁷ However, the presence of Gal f in the core region of *B. eggerthii* 1_2_48FAA and of the other gut species, such as *B. vulgatus* mpk⁵ and *Veillonella parvula*,²⁸ raises important questions about its role and

impact in the crosstalk between the gut microbiota and the host immune system, which requires further investigation. Likewise, the presence of fucose in the inner part of *B. eggerthii* 1_2_48FAA core OS deserves a few more words. Indeed, binding studies executed on *ad hoc* synthesized *B. vulgatus* mpk LPS derivatives,²⁹ revealed that fucose and the directly linked Gal residues, as well as Gal β and Kdo, are involved in LPS core recognition by DC-SIGN.²⁹ This is a C-type lectin known for mediating host immune recognition of bacterial envelope glycans where it preferentially binds to fucosylated glycans and mannose-exposing ligands but exerting an opposite ligand-based immunological outcome: an anti-inflammatory response when it binds to fucose-expressing ligands and a pro-inflammatory response when it encounters mannose-expressing ones.³⁰ As a matter of fact, fucose residue was shown to be crucial in the interaction with *B. vulgatus* mpk LPS, being located in the protein binding site where it acts as anchor to the DC-SIGN protein.²⁹ It is worth underlining that it was proposed a complex binding epitope for DC-SIGN that involved both the core and the O-antigen portion of *B. vulgatus* mpk LPS, i.e. the terminal α -Fucose located in the inner core region and a terminal β -mannose linked to an internal L-configured Rhamnose of the O-antigen.²⁹ Overall, in *B. vulgatus* mpk LPS the presence of both the typical ligands of DC-SIGN, i.e. mannose and fucose, led to hypothesizing that this peculiar LPS contributes to the establishment of gut immune homeostasis by interacting with DC-SIGN and hence assuring a balanced host response.²⁹ Here, the lack of an O-antigen and/or of a mannose residue in *B. eggerthii* 1_2_48FAA LOS certainly boosts future research on the mechanism of binding between DC-SIGN and this novel LOS structure and the role exerted by its sugar moieties.

In this study, we also showed that *B. eggerthii* 1_2_48FAA LOS was a poor activator of TLR4-mediated signaling, which was in accordance with the hypo-acylated nature of its lipid A component, i.e. it possesses only four or five acyl chains and not six as other immunostimulatory LPS.¹⁵ Of note, the presence of an additional phosphate and/or of an EtN had no effect on the TLR4 activation, likely due to their presence only in very minor lipid A species. More interestingly, *B. eggerthii* 1_2_48FAA LOS displayed a reduced capacity to induce co-stimulatory surface markers like CD40, CD80 and CD86, which typically indicate DC and other antigen-presenting cells maturation. Maturation of DCs, which is key for efficient T-cell stimulation, involves a series of functional and phenotypic changes that enable them to efficiently trigger immune cascades and therefore cytokine production.^{30,31} The lack of a significant maturation of DCs upon stimulation with *B. eggerthii* 1_2_48FAA LOS tempting suggests that it may be a strategy adopted by the bacterium to evading/dampening the host immune system, which however points to the importance of analyzing the functional properties of DCs at the single cell level. Therefore, *B. eggerthii* 1_2_48FAA LOS, like other LPS from *Bacteroides* ssp., appears to have the capacity to dampen TLR4 activation and to only poorly activate an immune response.^{4,5,32,33} An understanding at the molecular level of this impaired TLR4 activation and the possible crosstalk/involvement of other immune receptors, such as lectins, is of unquestionable importance.

In conclusion, the discussed results contributed to the investigation of *B. eggerthii* LPS and its interactions with the host. In addition, it sheds light on the role of LPS, not as a toxin, but as a potential glycoconjugate that helps modulate the immune response. Therefore we could gain information on structural motifs of beneficial and harmless gut bacteria, in order to understand their role in the immunomodulation of our immune system.

3.7. References

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CHAPTER IV

The lipooligosaccharide of the intestinal symbiont *Akkermansia muciniphila* exhibits a remarkable structure and TLR signaling capacity

4.1. Introduction

The mucosal symbiont *Akkermansia muciniphila*^{1,2} has a Gram-negative architecture and was found to play an important role in maintaining this intestinal barrier function. It has developed into one of the few intestinal microbes with evidence of generating a beneficial host response in mice and humans.³ Administration of *A. muciniphila* cells increases barrier function, decreases serum LPS levels, and protect from weight gain and glucose intolerance in diet-induced obese mice.⁴ This was confirmed in various other studies that also revealed increased immune signalling by *A. muciniphila* via the expansion of regulatory T cells (Tregs).^{3,5,6}

Pasteurized *A. muciniphila* cells were found to be as active, or even more, as live cells in mouse models. This was attributed to the signalling of a heat-stable outer membrane protein, Amuc_1100 that is thought to signal through the TLR2 receptor.^{3,7,8} These results were partly replicated in a human study with subjects at risk for type 2 diabetes, in which pasteurized and live *A. muciniphila* cells increased gut barrier function and decreased serum LPS levels.⁹ However, *A. muciniphila*, as a Gram-negative bacterium belonging to the *Verrucomicrobia*, also produces heat-stable LPSs.¹⁰ This generates a paradox since a beneficial symbiont harbours a potentially inflammatory component. This provided the basis for the present study that shows the *A. muciniphila* strain Muc^T to produce a lipooligosaccharide (LOS), i.e., an LPS devoid of the O-antigen polysaccharide. The LOS consists of a complex glycan chain bearing two distinct undeca- and hexadecasaccharide units each containing three 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) residues. The lipid A moiety appears as a mixture of differently phosphorylated and acylated species and carries either linear or branched acyl moieties. Peritoneal injection of the LOS in mice increased higher gene expression of liver TLR2 than TLR4 (100-fold) and induced high IL-10 gene expression. *A. muciniphila* LOS was found to signal both through TLR4 and

TLR2, whereas lipid A only induced TLR2 in a human cell line. We propose that the unique structure of the *A. muciniphila* LOS allows interaction with TLR2, thus generating an anti-inflammatory response as to compensate for the canonical inflammatory signalling associated with LOS and TLR4, rationalizing its beneficial host interaction.

4.2. The chemical structure of the *A. muciniphila* LOS carbohydrate domain reveals an unprecedented complexity

To determine the full structure of *A. muciniphila* Muc^T LOS, including its carbohydrate (core oligosaccharide) and glycolipid (lipid A) moieties, a combination of organic chemistry, high-resolution mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy was employed. LOS was isolated, purified and fully de-lipidated to obtain the core oligosaccharide **OS** (composed of **OS**_{1deAc} and **OS**_{2deAc}) covalently connected to lipid A sugar skeleton (**Figure 4.1**). Due to its high complexity, the NMR assignment of core **OS** required the use of an ultra-high magnetic field at 1.2 GHz, which allowed to attribute all spin systems and to establish the saccharide sequence (**Figure 4.1**). NMR spectra unveiled the occurrence of 26 sugar units (including the lipid A sugar backbone), all present as pyranose rings. Their glycosylated positions were identified by downfield shift of carbon resonances; *inter*-residual NOE contacts, together with the long-range correlations derived from the HMBC spectrum, allowed to determine the **OS** structure as sketched in **Figure 4.1**. The sugar units were distributed along two glycan chains, the longest composed of a hexadecasaccharide, the shortest of an undecasaccharide chain (**OS**_{1deAc} and **OS**_{2deAc}, and **Figures 4.1 and 4.2**), both starting with a Kdo (2-keto-3-deoxy-D-*manno*-octulosonic acid) disaccharide unit, in turn connected to an additional, highly branched Kdo residue carrying the whole glycan chain. To further elucidate and corroborate the structure of *A.*

muciniphila OS, an in-depth structural characterization was performed by ultrahigh-resolution ESI FT-ICR MS and collision-induced dissociation (CID) (**Figure 4.2**). The OS MS showed a high heterogeneity resulting from the presence of both the undeca- and hexadecasaccharides and their variants generated from incomplete deacylation/deacetylation and/or in-source fragmentation. The two oligosaccharides were detected at m/z 817.9286 and m/z 812.5114 and with a mass measurement error of 0.37 and 0.12 ppm, respectively (OS1_{deAc} and OS2_{deAc}, **Figure 4.2**). CID fragmentation analysis on selected precursor ions confirmed the final resulting structures, with the presence of three Kdo residues per oligosaccharide and a specific fucose acetylation. In parallel, a mild acid hydrolysis on the LOS allowed to split the lipid A from the core portion; the resulting mixture was further purified and, additionally to the lipid A, two isolated oligosaccharides were indeed identified by NMR as the expected tetradecasaccharide and nonasaccharide (OS₁ and OS₂), thus fully confirming the structural assignment. The structure of *A. muciniphila* lipid A was defined by merging data from fatty acid compositional analysis, MALDI-TOF MS and MS/MS investigation (**Figure 4.2**). A heterogenous blend of lipid A species was identified, differing in acyl chain length and phosphorylation pattern. Three main families of peaks were identified as tetra-, penta- and hexa-acylated species with representative peaks at m/z 1922.7, 1670.6, and 1430.5 respectively, in approximately comparable area similarly to the corresponding *mono*-phosphorylated species. The main peak at m/z 1922.7 matched with a *bis*-phosphorylated lipid A carrying *iso* (i) C15:0 (3-OH) as primary directly *O*- and *N*-linked fatty acids, and *i*-C14:0 and C17:0 (or *i*-C17:0 or *a*-C17:0) as secondary acyl substituents. The peak at m/z 1670.6 was assigned to a *bis*-phosphorylated penta-acylated lipid A species lacking the C17:0 unit, whereas the *bis*-phosphorylated tetra-acylated lipid A species at m/z 1430.5 was devoid of both C17:0 and the primary *O*-linked *i*-15:0 (3-OH) decorating the non-reducing

glucosamine unit (**Figure 4.2**). In summary, *A. muciniphila* lipid A is made up of a complex blend of *mono*- and *bis*-phosphorylated, tetra- to hexa-acylated lipid A species, whose forms displaying the highest acylation degree has a 4 + 2 symmetry of the acyl chains with the respect to the glucosamine backbone (**Figure 4.2**).

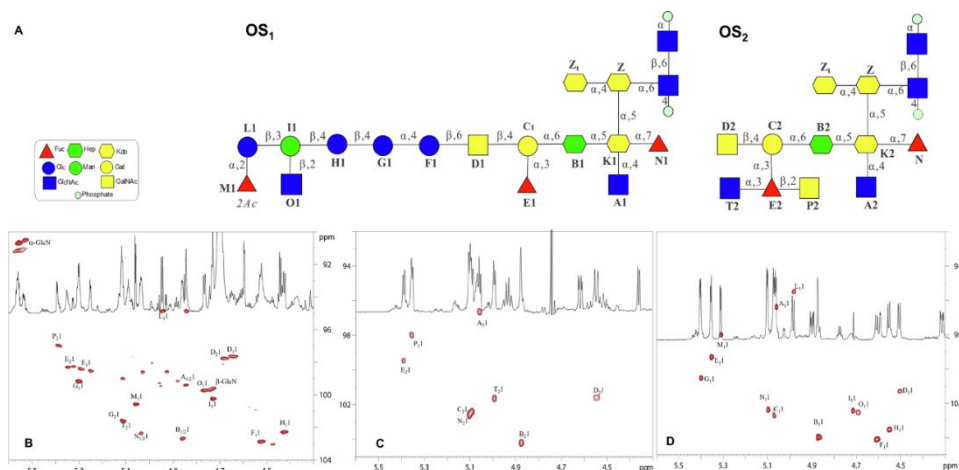


Figure 4.1. (A) The glycan components of the LOS from *A. muciniphila* sketched according to the symbology of monosaccharide residues (in the inset); the structure of the core region has been assigned by HR MS and NMR analyses of the acetic acid and full deacylated products; (B), (C), (D) multiplicity edited HSQC NMR spectra of (from left to right) *A. muciniphila* OS (full deacylated LOS, 1.2 GHz), OS₁ and OS₂ (from acetic acid hydrolysis, at 950 MHz): zoom of the anomeric region.

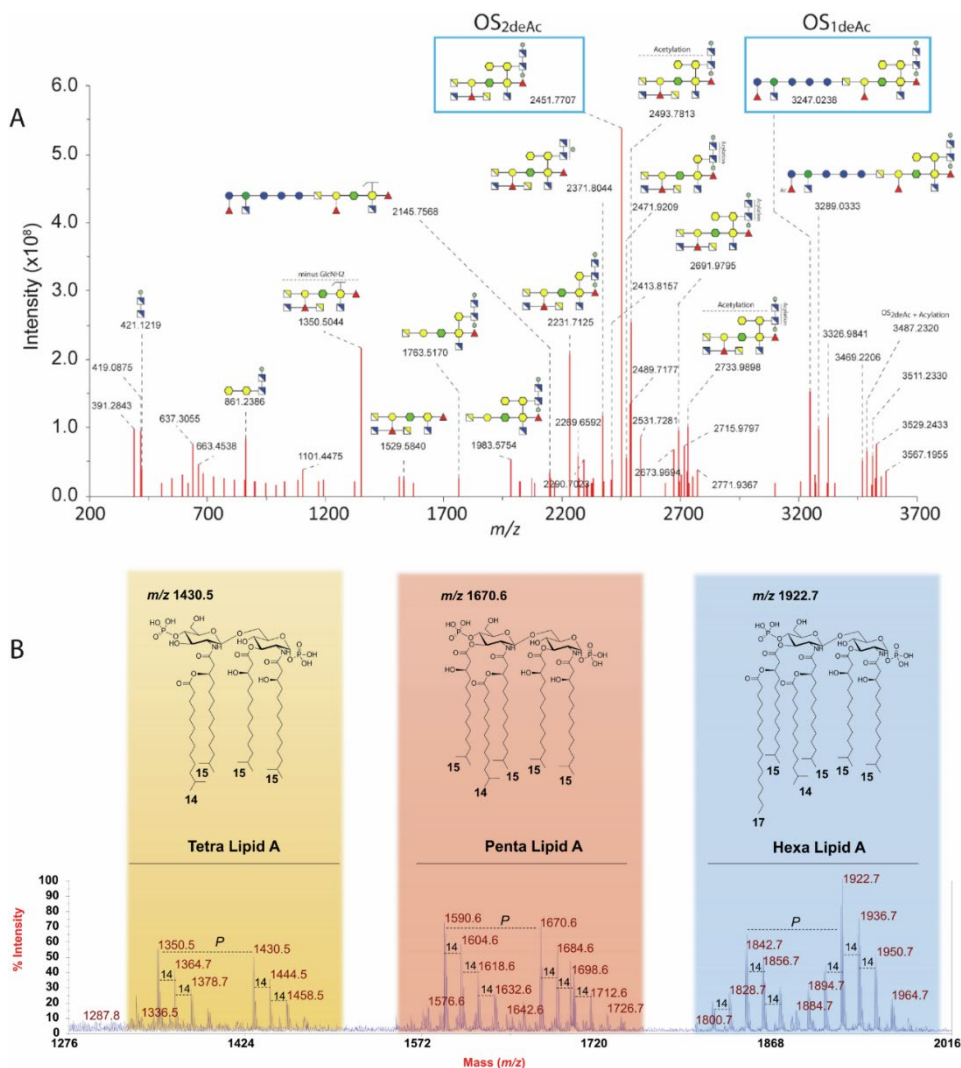


Figure 4.2. (A) HR and sub-ppm mass measurement of Nona- and Tetradeca-oligosaccharides from the deacylated LOS performed by ESI-FT-ICR MS (mass spectrum deconvoluted to $[M + H]^+$). (B) *A. muciniphila* lipid A. Negative ion MALDI-TOF mass spectrum of the lipid A from *A. muciniphila*. The families of lipid A species that differ in the degree of acetylation are indicated as “Hexa”, “Penta”, and “Tetra” Lipid A (P: phosphate group). The structure of the main bis-phosphorylated lipid A species for each family (Tetra, Penta and Hexa) has been sketched in each colored panel. The C17:0 moiety has been depicted in its linear form for description purposes only.

4.3. Injection of *A. muciniphila* LOS in mice triggers TLR2 mRNA and interleukin 10 expression

To examine its functional importance, the purified LOS of *A. muciniphila* and a vehicle (buffer) were injected intraperitoneally in two groups of mice ($n = 7-8$) which were sacrificed 4 h later (**Figure 4.3A**). LOS injection showed no effect on body weight or weight of organs, such as liver, cecum or cecal content, although a small increase in spleen weight was reported compared to the vehicle injection, possibly indicating an effect on the immune system. Subsequently, liver samples were analyzed for expression of genes involved in immune signaling in response to the *A. muciniphila* LOS injection as compared to that of the vehicle control (**Figure 4.3B**). The expression of several genes involved in the attenuation of inflammation was increased, including LPS binding protein (LBP), the MCP1 complement regulatory protein and interleukins IL1 β (mediator of inflammatory response) and IL10 (anti-inflammatory cytokine). As expected, the LOS triggered the expression of TLR4 mRNA, the canonical receptor of all bacterial LOS and LPS. However, most noteworthy was the very strong (over 100-fold) surge in relative TLR2 mRNA expression, suggesting that *A. muciniphila* LOS also can interact via this receptor.

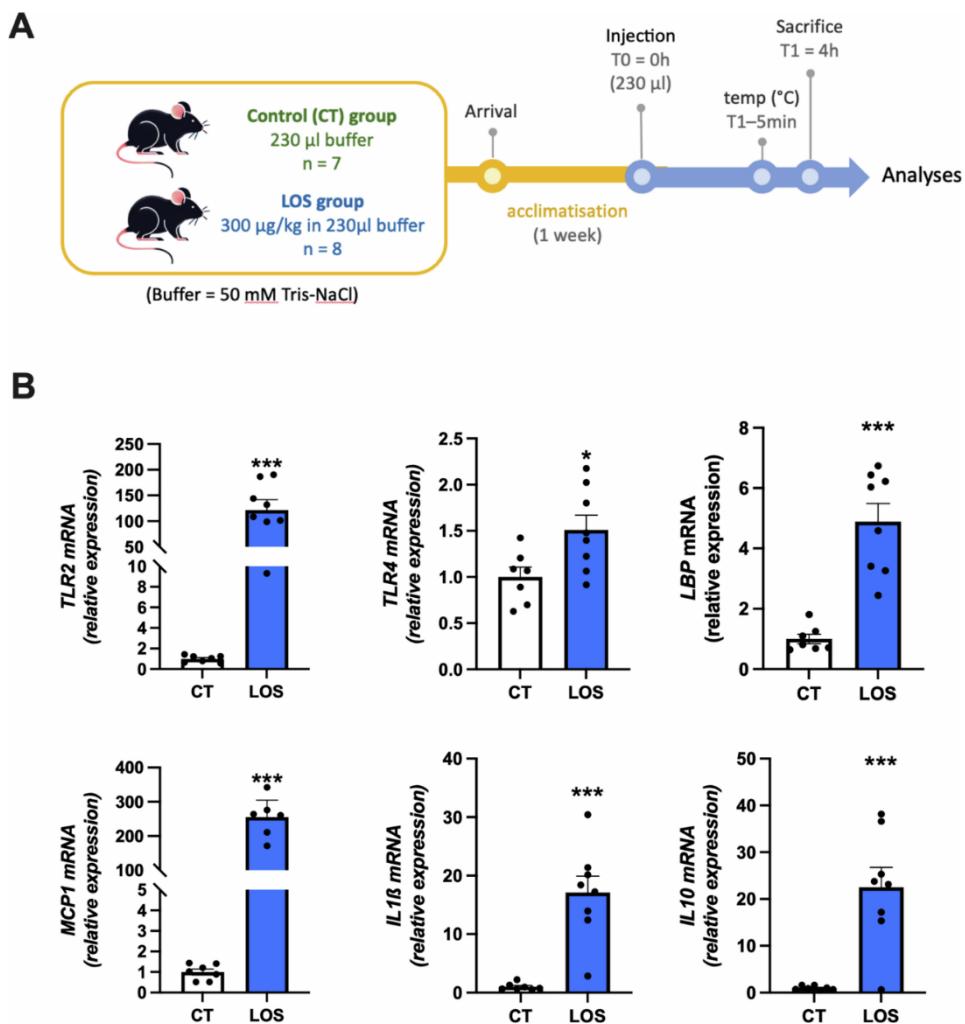


Figure 4.3. (A) Outline of the experimental setup. Two groups of mice were injected interperitoneally with 230 μ l of LOS solution (generating a final concentration of 300 μ g/kg body weight; LOS group, $n = 8$) or control group (CT; 50 mM Tris-NaCl buffer pH 7; CT group, $n = 7$). **(B)** Expression of relevant genes such as Toll like receptor 2 and 4 (TLR2, TLR4), monocyte chemoattractant protein 1 (MCP1), Lipopolysaccharides binding protein (LBP), interleukin 1beta and 10 (IL1 β and IL10) were determined in the liver by qPCR, RPL19 (ribosomal protein L19) was chosen as housekeeping genes. All the data were compared to the CT group as fold changed (CT group set at 1). Data are expressed as mean $\pm$ SEM and compared using two-tailed Mann Whitney test (* p -value < 0.05 ; *** p -value < 0.0005). Source data are provided as a Source Data file.

4.4. *A. muciniphila* LOS signals through both TLR4 and TLR2, with a key role for lipid A

The capacity of *A. muciniphila* LOS and of its components, the isolated core oligosaccharide (OS) and lipid A to signal via TLR2 and TLR4 receptors was further investigated using HEK-Blue hTLR2 and hTLR4 reporter cell lines, respectively (**Figure 4.4**). *A. muciniphila* LOS was found to activate the TLR4 receptor only slightly less than the LPS of *Escherichia coli* O111:B4 (a Gram-negative bacterium used as positive control for TLR4 activation) (**Figure 4.4B**). Remarkably, in contrast to the LPS of *E. coli* O111:B4, the *A. muciniphila* LOS was also capable of efficient signal transduction via the TLR2 receptor (**Figure 4.4C**).

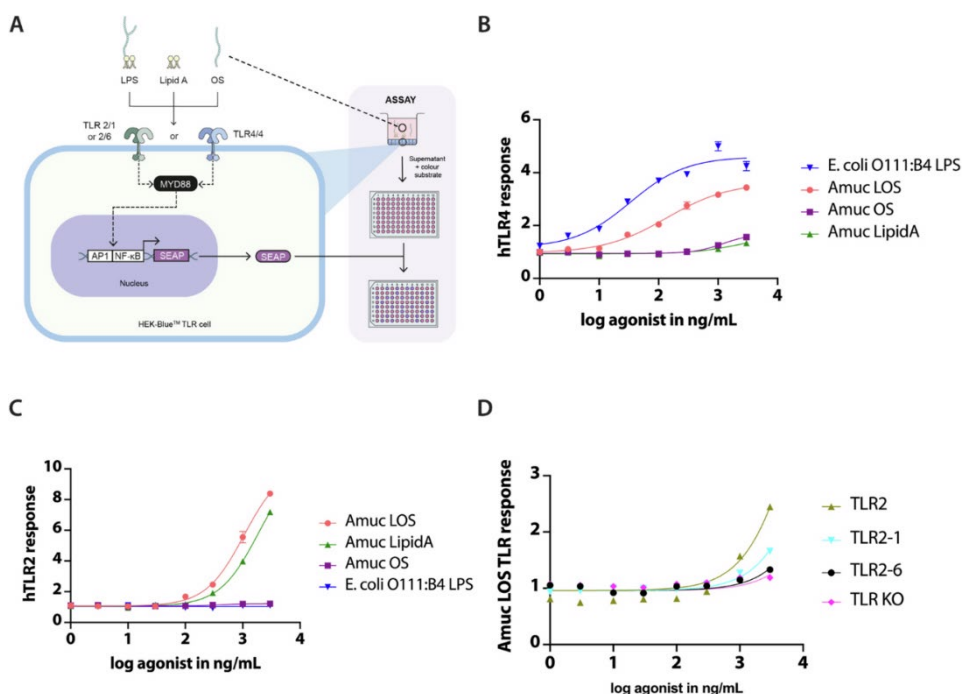


Figure 4.4. The signaling capacity of *A. muciniphila* LOS, OS and lipid A was evaluated by incubating them in HEK-Blue reporter cell lines (**A**), expressing hTLR4 (**B**) and hTLR2 (**C**). A comparative analysis of LOS signaling to the different hTLR2-1, hTLR2-6, and hTLR2 KO/6 cell lines was made, as the hTLR2 reporter cell line expresses both TLR1 and TLR6, allowing hetero- and homodimerization of TLR2 with TLR1, TLR6 and TLR2

(D). All experiments were performed at least in three biological replicates and representative experiments of three technical replicates are shown. The dose-response curves depict normalized mean TLR responses of three replicates $\pm$ SD, and log of the agonist in ng/mL. (Abbreviations used: LOS = lipooligosaccharide, OS = oligosaccharide, hTLR = human Toll-Like Receptor, TLR4 = TLR4 expressing cell line, TLR2 = TLR2 expressing cell line also harboring TLR1 and TLR6, TLR2-1 = cell line only harboring TLR2 and TLR1 heterodimers, TLR2-6 = cell line in which TLR2 and TLR6 are expressed and can form heterodimers, TLR KO = cell line in which all TLR receptors are knocked out, except TLR2).

Next, we determined the signalling activity of the *A. muciniphila* LOS components, OS and lipid A, to elucidate which part of the molecule contributes to the recorded TLR4 and TLR2 activation. Unexpectedly, both lipid A and OS showed little to no signalling activity with hTLR4-expressing cells, indicating the need for intact LOS to mediate signalling through TLR4 (**Figure 4.4B**). However, Lipid A showed comparable signalling through the hTLR2 cell line as the complete *A. muciniphila* LOS, while OS showed no activity (**Figure 4.4C**). Hence, we conclude that lipid A is the structural *A. muciniphila* LOS component involved in TLR2 signalling.

As the TLR2 receptor functions as a heterodimer formed with either TLR1 or TLR6 and both are present in the hTLR2 reporter cell line (TLR2, TLR1 and TLR6 expression), we tested the LOS in a comparative analysis using the hTLR2-1 (only TLR2 and TLR1 present), hTLR2-6 (only TLR2 and TLR6) and hTLR2 KO1/6 cell lines (only TLR2 present). A slight preference for signaling of *A. muciniphila* LOS via the TLR2-1 heterodimer was observed compared to the TLR2-6 heterodimer, whereas virtually no activation of the hTLR2KO1/6 cell line was detected, indicating that a potential TLR2 homodimer does not recognize LOS (**Figure 4.4D**).

In conclusion, these results confirm and extend the structural and mice experimental data and show that the *A. muciniphila* LOS is a weak agonist of TLR4, with the lipid A moiety to be virtually silent whilst showing an unusual strong activation of the TLR2 receptor, likely via TLR2-6 heterodimer.

4.5. Discussion and conclusions

A. muciniphila and its health benefits have attracted considerable attention in the last decades offering the possibility of not only using it as a next-generation therapeutic approach but also as a diagnostic and prognostic tool for a plethora of different inflammatory and metabolic disorders.³ In addition to metabolites produced by *A. muciniphila*, particular attention has been given to cell envelope components, such as the exposed outer membrane protein Amuc_1100 that induces the production of the anti-inflammatory cytokine IL-10 through the TLR2 signalling pathway, likely playing a critical role in maintaining the host immune homeostasis and intestinal mucosa.^{3,7,8}

Here the structural and functional elucidation of the LPS, the main constituents of the Gram-negative outer membrane that *A. muciniphila* presents as LOS, thus lacking the O-antigen portion has been described. Strikingly, the *A. muciniphila* LOS contains both core oligosaccharide and lipid moieties of a unique chemical architecture and structure with no chemical or biosynthetic correlation/analogy with any other LOS/LPS.^{11,12} *A. muciniphila* LOS lipid A contains a complex blend of similar amounts of tetra-, penta- and hexa-acylated lipid A species. Notably, hexa-acylated lipid A species have been associated with a strong activation of the TLR4-dependent response.^{12,13} The observation that the purified lipid A fraction of *A. muciniphila* is virtually silent in TLR4 signalling may be attributed to the unusual presence of a significant amount of *mono*-phosphorylated and hypo-acylated lipid A species as well as to the chemical nature and length of the acyl moieties. While *A. muciniphila* OS fraction was also silent in TLR4 signalling, the entire LOS shows some TLR4 activation but much less than the inflammatory LPS of *E. coli* O111:B4.

A special finding was the observation that the *A. muciniphila* LOS not only signals to TLR4 but activates the TLR2 receptor even much more efficiently. This

is highly relevant since the latter signalling has been recognized as the main facilitator of the anti-inflammatory effects of *A. muciniphila*.³ Detailed analysis showed that the LOS signal transduction pathway to TLR2 signalling can be mainly ascribed to the TLR2 activation by its lipid A moiety that consists of branched fatty acids with 15–17 carbon atoms. Of note, several studies reported that lipid A species of intestinal *Bacteroides* spp. have unique properties¹⁴ and also contain such branched aliphatic chains and contribute to the TLR2 activation of the LPS together with glycine lipids.^{15,16} The latter are made up of the same or very similar acyl components that in *Poryphyromonas gingivalis* were found to activate TLR2.^{16–18} Glycine lipids as detected in these inflammatory bacteria have not been reported in *A. muciniphila* cells. In contrast, *A. muciniphila* was found to be a major source of ornithin lipids that dampen the *E. coli* LPS-induced inflammation by upregulating the expression of ATF3, a master regulator of metabolic and immune homeostasis.¹⁹ This is another example of how specific cell-envelope located molecules of *A. muciniphila* affect the inflammatory response.

It has been well established that the TLR2 and TLR4 signalling pathways shape the development and function of the gut and enteric nervous system, providing a link between the gut-brain axis.²⁰ Signalling between intestine and hypothalamus was recently demonstrated to take place in a mouse model by the action of pasteurized *A. muciniphila*.²¹ As these pasteurized cells are metabolically inert, this obviates the involvement of in situ-produced metabolites but is likely to include the activity of heat-stable components such as the LOS described here.

An unexpected finding was that the LOS oligosaccharide moiety is a unique chemical structure possessing a mixture of two complex glycan units with no repeating motif and including a distinctive fucosylation pattern. Interestingly, the highly branched fucose unit (residue E₂ **Figure 4.1**) present in the shorter undecasaccharide chain (OS₂) might act stopping a further elongation of the

saccharide moiety. Indeed, the same fucose (residue E₁) is terminal in the longer hexadecasaccharide chain (OS₁). Scrutinizing the *A. muciniphila* genome and its observed transcriptome and proteome during growth on mucin, revealed a large set of expressed genes and proteins predicted to be involved in the LOS synthesis. It is tempting to hypothesize that the occurrence of fucose as well as the large number of GalNAc and GlcNAc residues, might be a way for the *A. muciniphila* LOS to be recognized by host receptors, such as by specific lectins, as *self*. In this way *A. muciniphila* would adopt the same strategy as some human pathogens that decorate their LPS with glycan motifs mimicking host structures to escape immune surveillance. This stealth protection mechanism may tune down the inflammatory signalling capacity of the cell-envelope located LOS, as proposed for other mutualistic gut-associated bacteria.²²

The complex signalling unravelled here further addresses the paradox that *A. muciniphila* is an abundant mucosal symbiont while being a Gram-negative bacterium with the potential to activate an inflammatory signal via TLR4. Using *in vivo* mice experiments, we show that when LOS is introduced in the circulation via intraperitoneal injection, the liver expression of the gene encoding TLR2 is hundred-fold more increased than that encoding TLR4. We propose that this leads to an amplification of the beneficial LOS signalling via the TLR2 rather than the TLR4 receptor. This would explain the observed large increase of IL10, which represents the hallmark of anti-inflammatory activity attenuating inflammation. It is worth noting that IL10 is well known to inhibit the production of proinflammatory cytokines including IL1 β .²³ A recent study using various mouse models showed that *A. muciniphila* strain Muc^T signals via TLR2-dependent pathway when administered intraperitoneally.²⁴ Altogether, our *in vitro* data and these *in vivo data* support the hypothesis that *A. muciniphila* membrane components including LOS are activating TLR2-dependent pathways.

Our study has some limitations as we have used the highly purified and characterized *A. muciniphila* LOS components but not synthesized these de novo as their chemical complexity is very high. Indeed, although we strongly deem that such structural complexity and heterogeneity are responsible for the peculiar immunological behaviour of *A. muciniphila* LOS, the lack of synthetic derivative(s) also precludes a structure activity relationship study of the LOS moieties. Furthermore, this also impaired addressing intricate details of the immune signalling, like the elucidation of in vivo inflammatory response or lack thereof upon LOS exposure or their comparison to other LOS structures for microbiota-relevant bacteria like *B. thetaiotaomicron*.²⁵

This study published in *Nature Communications* in 2024 (DOI: 10.1038/s41467-024-52683-x) paves the way to a new concept of LPS, not anymore only as a foe but instead a friendly glycolipid. Indeed, we here clearly show that *A. muciniphila* produces an intricate LOS that, due to its uncommon and complex chemical structure with an exceptionally large non-repetitive carbohydrate backbone, might provide a decisive contribution to the health-promoting effects of this gut symbiont by uniquely modulating the immune and potentially the gut-brain response via TLR2 and TLR4. Future structure to function studies will be pivotal for identifying the direct role of LOS in the crosstalk between *A. muciniphila* and the human host. In this context, studies aimed to fully dissect the chemical features and the epitopes responsible of immune recognition will be crucial in expanding the potential use of *A. muciniphila* and its constituents to conceive new health-promoting therapeutic avenues.

4.6. References

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CHAPTER

V

Deciphering the structure
and immunological properties of
Pseudoruminococcus massiliensis E10-96H
capsular polysaccharide

5.1. Introduction

More than a thousand bacterial species have been found in the human gut microbiota, many of them have been studied but a significant number remain uncharacterized.¹ It has now been confirmed that the microbiota can affect almost all aspects of the host, while its dysbiosis is linked to a broad spectrum of diseases.² Thanks to advanced research technologies, we can closely examine how the microbiota maintains human health and contributes to pathogenesis.^{3,4} Because the human gut is involved in a wide range of physiological functions, its modulation is expected to prevent or treat corresponding diseases. A greater understanding of this host-microbiota relationship has allowed the development of microbiota-based therapies such as fecal microbiota transplantation and bacterial modulation.^{5,6} Fecal microbiota transplantation (FMT) is a treatment that has proven effective for recurrent *Clostridioides difficile* infection (rCDI) and continues to be investigated for its therapeutic possibilities to treat other systemic infections.^{7,8} The main bacterial species associated with a positive outcome in fecal microbiota transplantation have not yet been identified.

A new species recently isolated from a healthy male fecal donor, *Pseudoruminococcus massiliensis*,⁹ has been described as strictly anaerobic and belonging to the family Ruminococcaceae, its closest relative being *Ruminococcus bromii*, a gram positive bacteria which is considered a keystone species in the intestinal microbiota, as it is able to degrade resistant starch and contributes to colonic fermentation.^{10,11} In recent studies, increased populations of *R. bromii* and other gram-positive bacteria have been observed when diets increased consumption of resistant starch, with the action of these bacteria being key to releasing products that can be used by other bacteria.^{10,12} *P. massiliensis* expresses a carbohydrate-containing cell-surface structure, the capsular polysaccharide (CPS). CPS generally have very high molecular weights (often ≥ 100 kDa) and extend beyond peptidoglycan and other cell wall components,

generating a thick protective layer around the entire bacterial cell.¹³ Capsules protect bacteria from desiccation, phages, and immune cells.¹⁴ Emerging data indicate that polysaccharides play extensive roles in host-gut microbiota symbiosis, not only escaping the host immune system recognition but also directly modulating its effects.^{15,16} The chemical characteristics of CPS in the human gut microbiota and the mechanisms that prevent pro-inflammatory responses remain largely unknown. Therefore, elucidation of the fine chemical structures of these microorganisms and their immunological properties is essential to understand their role in health and disease.

With this aim, in this chapter we studied the structure and immunological properties of the CPS from *Pseudoruminococcus massiliensis* E10-96H. In a recent study,¹⁷ the complete genome sequence of E10-96H was shown to have than 98% similarity to the type strain. E10-96H contains 20 genes encoding glycoside hydrolase, it degrades starch *in vitro*, and may contribute to fiber degradation, contributing to the feeding of other species and the production of butyrate in the intestinal ecosystem. The strain has pilus-like structures and adheres to enterocytes *in vitro*, but without eliciting a proinflammatory response.¹⁸ *P. massiliensis* appears to have a commensal behaviour with the host epithelium and its role in intestinal ecology should be further investigated. The study of the role of *P. massiliensis* in the intestinal microbial ecosystem is important providing the opportunity for novel additional studies for the possible beneficial interactions of *P. massiliensis* with the human host. Here, I report on the structural investigation of the CPS from *Pseudoruminococcus massiliensis* E10-96H and its immunological properties in *in vitro* models. We found that the CPS contains a homopolymer made up of a β (1-6) hexofuranose sugar unit carrying at O-2 aglycon. We then show that the CPS of *P. massiliensis* elicits significantly lower expression of maturation markers CD40, CD80, CD83 and CD86 on monocyte-derived dendritic cell compared to pro-inflammatory *Escherichia coli* LPS.

Further, we observe that the CPS acts as a weak activator of TLR4-mediated signaling. Together, these observations provide new structural and biological insights into CPS from gut bacteria, showing the importance of structural features in modulating host immunity.

5.2. *P. massiliensis* E10-96H isolation and chemical analyses

P. massiliensis E10-96H was isolated with the hot-phenol water extraction protocol and purified with enzymatic digestion and gel-filtration chromatography. The analysis of the monosaccharides showed the presence of D-mannose (D-Man), D-glucose (D-Glc), D-galactose (D-Gal), D-glucosamine (D-GlcN) and at high elution times a substituted monosaccharide identified following NMR analyses. Interestingly, no traces of Kdo or hydroxylated fatty acids were found, markers of Gram-negative bacteria, which contributed to the hypothesis that this bacterium doesn't have a classical Gram-negative bacteria structure.

5.3. Structure of *P. massiliensis* E10-96H repetitive unit

In order to study the envelope component of this bacteria, the material isolated from the hot-phenol water extraction was further purified with a centrifugation protocol (9000 rpm, 5 h, 4 °C). In order to understand the nature of the phases both, the precipitate and the supernatant, were studied via NMR spectra. The carbohydrate-containing supernatant was then selected for further study with NMR spectroscopy. One spin system was identified (**A**, **Figure 5.1**) presented in furanose form, as evidenced by the low-field shifted carbon signals and corroborated by the intra-residue long-range correlation between positions 1 and 4. As indicated by the downfield carbon shift, this unit **A** is glycosylated at O-6, and substituted at O-2 by an aglycon. This substituent is a 4-hydroxypentanoic acid and is linked to the galactofuranose skeleton in position C-2 (**E**, **Table 5.1**).

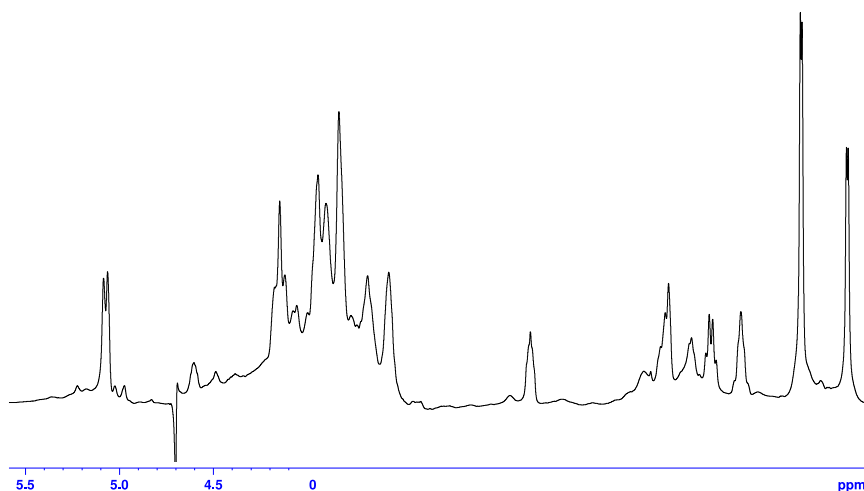


Figure 5.1. NMR spectra from *P. massiliensis* purification of the supernatant via centrifugation protocol. (A) ^1H -NMR (B) ^1H -NMR and ^1H , ^{13}C -HSQC (blue, red) spectra.

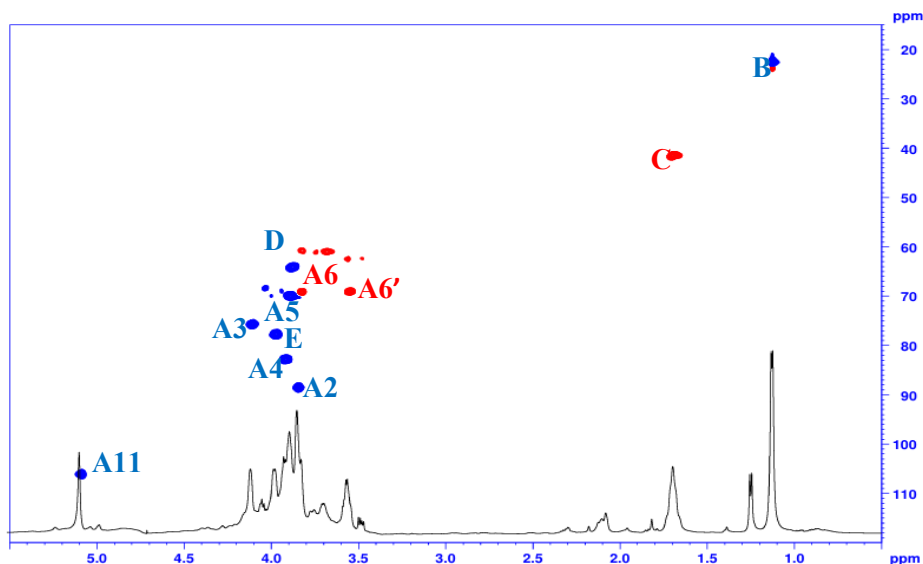


Figure 5.2). The analysis of *intra*-residual NOE contacts and long-distance correlations in the HMBC spectra were key to completing the sequence

Table 5.1. ^1H and ^{13}C chemical shifts of *P. massiliensis* E10-96H homopolymer

	1	2	3	4	5	6
A 2,6- β -Gal	5.09	3.84	4.10	3.91	3.89	3.82 / 3.55
	106.0	88.5	75.8	82.9	70.0	69.2
	F -COOH	E -CH-	C -CH ₂ -	D -CHOH-	B -CH ₃	
	----	3.97	1.68	3.87	1.12	
	180.6	77.8	41.6	64.1	22.4	

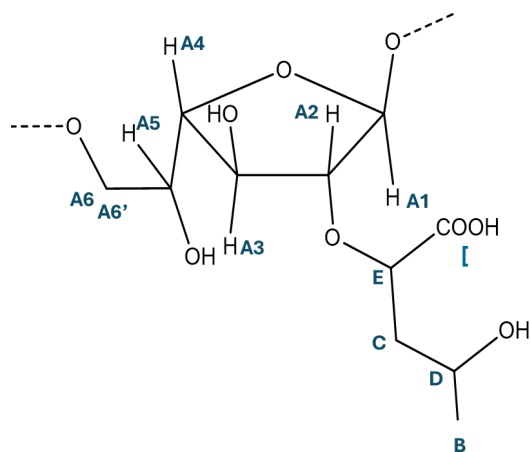


Figure 5.2. Structure of the homopolymer produced by *P. massiliensis* CPS.

5.4. Structure of *P. massiliensis* E10-96H repetitive unit after mild acid hydrolysis

In parallel, an aliquot of the material obtained after the hot water-phenol extraction was subjected to mild acid hydrolysis and purified with gel filtration chromatography. Remarkably, the NMR study of the obtained material revealed the presence of a structure derived from the original thanks to a lactonization

process. In the HSQC spectrum, the presence of both species can be observed, both the previously described structure (the same assignment is maintained) and the structure resulting from hydrolysis (letters **Y** and **B'-F'**) (**Figure 5.3** and **Table 5.2**). The spin system was identified as previously described, with the aglycone being a dihydrofuran-2(3*H*)-one substituted in position 5 by a methyl and in position 3 by the Galf skeleton. In this case, the shift of positions **E'** and **D'** stands out, since they are involved in the formation of the lactone (**Figure 5.4**). The analysis of *intra*-residual NOE contacts and long-distance correlations in the HMBC spectra were crucial to completing the sequence.

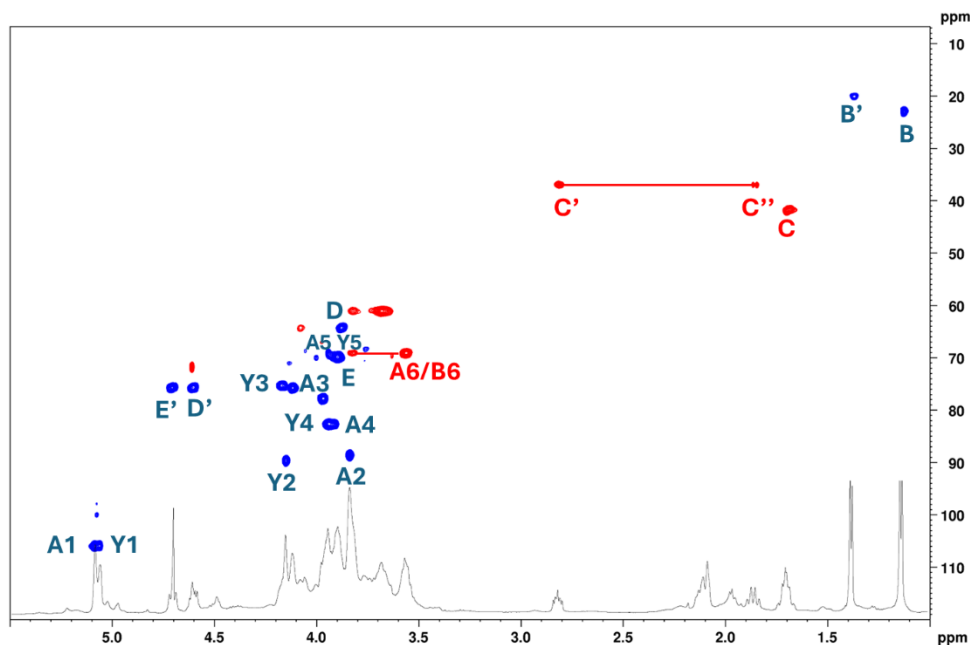


Figure 5.3. NMR spectra from *P. massiliensis* purification of the supernatant via gel-filtration chromatography. (A) ^1H -NMR (B) ^1H -NMR and ^1H , ^{13}C -HSQC (blue, red) spectra.

Table 5.2. ^1H and ^{13}C chemical shifts of *P. massiliensis* E10-96H homopolymer.

	1	2	3	4	5	6
X 2,6- β -Gal	5.08	4.15	4.17	3.94	3.90	3.82/3.56
	106.1	89.7	75.5	82.7	69.9	69.2
	F' -COO-	E' -CH-	C' -CH ₂ -	D' -CH-	B' -CH ₃	
	----	4.70	2.82/11 .86	4.62	1.37	
	172.9	75.8	64.4	75.5	20.1	

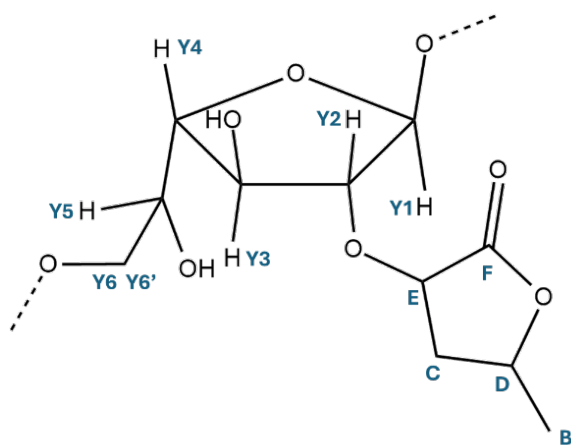


Figure 5.4. Structure of the homopolymer produced by *P. massiliensis* CPS after mild acid hydrolysis.

5.5. Identification of potential galactofuranose related biosynthetic enzymes in the genome of *P. massiliensis*

In this analysis, bioinformatic methods were employed to identify potential galactofuranose (Gal_f)-related biosynthetic enzymes in *Pseudoruminococcus massiliensis* (taxid:2086583), using homology search techniques. Specifically, the “BLASTp tool” on the NCBI platform was used to compare known sequences of enzymes involved in Gal_f biosynthesis, such as the well-studied UDP-galactopyranose mutase from *Escherichia coli* K12 (UniProt ID: P37747) and the Galactofuranosyltransferase GlfT2 from *Mycobacterium tuberculosis* (UniProt ID: O53585), against the *P. massiliensis* protein database (**Table 5.3**). The purpose of this search was to determine if homologous enzymes related to Gal_f biosynthesis are present in *P. massiliensis*, given the structural and biochemical importance of Gal_f in bacterial cell wall and outer membrane components.

The BLASTp analysis identified several putative homologs within the *P. massiliensis* genome, specifically UDP-galactopyranose mutase and Galactofuranosyltransferase. The details of these enzymes are presented below (**Table 5.3**), listing their accessions and E-values, which indicate high-confidence matches. These matches suggest the presence of a pathway potentially similar to that in *E. coli* and *M. tuberculosis*, where Gal_f plays critical structural roles.

Table 5.3. BLASTp analysis for galactofuranose related biosynthetic enzymes in the non-redundant protein sequences database maintained by NCBI.

Description	Accession	E-value
UDP-galactopyranose mutase	WP_337665801.1	8e-175
UDP-galactopyranose mutase	WP_022264184.1	1e-174
UDP-galactopyranose mutase	WP_118366892.1	5e-174
UDP-galactopyranose mutase	WP_181013442.1	2e-173
Galactofuranosyltransferase	WP_106764251.1	3e-44
Galactofuranosyltransferase	WP_270750641.1	3e-44
Galactofuranosyltransferase	WP_022264185.1	5e-44

5.6. TLR4 and TLR2 activation by *P. massiliensis* E10-96H CPS and moDC maturation

To assess the immunological properties of *P. massiliensis* E10-96H CPS, different concentrations of purified CPS were tested in the HEK-Blue™ cells transfected with human TLR4, MD-2, and CD14 genes or HEK-Blue™ hTLR2 cells. *P. massiliensis* CPS exhibited a significantly weaker capability to activate TLR4 signaling compared to *E. coli* LPS used as a positive control (10 and 100 ng, **** $p < 0.0001$) (**Figure 5.5A**). In parallel, no significant activation compared to untreated cells was observed in HEK-Blue™ hTLR2, indicating that *P. massiliensis* CPS is unable to activate TLR2 signaling (**Figure 3B**).

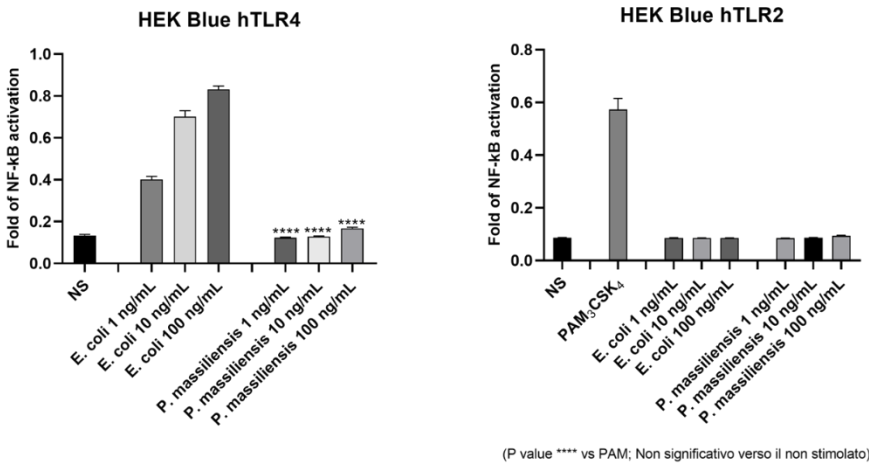
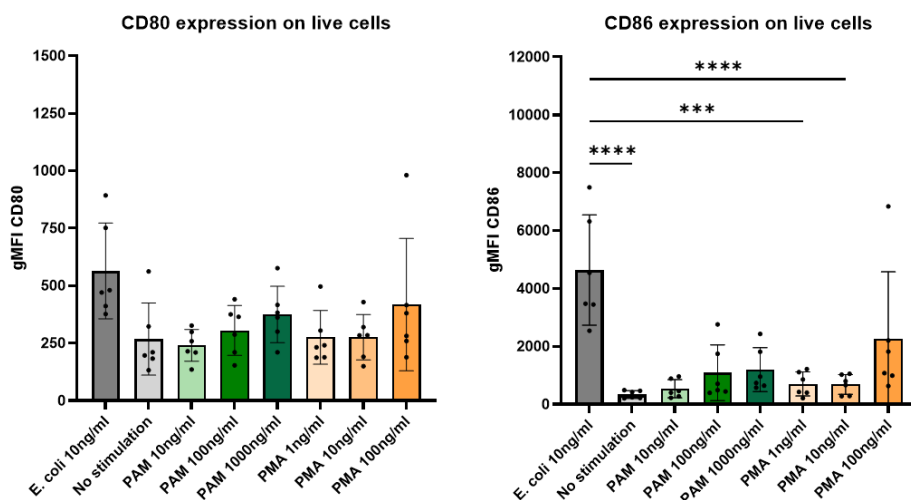


Figure 5.5. Analysis of TLR activation by *P. massiliensis* CPS (**A**) Stimulation of HEK Blue™ hTLR4 and (**B**) HEK Blue™ hTLR2 cells. Secreted embryonic alkaline phosphatase (SEAP) levels (OD) upon stimulation with LPS from *E. coli* and *P. massiliensis* CPS at the indicated concentrations (1, 10, 100 ng/mL). In (**A**) Ultrapure LPS from *E. coli* 0111:B4, a potent TLR4 agonist, was used as a positive control, whereas in (**B**) it was used as a negative control, due its known inability to activate the TLR2 signaling. The positive control in (**B**) was Pam3CSK4 (500 ng/mL), a synthetic triacylated lipopeptide agonist for TLR2 signaling. (**A**) Significant difference between *P. massiliensis* CPS generated values and the corresponding *E. coli* LPS (*P. massiliensis* CPS vs. *E. coli*, **** $p < 0.0001$) or untreated cells (*P. massiliensis* CPS vs. untreated cells (NS), non significative) values by the Student *t*-test are indicated. Data are expressed as mean $\pm$ SD of three independent experiments.

The next step to study the immunological potential of *P. massiliensis* CPS, we observed its ability to induce maturation of monocyte derived dendritic cells (moDCs) by flow cytometry. Interestingly, *P. massiliensis* CPS stimulation showed significantly reduced expression of maturation markers CD86 and CD83 on moDCs (**** $p<0.0001$) compared to *E. coli* LPS (10 ng/mL) and no differences with PAM (10, 100, 1000 ng/mL) (Figure 5.6). These findings revealed that *P. massiliensis* CPS may elicit a dose-response effect, but its ability to induce moDC maturation is consistently weaker than the LPS from *E. coli*. This indicates a distinctly less potent immunostimulatory profile for *P. massiliensis* homopolymer.



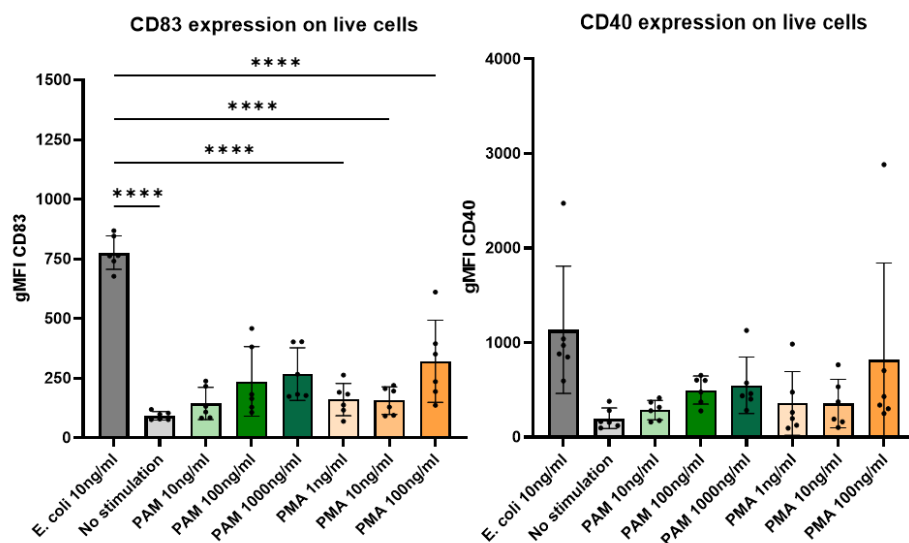


Figure 5.6. Analysis of moDC cell surface marker expression. The expression of cell surface markers CD40, CD80, CD83 and CD86 upon *P. massiliensis* CPS (1, 10, 100 ng/mL) here named as PMA, Pam3CSK4 a synthetic triacylated lipopeptide here called PAM and *E. coli* LPS (10 ng/mL) 18h-stimulation of moDCs was quantified by flow cytometry. Data is presented as geometric Mean Fluorescence Intensity (GMFI) and represent the results of six independent donors (*P. massiliensis* CPS vs. *E. coli* LPS) (* $p < 0.0001$).

5.7. Discussion and conclusions

The capability to break down complex carbohydrates is a critical characteristic found within a specific group of bacteria in the gut environment.¹⁹ The first degraders of insoluble polysaccharides, such as *R. bromii*, *Eubacterium rectale* and *Bifidobacterium* spp.¹⁹ work to decompose indigestible food particles in the colon, consequently releasing energy sources for the microbial community as part of the bacterial cross-feeding system. The bacteria's ability to ferment resistant starch serves a crucial role in supporting other species that produce butyrate, an essential bacterial metabolite that regulates gut homeostasis by enhancing the barrier function of the intestinal lining and mitigating inflammation.¹⁸

Finding the causes of and cures for microbiome-involved diseases requires establishing the molecular mechanisms connecting microbes to disease. To date, different studies have demonstrated the benefits of FMT but the main bacterial species associated with a positive outcome are still unknown.²⁰ The mechanisms responsible for these correlations are largely lacking in animal models and in patients.²¹ Here, we investigated a bacterium that could contribute beneficially in FMT treatment, *P. massiliensis*. We found that the isolate exhibited a structure with a repeating unit forming a capsule, which was used for *in vitro* studies. Some bacteria possess a protective capsular polysaccharide, which covers them and allows evasion of immune recognition by the host. Thus, *Ruminococcus gnavus*, another member of the *Ruminococcus* genus and a prevalent species of the human gut, has strains that possess a protective capsule that prevents an inflammatory response from occurring, while strains that lack this capsule and have their cell walls exposed elicit a robust immune response in *in vivo* experiments with mice.²²

P. massiliensis E10-96H presents extracellular appendages that may be beneficial for the settlement of the bacteria in processes such as a FMT. A study showed that *P. massiliensis* E10-96H adheres to the enterocyte cell lines Caco-2 and HT-29 in a similar way as other intestinal bacteria such as *Bacteroides*, *Sutterella* and *Akkermansia* spp.^{23–25} These data obtained on *P. massiliensis* and taking into account the ability of its closest relative *R. bromii* to break down resistant starch,²⁶ may encourage the assumption of the fundamental role of *P. massiliensis* in breaking down complex carbohydrates to favor the cross-feeding of other species. Furthermore, *P. massiliensis* E10-96H does not provoke the pro-inflammatory response in enterocytes which also corroborates the hypothesis of its role as a commensal in the host epithelium.¹⁷

In the past, it has been demonstrated that certain human gut microbes have capsules that promote immune tolerance. Notably, one of the most known examples is from *Bacteroides fragilis* which capsule stimulates the proliferation

of regulatory T-cells and the production of IL-10.²⁷ This finding indicates that capsule serve as agents of immune tolerance for specific members of the human gut microbiota. Our study shows that the capsule from *P. massiliensis* E10-96H possesses a tolerogenic capsule formed by repeating units of an aglycone-substituted hexofuranose linked by a $\beta(1\rightarrow6)$ bond. In addition of our structural characterization, we tried to identify galactofuranose related biosynthetic enzymes that could be implicated in the biosynthesis of galactofuranose containing outer membrane component of *P. massiliensis*. Reports indicate the presence of Galf residues in the glycans of a variety of human pathogens, like *Mycobacterium tuberculosis* where Galf is a critical component of the arabinogalactan. Furthermore, Galf has been shown to be present in surface glycoconjugate structures that seems to play key roles in pathogenesis of some protozoan parasites, for example the lipopeptidophosphoglycan, the glycoinositolphospholipids and certain mucins of *Trypanosoma cruzi* and the lipophosphoglycan of *Leishmania* spp.^{28–31} Remarkably, Galf has not been reported to exist in mammals, instead Galf-containing epitopes have been shown to be highly antigenic.^{32,33} Thus, the metabolism of Galf has become an attractive candidate as a target for novel antimicrobial drugs. The human lectin intelectin-1 (ITLN-1), produced by intestinal epithelial cells and elevated in certain conditions, specifically recognizes exocyclic 1,2-diols, including Galf residues on microbial surfaces.^{34,35} This interaction promotes the phagocytic removal of pathogens by enhancing their sequestration and directing them towards neutrophils. Nevertheless, the existence of Galf in the core region of gut-related species, as explained in *B. eggerthii* 1_2_48FAA (**Chapter III**), prompts critical questions about its role in the dialogue between gut microbiota and the host immune response, warranting further study.

Furthermore the *in vitro* immunological essays showed that the CPS does not elicit the TLR4 activation, which was in accordance with the absence of the lipid

A component. Interestingly, the CPS from *P. massiliensis* does not induce the expression of surface maturation markers like CD40, CD80, CD83 and CD86 on moDCs. The maturation of this DC is necessary for the stimulation of the T-cells which contributes to the activation of immune cascades and consequently cytokine production.³⁶ These results, the lack of TLR4 activation and the lack of significant maturation of DCs upon the stimulation with *P. massiliensis* CPS, contribute to the hypothesis that this bacterium evades the host immune recognition. Additional studies are required to understand what role this encapsulated *P. massiliensis* plays in the gut and, potentially, its beneficial contribution to FMT treatments as *R. bromii* does.³⁷ Our work provides a comprehensive structural characterization of its CPS and some approaches to its immunological properties. From a wider perspective, it highlights the ability of bacterial glycan to elucidate how members of the gut microbiota can shape human health and disease.

As a follow-up to this project, the absolute configuration of the repeating unit will be investigated and the isolated capsular polysaccharide, will be used in immunological tests to understand how it affects cytokine production in macrophages, in collaboration with the group of Professor Reeta Satokari from the University of Helsinki.

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CHAPTER VI

Insights into the structural and immunological
profiling of lipopolysaccharide from
Fusobacterium gonidiaformans

6.1. Introduction

The genus *Fusobacterium* spp. encompasses a variety of gram-negative anaerobic bacilli. While *Fusobacterium* infections are uncommon, they may occur following pathogen entry through compromised mucosal surfaces of the host, often due to trauma, tumors, or previous infections.¹ This progression can lead to severe disease within? metastatic sites.^{2,3} The distinctive ability of *Fusobacterium* to invade as a pathogen among gram-negative anaerobes and its broader clinical spectrum is undeniable noteworthy.⁴ In particular, two *Fusobacterium* species, *F. nucleatum* and *F. necrophorum*, stand out as the most frequently identified pathogens within this genus and are widely documented in the literature.^{5,6} A less studied species, *Fusobacterium gonidiaformans*, is commonly isolated from the urogenital and intestinal tracts in humans⁷ and is often associated to preterm-delivery, puerperal infections and bacteremia, and in few reports,⁸ to Fournier's gangrene and peritonsillar infections;⁹⁻¹² a study by Wu et al. highlighting report on its abundance in patients with colorectal cancer.¹³

The closest genetic relative of *F. gonidiaformans* is *F. necrophorum*.⁴ *F. necrophorum* a normal inhabitant of the human oropharynx, although it may be present during inflammatory diseases and has been implicated in peritonsillar infections and is the etiological agent of Lemierre syndrome.¹⁴⁻¹⁶ The pathogenetic mechanism of *F. necrophorum* is complex and not fully understood. Several toxins such as leukotoxin, endotoxin, hemolysin, hemagglutinin and adhesin have been identified as virulence factors.¹⁷⁻¹⁹ Among these, leukotoxin and endotoxin are thought to play a crucial role in evading host defenses and causing infection. *F. necrophorum* is commonly found in mixed infections, suggesting possible synergisms with other pathogens in the pathogenesis of infections.

As a potential pathobiont, the specific role of *Fusobacterium gonidiaformans* in inflammation-related diseases requires further investigation. In this context, we

focus on the main component of its outer membrane, the lipopolysaccharide (LPS). Specifically, as mentioned in **Chapter I**, in cases where the O antigen is absent, it is referred to as lipooligosaccharide (LOS) or rough-type LPS (R-LPS). The immunostimulatory properties of LPS are determined by their structure, with variations leading to different degrees of immune activation and consequently the production of proinflammatory mediators.²⁰ It is essential to understand the detailed molecular interactions between LPS and their receptors, as well as the resulting signalling pathways that influence disease or tolerance, this will advance our knowledge into the structure to function relationship of such important glycoconjugates. Therefore, we extracted and purified *Fusobacterium gonidiaformans* LOS. We employed a combination of chemical, spectroscopic, and spectrometric techniques to determine the structure of the LOS, and furthermore, we evaluated the impact on the innate immune system using *in vitro* human cell models.

6.2. *F. gonidiaformans* LOS isolation and chemical analyses

The LOS from *F. gonidiaformans* was isolated using the hot phenol-water extraction and further purified via enzymatic treatments and gel-filtration chromatography. Monosaccharide analyses were done to confirm the presence of D-galactose (D-Gal), D-glucose (D-Glc), D-mannose (D-Man), D-glucosamine (D-GlcN), L-glycero-D-manno-Heptose (Hep), 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo). Methylation analysis showed the presence, in pyranose form, of terminal GlcN, 4-substituted and 6-substituted GlcN (4-GlcN and 6-GlcN), 2-substituted Glc (2-Glc), 6-substituted Gal (6-Galp), 2-substituted heptose (2-Hep), 7-substituted heptose (7-Hep), 3,4-substituted heptose (3,4-Hep), terminal heptose (*t*-Hep), terminal mannose (*t*-Man). Fatty acid compositional analysis revealed the presence of tetradecanoic acid (C14:0), 3-hydroxytetradecanoic

acid [C14:0(3-OH)], hexadecanoic acid (C16:0), *anteiso*-heptadecanoic acid (*a*C17:0 and *i*C-17:0).

6.3. Structure of *F. gonidiaformans* fully de-acylated LOS (OS_{deAc})

An aliquot of *F. gonidiaformans* LOS was fully de-acylated and purified by gel-filtration chromatography, to obtain the pure saccharide fraction (OS_{deAc}) which was further studied with NMR spectroscopy (**Figures 6.1 and 6.2**). The anomeric configuration of the monosaccharide units was determined evaluating $^3J_{H1,H2}$ and $^1J_{C1,H1}$ coupling constants, and further confirmed by *intra*-residue NOE contacts (**Table 6.1**). For the relative configuration the analysis of the vicinal $^3J_{H1,H2}$ coupling constants and *intra*-residue NOE contacts was used for the elucidation. ^{31}P NMR experiments were key to establishing the position of the phosphate groups. Twelve spin systems were identified (**A-N; Figures 6.1 and 6.2, Table 6.1**). All the monosaccharide residues were present as pyranose rings, as indicated by the ^{13}C chemical shift values and the long-range correlations in the HMBC spectrum between positions 1 and 5 (for Kdo between C-2 and H-6).²¹

Table 6.1. ^1H , ^{13}C and ^{31}P chemical shifts of *F. gonidiaformans* OS_{deAc}

	1	2	3	4	5	6	7	8
M 4-β-GlcN	4.77	3.19	3.65	3.66	3.65	3.94 / 3.78		
	99.4	56.4	73.0	69.7	74.5	60.3		
N 6-α-GlcN (1P)	5.65	3.37	3.91	3.42	4.19	4.27 / 3.85		
	90.9	54.4	70.0	70.1	72.8	68.9		
H 6-β-GlcN (4P)	5.01	3.04	3.71	3.86	3.84	3.68 / 3.56		
	99.4	55.6	74.1	74.0	73.7	62.5		
K 5-α-Kdo (4P)	-----	-----	2.16 / 1.87	4.23	4.12	3.84	3.82	3.98 / 3.72
	175	99.25	34.6	65.3	74.8	71.8	71.6	63.8

A 3,4- α -Hep (2P)	5.29	4.44	4.27	4.10	4.45	4.10	3.60 / 3.72	
	99.1	73.6	74.9	72.1	71.3	68.3	63.5	
F <i>t</i> - α -Man	5.20	4.09	3.88	3.88	3.86	4.04		
	97.6	69.7	70.4	70.6	74.2	65.8		
B 2- α -Gal	5.74	3.66	3.89	3.79	3.48	3.95 / 3.73		
	96.2	75.4	70.6	72.8	69.4	63.3		
D 6- β -Gal	4.45	3.55	3.66	3.93	3.73	4.04		
	103.2	71.1	72.4	68.6	75.4	64.0		
E 4- β -GlcN	4.79	3.05	3.65	3.75	3.84	3.99 / 3.84		
	99.3	55.7	75.1	78.4	71.2	59.8		
G 2- α -Hep	5.61	4.15	3.67	3.74	3.85	4.04	3.72/3. 95	--
	99.3	80.3	70.1	71.6	71.5	65.8	63.2	--
C 7- α -Hep	5.17 / 5.14	3.99 / 4.00	3.81	4.08	3.72 / 3.59	4.21	4.06/3. 90	
	101.8/ 101.9	70.7	71.5	71.5	63.6	67.2	71.9	

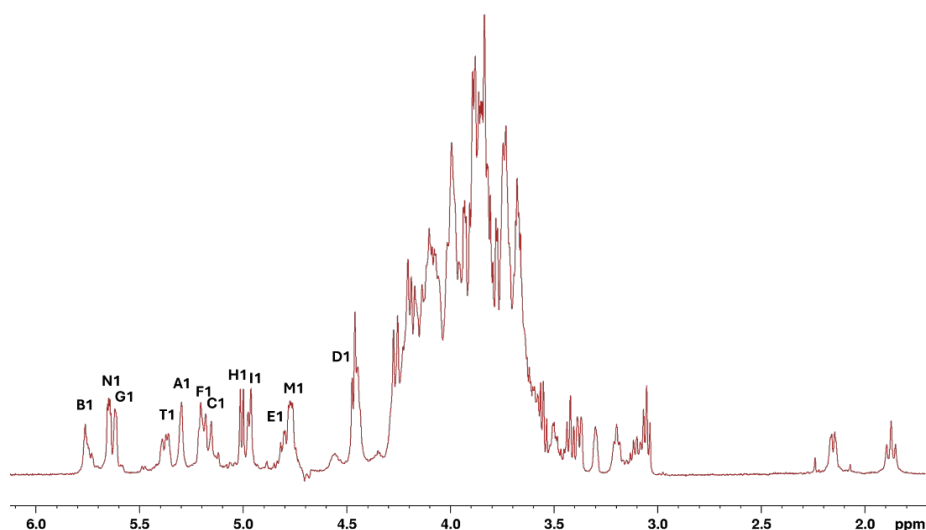


Figure 6.1. ^1H NMR of fully deacylated LOS (OS_{deAc}). Anomeric signals in the inset are as attributed in Table 1.

Spin systems **E**, **H**, **N**, **M** were all *gluco* configured sugar residues, based on the large $^3J_{\text{H,H}}$ ring coupling constants (8-10 Hz). Furthermore, they were identified as GlcpN residues based on the correlation of their H-2 resonance to nitrogen bearing carbon signals (**Figure 6.1** and **Table 6.1**). The *intra*-residue NOE contact of H-1 with H-3 and H-5, $^3J_{\text{H1,H2}}$ and $^1J_{\text{CH}}$ values were indicative of β -anomeric configuration of **E**, **H** and **M**. Conversely **N** was identified as an α -GlcNp, whose anomeric configuration was determined according to $^3J_{\text{H1,H2}}$ and $^1J_{\text{CH}}$ values and the sole H1-H2 *intra*-residual NOE contact. Spin systems **D** and **B** were assigned to *galacto*-configured sugar units as observed in the $^3J_{\text{H3,H4}}$ and $^3J_{\text{H4,H5}}$ values (3 Hz and <1 Hz, respectively). Both $^3J_{\text{H1,H2}}$ and $^1J_{\text{CH}}$ coupling constants, together with *intra*-residual NOE contact of H-1 with H-2, were diagnostic for the α -configuration of **B**. Spin system **D** was identified as α -Gal. Spin system **K** was assigned to the Kdo residue based on the diastereotopic methylene proton signals resonating in the high-field region of the NMR spectrum; its α -anomeric configuration was assigned on the basis of the H-3

chemical shift values and the $^3J_{\text{H-7,H8a}}$ and $^3J_{\text{H-7,H-8b}}$ coupling constants (**Table 6.1**).^{21–23} The TOCSY spectrum allowed the identification of all other resonances of ring protons up to H-7 proton signals from H-2 each residue, leading to the identification of these spin systems **A**, **G**, and **C** as α -heptoses.

The down-field shifted carbon signals were indicative of glycosylation at position O-6 of **D**, **N** and **H**, O-5 of **K**, O-4 and O-3 of **A**, O-7 of **G**, O-4 of **E**, O-2 of **G** and **B** whereas **M**, **I** and **F** were terminal sugar residues, in full accordance with the methylation data.

The analysis of the *inter*-residual NOE contacts and the long-range correlations in the HMBC spectrum (**Figure 6.2A**) allowed the definition of the complete sugar sequence of the LOS from *F. gonidiaformans* (**Figure 6.2B**). In particular, the lipid A skeleton was composed of residues **H** and **N**. Kdo unit was, in turn, substituted at O-5 by the heptose residue **A**, as suggested by the corresponding *inter*-residue NOE contacts, together with the HMBC correlations **A1-K5** contacts. Heptose **A** was substituted at O-3 by the terminal GlcN **M** as shown by the NOE exhibited between the anomeric signal from **M** and H-3 from **A**. Also, **A** was further substituted at O-4 by the heptose **G** that was also substituted at O-2 by **C**. The heptose **C** was substituted at O-7 by the GlcN **E** whereas **E** residue was glycosylated at O-4 by the β -Gal residue **D**. Residue **D** was in turn substituted at O-6 by the terminal heptose **I** (**Figure 6.3**).

The ^{31}P NMR spectrum showed the presence of four monophosphate monoester signals (**Table 6.1**). The substitution pattern was deduced by the ^1H , ^{31}P HSQC spectrum, which showed correlations of ^{31}P signals located on both GlcN of the lipid A backbone **H** and **N**, on O4 of the Kdo sugar unit **K** and the O2 of the α Hep **A** (**Table 6.1**).

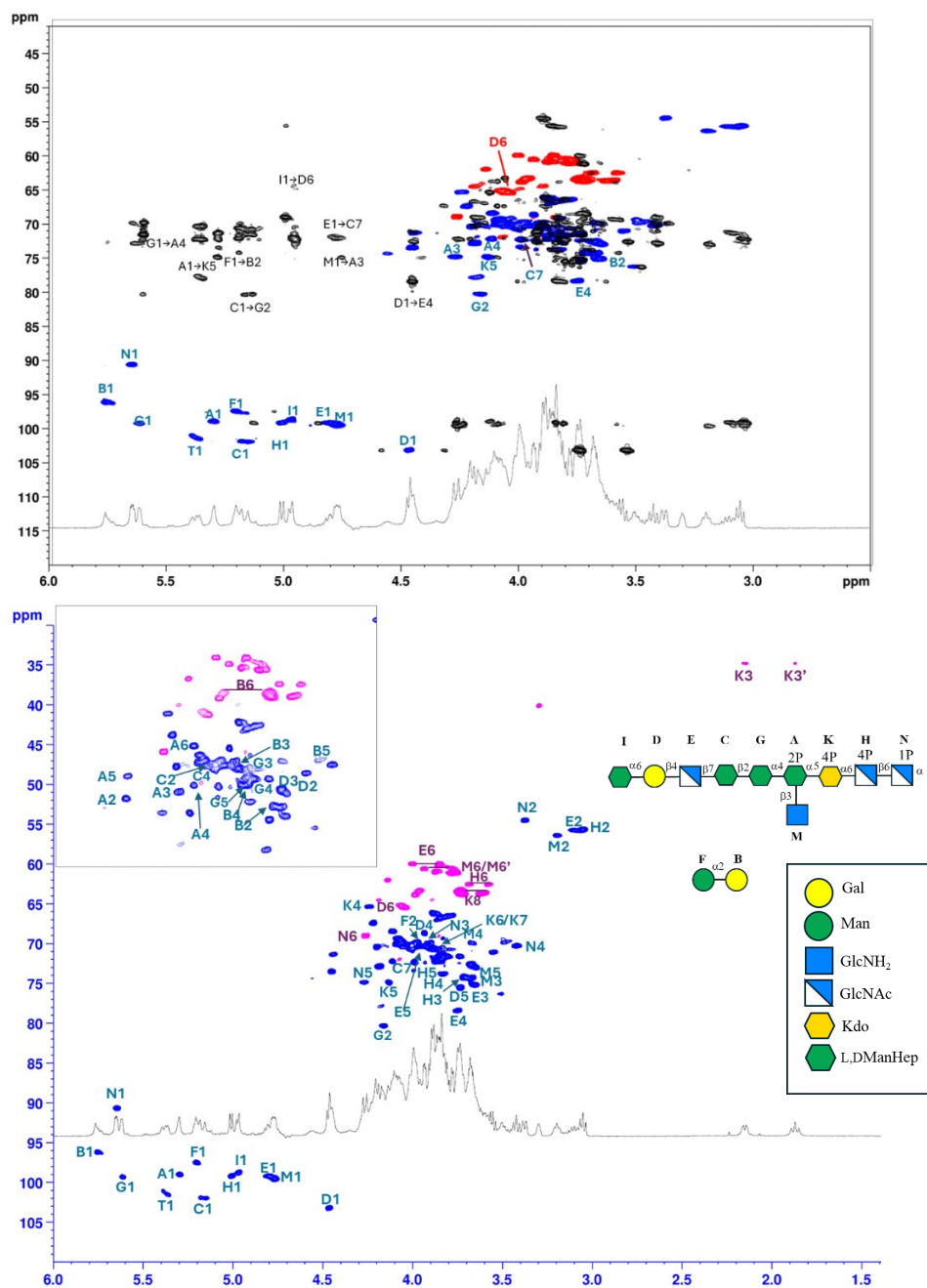


Figure 6.2. (A) NMR structural assessment of OS_{deAc} that was obtained upon full decylation of an aliquot of purified *F. gonidiaformans* LOS. Zoom of the HMBC (black) and HSQC (blue and red) and ^1H NMR spectra of the OS_{deAc} have been reported in (A). The key *inter-residue* long-range correlations involving sugar moieties (A-N) are

indicated; letters are as in Table 1. **(B)** NMR structural assessment of **OS_{deAc}** *F. gonidiaformans* LOS. Multiplicity edited HSQC (blue and pink) NMR spectrum, assigned as attributed in Table 1.

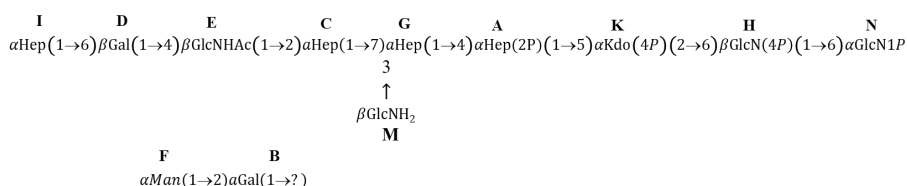


Figure 6.3. Structure of the **OS_{deAc}** from *F. gonidiaformans* LOS derived from the combination of chemical, NMR and MS investigations. In red the portion identified by merging data from chemical analyses and MS investigation

6.4. Structure of *F. gonidiaformans* core oligosaccharide (OS) and lipid A moieties

The LOS underwent mild acid hydrolysis that exploited the lability of the glycosidic linkage of Kdo and therefore split the lipid A and the core oligosaccharide (**OS**). NMR analysis of **OS** fully confirmed the above data, as well as the *N*-acetylation on the aminosugar residue **E** (**Figure 6.4**).

The lipid A will undergo further compositional and MS investigations to complete and confirm the structure, as the linkage between the two moieties characterized is still pending.

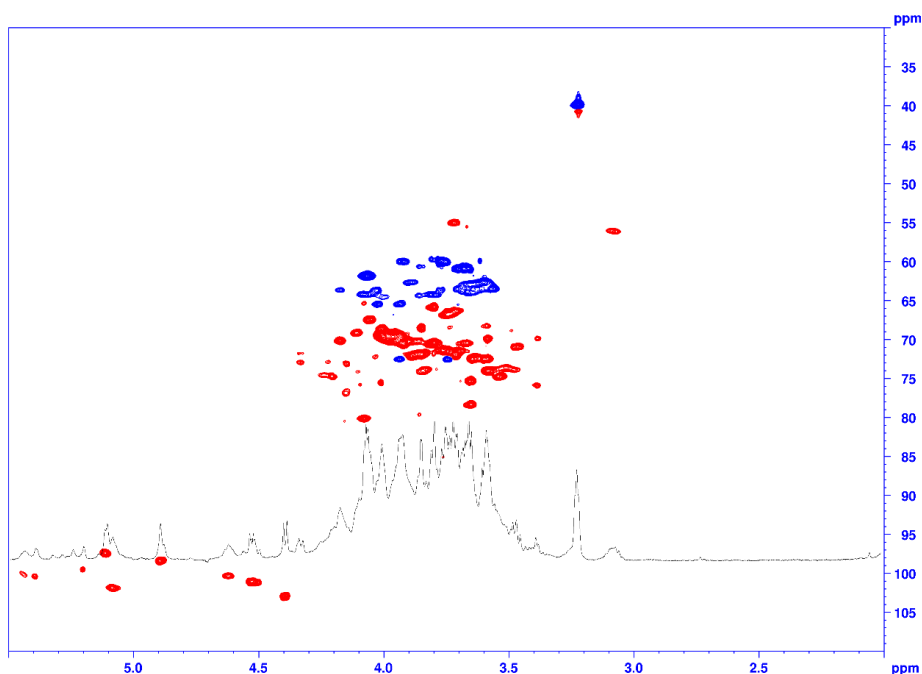


Figure 6.4. OS that was obtained after acid treatment of an aliquot of purified LOS. Multiplicity edited HSQC (blue and red) NMR spectrum.

6.5. TLR4 and TLR2 activation by *F. gonidiaformans* LOS and moDC maturation

To assess the immunological properties of *F. gonidiaformans* LOS, different concentrations of purified LOS were tested in the HEK-Blue™ cells transfected with human TLR4, MD-2, and CD14 genes or HEK-Blue™ hTLR2 cells. *F. gonidiaformans* LOS exhibited a weaker capability to activate TLR4 signaling compared to *E. coli* LPS used as a positive control (10 and 100 ng, *** $p < 0.001$) (**Figure 6.5A**). In parallel, no significant activation compared to untreated cells was observed in HEK-Blue™ hTLR2, indicating that *F. gonidiaformans* LOS is unable to activate TLR2 signaling (**Figure 6.5B**).

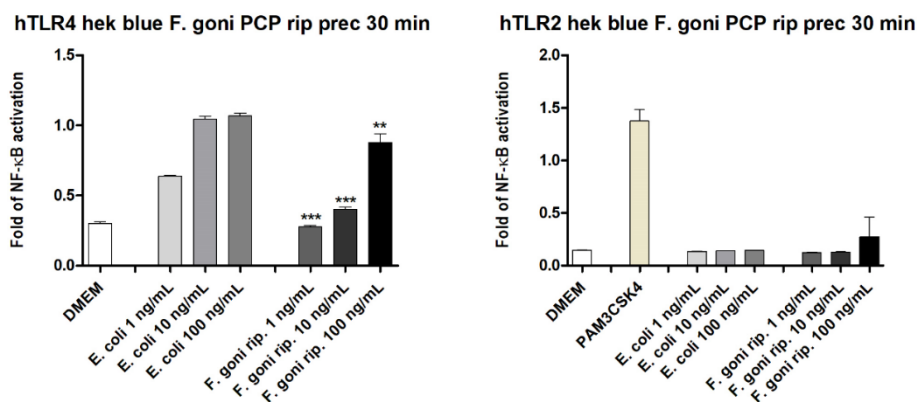


Figure 6.5. Analysis of TLR activation by *F. gonidiaformans* LOS. **(A)** Stimulation of HEK Blue™ hTLR4 and **(B)** HEK Blue™ hTLR2 cells. Secreted embryonic alkaline phosphatase (SEAP) levels (OD) upon stimulation with LPS from *E. coli* and *F. gonidiaformans* LOS at the indicated concentrations (1, 10, 100 ng/mL). In **(A)** Ultrapure LPS from *E. coli* 0111:B4, a potent TLR4 agonist, was used as a positive control, whereas in **(B)** it was used as a negative control, due its known inability to activate the TLR2 signaling. The positive control in **(B)** was Pam3CSK4 (500 ng/mL), a synthetic triacylated lipopeptide agonist for TLR2 signaling. **(A)** Significant difference between *F. gonidiaformans* LOS generated values and the corresponding *E. coli* LPS (*F. gonidiaformans* vs. *E. coli*, *** $p < 0.001$) or untreated cells (*F. gonidiaformans* vs. untreated cells (NS), #### $p < 0.001$) values by the Student *t*-test are indicated. Data are expressed as mean $\pm$ SD of three independent experiments.

To further explore the immunological potential of *F. gonidiaformans* LOS, we evaluated its ability to induce maturation of monocyte derived dendritic cells (moDCs) by flow cytometry. Interestingly, *F. gonidiaformans* LOS stimulation showed differences in the expression of maturation markers CD40, CD86, CD83 and CD80 on moDCs (*** $p < 0.0001$) compared to *E. coli* LPS (10 ng/mL) (**Figure 6.6**). These findings revealed that *F. gonidiaformans* does elicit a dose-response effect, but its ability to induce moDC maturation is lower than the LPS from *E. coli* at the same concentration. This indicates a lower immunostimulatory profile for *F. gonidiaformans* LOS in comparison with *E. coli* LPS.

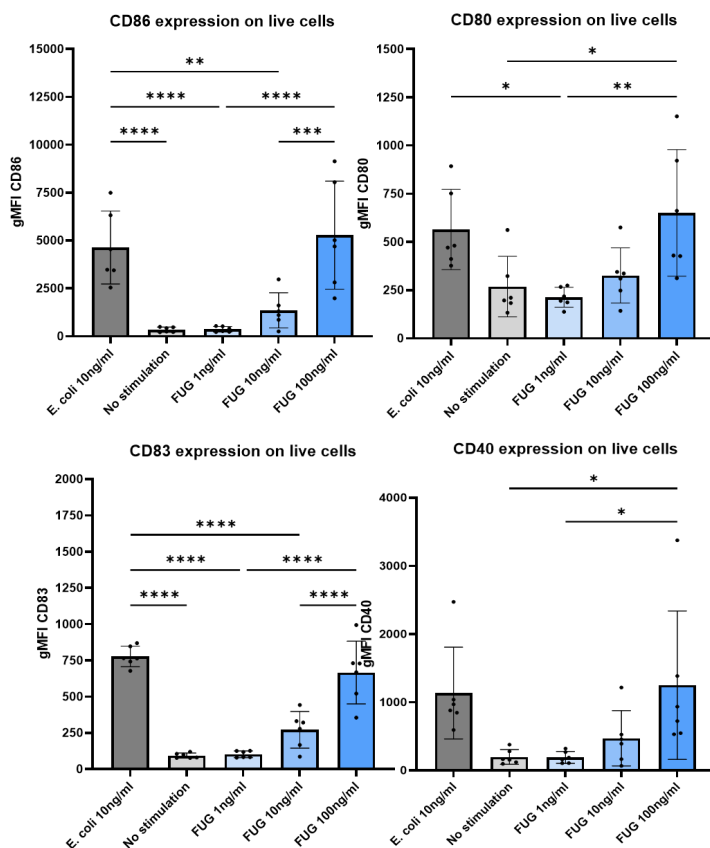


Figure 6.6. Analysis of moDC cell surface marker expression. The expression of cell surface markers CD40, CD80, CD83 and CD86 upon *F. gonidiaformans* LOS (1, 10, 100 ng/mL) and *E. coli* LPS (10 ng/mL) stimulation of moDCs was quantified by flow cytometry. Data is presented as geometric Mean Fluorescence Intensity (GMFI) and represents the results of six independent donors (*F. gonidiaformans* LOS vs. *E. coli* LPS) (**** $p < 0.0001$).

6.6. Discussion and conclusions

We here characterized the novel structure of the LOS obtained from *F. gonidiaformans*. And in common with its closest relative, *F. necrophorum*,⁴ the abundance of 3-hydroxy-tetradecanoic acid (C14:0 3-OH) stands out. Hydroxylated fatty acids are most frequently reported to occur in Gram-negative bacteria.²⁴ Interestingly, the LOS of *F. gonidiaformans* also contains four

phosphates, two of them located in the GlcN skeleton of the lipid A, being of particular interest since phosphorylation is an important factor for the toxicity of these molecules.²⁵ The core portion is comprised by a decasaccharide with a phosphorylated Kdo unit, replaced at O-5 by a branched α -Hep. The presence of Kdo and Hep are common among the different species of *Fusobacterium*, as is the case of *F. nucleatum*, *F. necrophorum*, *F. varium* and *F. mortiferum*.^{26,27} However, the Kdo of *F. gonidiaformans* in addition to being phosphorylated at O-4, is substituted at O-3 by a non-acetylated terminal β -GlcN, and at O-4 by another α Hep, in addition to the substitution at O-2 by a phosphate. The second Hep is in turn substituted by a β GlcN substituted at O-4 by a β -Gal and in turn the latter has at O-6 a terminal α -Hep.

Fusobacterium gonidiaformans was first described as *Bacillus gonidiaformans* in 1924 following isolation from tonsillar granules.²⁸ *Fusobacterium* infections are rare, but they can occur following entry of the infectious agent through damaged mucosal surfaces of the host due to trauma, tumour or prior infection, possibly progressing to severe disease with metastatic foci.²⁹ Their ability to invade in this way as primary pathogens make *Fusobacterium* remarkable amongst Gram-negative anaerobes.³⁰ *Fusobacterium gonidiaformans* is a rarely described species genetically similar to *F. necrophorum* which can cause sepsis in a young, otherwise healthy population. As *F. gonidiaformans* can be found in the genital tract, it should be considered a potential pathogen in the peripartum period and be considered when deciding upon antimicrobial administration.

In this study, we also reported that *F. gonidiaformans* LOS was an activator of TLR4-mediated signaling, which was in accordance with the hexa-acylated nature of its lipid A component, i.e. it possesses six acyl chains as other immunostimulatory LPS.³¹ The presence of the phosphates can be further discussed, the Kdo-lipid A bond is strengthened by the presence of the phosphate group. Phosphate groups and charged sugar residues in the inner core and lipid A

are assumed to contribute to the stability of the outer membrane via crosslinking cations. Phosphate groups in this lipid A region critically determine the biological effects of LPS,³² they affect the binding of LPS to TLR4 and subsequent NF κ B activation³³ and hence the host response towards LPS molecules^{34,35} and even mediate resistance to antimicrobial peptides.³⁶ Like other *Fusobacterium* species, such as *F. nucleatum* and *F. necrophorum*, *F. gonidiaformans* can be recognized by TLR4.^{37,38} Activation of this receptor leads to the release of inflammatory cytokines. The expression of these mediators has been reported to be also regulated by the NF- κ B pathway. Once activated, the p65 subunit of NF- κ B will be isolated from the inhibitory protein I κ B α and translocated to the nucleus where it can trigger the transcription of specific target genes, including TNF- α , IL-1 β , and IL-6.³⁹ Therefore, it would be interesting to examine the inflammatory cytokines TNF-A, IL-1 β , and IL-6 to better understand these molecular mechanisms in host cells.

F. gonidiaformans LOS induces surface maturation markers like CD40, CD80, CD83 and CD86 on moDCs. Maturation of DCs, which is key for efficient T-cell stimulation, involves a series of functional and phenotypic changes that enable them to efficiently trigger immune cascades and therefore cytokine production.^{40,41} Interestingly, our results suggest that *F. gonidiadormans* LOS may be recognized by the immune system, however the kind of immune response which it may elicit still needs to be elucidated. In particular, the results showed that the exposure of maturation markers were similar to the one produced by *E. coli* LPS 10 ng/mL when the concentration of *F. gonidiaformans* LOS was 100 ng/mL. Therefore, *F. gonidiaformans* LOS, like other LPS from *Fusobacterium* spp., appears to have the capacity to moderately activate the TLR4 and lead to immune recognition and moDC maturation, in a dose-response manner.

This chapter unveils a partial structure of LOS (or R-LPS) from a potential pathogen, to our knowledge, never characterized before. Furthermore, it presents

the initial results of the preliminary results on its function-relationship. The aim will be to define whether the structure of LOS from *F. gonidiaformans* presents any peculiarities in its immunological behavior. From the immunological point of view, the study of the interaction of the LOS with the host will be studied in various systems, including the production of pro-inflammatory cytokines (IL-6, TNF α ...) in moDCs. How and to what extent the structural features present in LOS from *F. gonidiaformans* are involved in its pathogenicity will be under investigation to shed light in this context.

6.7. References

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CHAPTER VII

General conclusion

The main focus of this PhD project has been to characterize diverse bacterial structures (LPS, LOS, CPS, EPS) and their structural determinants in interactions with host receptors. The characterized LPS from gut microbiota-constituting bacteria such as *Bacteroides eggerthii* and *Akkermansia muciniphila*, the CPS of *Pseudoruminococcus massiliensis*, the LPS of the potential pathogen *Fusobacterium gonidiaformans* have been reported. The results will contribute to understanding of the interactions of both commensal and pathogenic bacteria with the host. Investigating the unique structures of gut microbiota bacteria along with their immunological functions could be crucial for devising therapies aimed at restoring balance in the host gut environment. Additionally, examining pathogenic bacteria may enhance our understanding of disease mechanisms and support the creation of novel treatments to effectively address these harmful organisms.

In Chapter III, *B. eggerthii*, a novel LOS structure of a member of the *Bacteroides* family, species characterized by benefiting the host, was revealed. Lipid A is composed of tetra- and penta-acetylated species with varying degrees of phosphorylation, this heterogeneity being typical of gut bacteria. In addition, this LOS shares certain common features with other *Bacteroides* species, which contributes to the understanding of the motifs that may be beneficial or not harmful to the host.

In chapter IV, the highly complex structure of the LOS of *A. muciniphila* was determined. It contains three Kdo units, is rich in fucose and its lipid A contains branched fatty acids. LOS shows signaling through TLR4 and TLR2, whereas lipid A only induces TLR2. This may have an important role in the development of its beneficial effects since it has been related to protection from diet-induced obesity, increased mucosal barrier function and reduced insulin resistance and inflammation.

In chapter V, the CPS of *P. massiliensis* E10-96H has been reported. The capsule is formed by repeated units of an aglycone-substituted galactofuranose

connected by a $\beta(1\rightarrow6)$ linkage. Alongside our structural analysis, we aimed to identify the biosynthetic enzymes related to galactofuranose that may play a role in the formation of the outer membrane components containing this sugar in *P. massiliensis*. Our research offers an in-depth structural analysis of its CPS and explores aspects of its immunological characteristics.

In Chapter VI, we have examined the LOS of *F. gonidiaformans*. The core portion is comprised of a decasaccharide with a phosphorylated Kdo unit, replaced at O-5 by a branched α Hep. Given its potential to act as a pathobiont, understanding the specific contributions of *F. gonidiaformans* to inflammation-associated diseases is crucial. *F. gonidiaformans* LOS, like other LPS from *Fusobacterium* spp., appears to have the capacity to moderately activate the TLR4 and lead to immune recognition and moDC maturation, in a dose-response manner. It is vital to grasp the complex molecular interactions between LPS and their receptors, as well as the subsequent signaling pathways that can either promote disease or induce tolerance. This knowledge will enhance our understanding of the relationship between the structure and function of these significant glycoconjugates.

The findings from this study hold significant promise for the development of new molecules with potential clinical applications, such as analogs of beneficial bacterial LPS structures that could replicate their advantageous effects. Additionally, these insights may facilitate the creation of novel treatments targeting pathogens that interact with human host receptors via LPS.

In summary, understanding the architecture of bacterial envelope components is crucial for elucidating their interactions with the host and clarifying their functional roles. Currently, our grasp of the molecular determinants that define

bacterial behavior remains foundational. This Ph.D. thesis illuminates previously unrecognized structures and activities of bacterial envelope components, contributing valuable knowledge to the field. While our understanding of their characteristics, functions, and immunomodulatory properties is still evolving, the novel results presented herein significantly advance our comprehension and lay the groundwork for future research directions.

CHAPTER VIII

Appendix: Glycans from plant-associated bacteria

Structure and properties of the
exopolysaccharide isolated from
Flavobacterium sp. Root935

8.1. Introduction

The plant microbiota comprises an assorted community of microorganisms, including bacteria, viruses, fungi, protists and nematodes, and capable of thriving in various niches with plants. *Flavobacterium* sp. Root935 is a mesophilic, Gram-negative bacterium isolated from the roots of *Arabidopsis thaliana* ecotype Shakdara. Known for its ability to degrade complex organic compounds in diverse environments such as freshwater, marine, and soil, *Flavobacterium* is commonly identifiable by its characteristic yellow-orange colonies, attributed to carotenoid or flexirubin pigments.^{1,2} The family Flavobacteriaceae, along with other related families and taxa, is of a crucial importance in promoting plant growth and fitness by facilitating the mobilization of nutrients in the rhizosphere.³ Given these characteristics, there is growing interest in studying *Flavobacterium*, as harnessing beneficial bacteria for crop production could lead to more sustainable and environmentally friendly agricultural practices. Moreover, specific strains of *Flavobacterium*, if found to possess beneficial traits, could be utilized in biocontrol applications.^{4,5}

Despite *Flavobacterium* high capability to proliferate in root rhizosphere, little is known of mechanisms enabling their colonization of root surface, and consequently of their ability to proliferate and persist in such a competitive environment. Evasion or suppression of plant immune response is fundamental not only to pathogens to infect the host but also to commensals to variously colonize plant niches and establish symbiotic relationships. Nevertheless, mechanisms exploited i) by plant immune system to discriminate between advantageous and pathogenic species and ii) by beneficial microbes to evade plant recognition remain largely unknown.

The initial steps of bacterial colonization are mediated by bacterial envelope components, including lipopolysaccharide (LPS),^{6,7} exopolysaccharide (EPS),⁸ and adhesins. These bacteria-specific molecules, referred to as microbe-associated

molecular patterns (MAMPs), undergo plant immune recognition through the action of pattern recognition receptors.⁹ In addition, EPSs, produced by both pathogenic and symbiotic bacteria during interaction with plants, have several functions, including sequestration of calcium ions, reactive oxygen species (ROS) scavenging, prevention of cellulose-mediated cell agglutination, tolerance to acidic pH, participation in biofilm formation, and host surface attachment.^{8,10–13} Interestingly, the immunosuppressive effect of EPSs occurs through the reduction of cytosolic calcium influx and the decrease of ROS generation, rather than through receptor-mediated signaling.¹⁴

Here we report on a comprehensive analysis of the structure, conformation, morphological characteristics and preliminary host recognition properties of the EPS isolated from *Flavobacterium* sp. Root935, member of the *Arabidopsis thaliana* root microbiota and isolated from roots of *Arabidopsis thaliana* ecotype Shakdara. To this aim we combined structural biology methodologies, NMR, biophysical and computational approaches, lectin microarray and preliminary immunological assays.

8.2. *Flavobacterium* sp. Root935 EPS isolation and chemical analyses

Following the hot phenol-water extraction, *Flavobacterium* sp. Root935 exopolysaccharide (EPS) was isolated in the phenol phase and further purified by enzymatic treatments and gel filtration chromatography. Compositional and linkage analyses revealed the occurrence of terminal 4-amino-D-fucose (*t*-Fuc4N), 3-substituted L-rhamnose (3-Rha), 3,4-substituted D-glucosamine (3,4-GlcN), 3-substituted D-Galactose (3-Gal), 3-substituted D-Quinovosamine (3-QuiN), 3-substituted L-Fucose (3-Fuc), 6-substituted D-Mannose (6-Man).

8.3. *Flavobacterium* sp. Root EPS structure determination and conformational analysis

The high viscosity of the isolated EPS (**Figure 7.1**), ascribable to the extensive decoration of the glycan chain (see below), impaired a detailed NMR analysis on the intact saccharide chain. Therefore, a mild alkaline treatment was conducted to free the hydroxyl groups and increase solubility and tumbling in solution of *Flavobacterium* EPS (EPS_{deO}), that therefore underwent extensive NMR investigation.¹⁵ The anomeric configuration of the monosaccharide units was assigned based on $^3J_{H1,H2}$ and $^1J_{C1,H1}$ coupling constants and confirmed by *intra*-residual NOE contacts; vicinal $^3J_{H,H}$ coupling constants and *intra* residual NOE contacts revealed the relative configuration of the sugar residues. Seven spin systems were identified (**Figure 8.1, Table 8.1**).

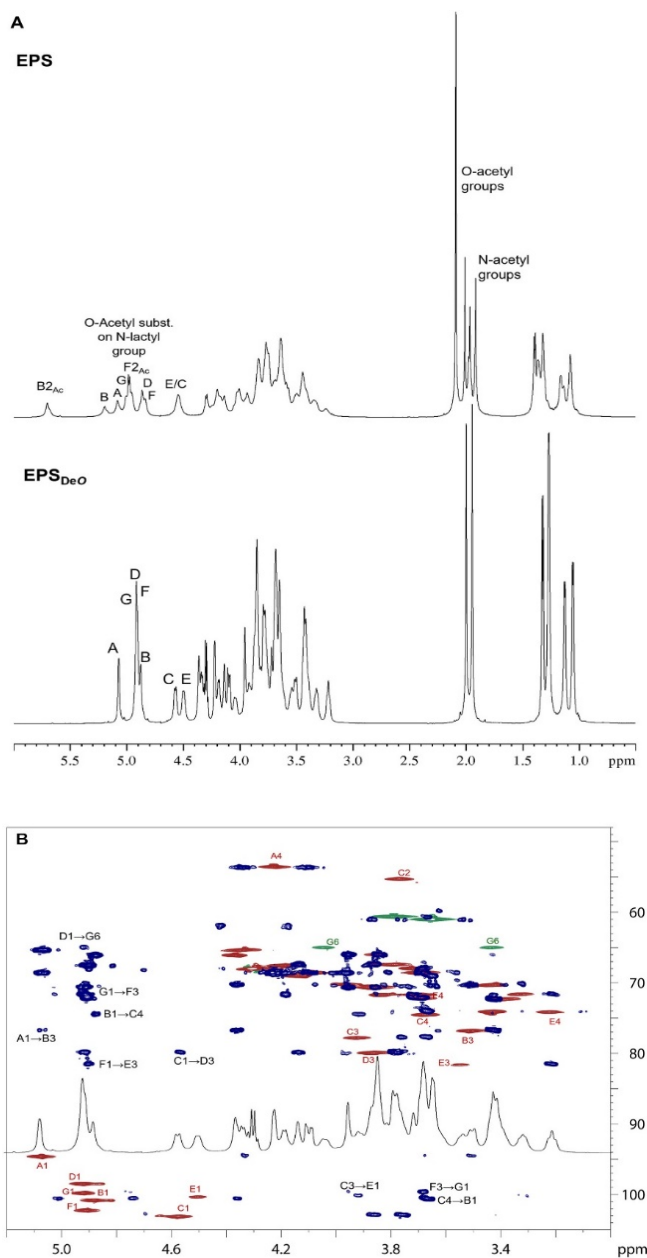


Figure 8.1. (A) DOSY NMR spectra of *Flavobacterium* sp. Root935 EPS and EPS_{deO}. (B) Multiplicity edited HSQC (red and green) and HMBC (blue) NMR spectra of *Flavobacterium* sp. Root935 EPS_{deO}; the key *inter-residual* correlations were indicated; letters are as in Table 1.

Table 8.1. ^1H and ^{13}C NMR chemical shift values of *Flavobacterium* EPS_{deO}. Three further acetylated positions were identified in EPS and are annotated in italics.

Unit	1	2	3	4	5	6
A	5.07	3.69	4.10	4.22	4.34	1.05
t-α-Fuc4N	94.3	68.5	68.6	53.6	65.3	15.8
	$^1J_{\text{C,H}} = 173.4$ Hz			N-lactyl residue CH ₃ -CHOH-C=O 1.32/20.4; 4.30/68.0; 178.5		
				<i>Acetylated Lactyl residue in EPS: 4.98/70.34; 2.01/20.04</i>		
B	4.88	4.36	3.50	3.42	3.39	1.28
3-β-Rha	100.4	66.1	76.6	70.2	72.2	16.7
	$^1J_{\text{C,H}} = 160.0$ Hz					
	5.18/98.7	<i>5.69/67.5 in EPS, acetylated at O2; Acetyl group: 2.09/20.4</i>				
C	4.57	3.76	3.92	3.67	3.42	3.76/3.79
3,4-β-GlcN	102.8	55.4	77.7	74.4	73.9	60.6
	$^1J_{\text{C,H}} = 163.8$ Hz	Acetyl CH ₃ -C=O 2.00/22.5; 173.8				
D	4.91	3.79	3.86	4.13	3.87	3.65
3-α-Gal	98.2	67.4	79.8	69.4	70.6	60.8
	$^1J_{\text{C,H}} = 172.5$ Hz					
E	4.50	3.62	3.55	3.21	3.32	1.27
3-β-QuiN	100.1	56.5	81.5	74.0	71.6	17.2
	$^1J_{\text{C,H}} = 164.1$ Hz	Acetyl CH ₃ -C=O 1.94/22.3; 173.8				
F	4.90	3.72	3.68	3.68	4.18	1.13
3-α-Fuc	102.0	68.0	72.1	71.6	67.5	15.3
	$^1J_{\text{C,H}} = 172.6$ Hz					
	4.84/99.9	<i>4.98/67.4 in EPS, acetylated at O2; Acetyl group: 2.01/22.5</i>				
G	4.91	3.95	3.85	3.85	3.84	3.43
6-α-Man	99.4	70.2	71.6	66.0	65.9	64.8
	$^1J_{\text{C,H}} = 173.0$ Hz					

Residues A, D and F corresponded to *galacto* configured sugar units, supported by $^3J_{\text{H-3,H-4}}$ and $^3J_{\text{H-4,H-5}}$ values below 1 Hz, both diagnostic of the equatorial orientation of H-4; their α -anomeric configuration was assigned by the $^1J_{\text{CH}}$ coupling constant value (above 170 Hz). Residue D matched with an α -Gal, while the scalar correlations of ring proton signals with a methyl group at position 6 allowed to identify residue F as an α -Fuc. Analogously, residue A was identified as an α -Fuc4N unit, according to the correlation of H-4 resonance to a nitrogen bearing carbon signal at 53.6 ppm, in turn bearing an N-lactyl residue. The *manno* configuration of residues B and G was established by $^3J_{\text{H-1,H-2}}$ and $^3J_{\text{H-2,H-3}}$, both below 2 Hz and diagnostic of the H-2 equatorial orientation. Residue G was identified as an α -Man while residue B was identified as a β -Rha, the anomeric configuration based on the $^1J_{\text{CH}}$ coupling constant value (160.0 Hz) and confirmed by the *intra*-residual NOE correlations between H-1 and H-3 and H-5. Residues C and E were identified as *gluco* configured sugar units, as indicated by the ring $^3J_{\text{H,H}}$ coupling constants (8–10 Hz). The *intra*-residual NOE contact of H-1 with H-3 and H-5, $^3J_{\text{H1,H2}}$ and $^1J_{\text{CH}}$ values were indicative of their β -anomeric configuration. Both residues C and E were identified as *N*-acetylated aminosugars, since in the HSQC spectrum H-2 resonance for both correlated to a nitrogen bearing carbon signal. Residue C matched with a β -GlcN sugar unit while residue E was identified as β -QuiN unit, as attested by the scalar correlations of ring protons to the methyl signal at position 6. The downfield shift of carbon resonances identified the following glycosylated positions: O-3 of B, D, E and F; O-3 and O-4 of C; and O-6 of G. Residue A was a terminal sugar unit. The *inter*-residual NOE contacts together with the long-range correlations derived from the HMBC spectrum (**Figure 8.1B**), between A1 and B3; B1 and C4, E1 and C3; C1 and D3, D1 and G6, G1 and F3 and finally F1 and E3.

Based on the structure obtained for the EPS_{deO}, the NMR spectra of intact EPS were evaluated and partially assigned in order to gain further information on the

substitution pattern of this complex glycan chain. Therefore, the down-field shift of H-2 B and H-2 D and of the hydroxyl group of the lactyl residue were all diagnostic of *O*-acetylation at these positions (**Table 8.1**) and matched with *Flavobacterium* EPS structure as depicted in **Figure 8.2**.

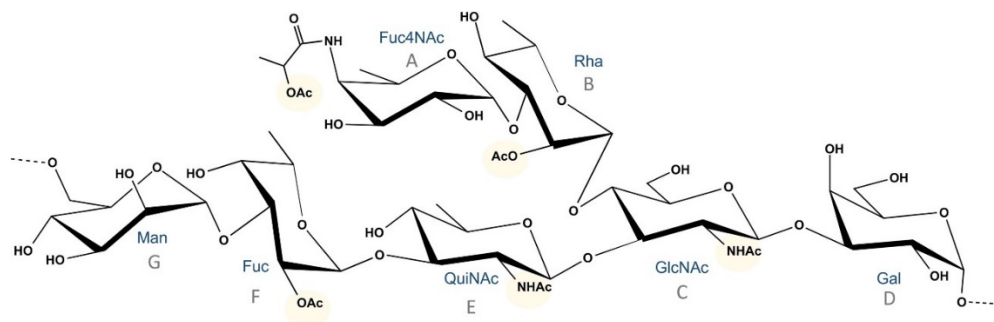


Figure 8.2. The repeating unit of *Flavobacterium* sp. Root935 EPS structure.

Molecular mechanics and dynamic simulations were employed to investigate the three-dimensional features of *Flavobacterium* sp. Root935 EPS. Initially, the conformational preferences of all the constituting disaccharide repeating units were determined by building their potential energy surfaces as a function of the glycosidic torsional angles Φ and Ψ . The calculated maps suggested that each disaccharide mainly adopted conformations according to the *exo*-anomeric orientation. Subsequently, starting from the favorite global minima, a heptasaccharide fragment representing the EPS repeating unit was built and its conformational behavior was disclosed by using molecular dynamic simulations in explicit solvent with AMBER18. The results of the MD cluster analysis revealed that *Flavobacterium* EPS oligosaccharide adopted a defined Y-like shape (**Figure 8.3A**).

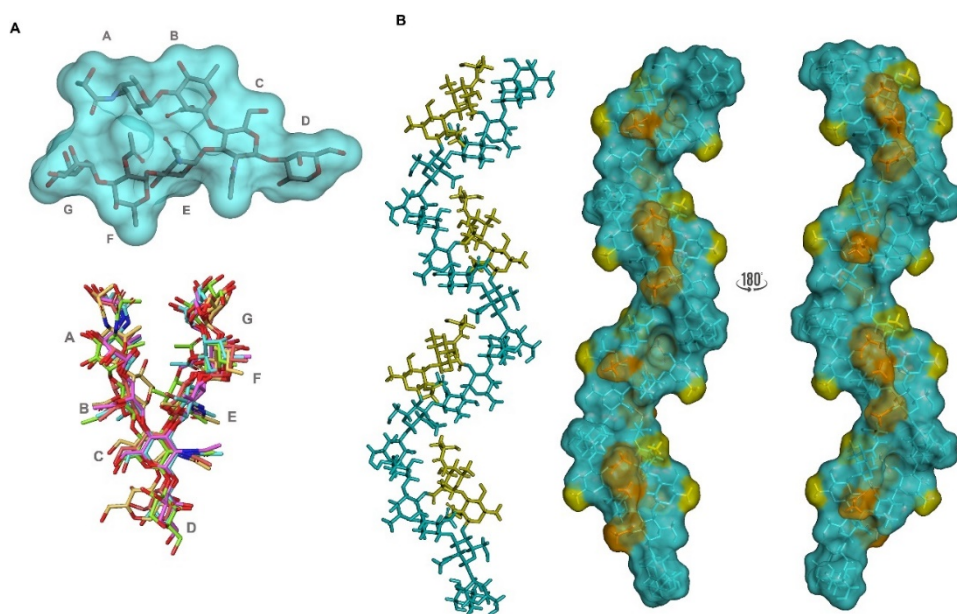


Figure 8.3. Snapshots of *Flavobacterium* sp. Root935 oligosaccharide from simulated trajectories in explicit solvent with AMBER18. **(A)** Superimposition of the main clusters from MD simulation on the heptasaccharide fragment representing the *Flavobacterium* sp. Root935 EPS repeating unit. **(B)** 3D model of *Flavobacterium* sp. Root935 EPS, including four OS repeating units with two additional residues, one at the reducing- and one at the terminal- end. The extended conformation of *Flavobacterium* EPS is shown with the acetyl and methyl groups colored in orange and yellow, respectively.

Modelling the structure of a longer *Flavobacterium* EPS, including three OS repeating units with two additional residues, one at the reducing- and one at the terminal- end, suggested that the polymer tends to adopt an extended conformation, characterized by a left-handed helicoidal structure, with 9 residues per turn and a pitch of around 31 Å. The Y-like arrangement found in the heptasaccharide fragment was maintained in the longer minimized polysaccharide. In addition, acetyl groups regularly surrounded the polymer backbone likely influencing the OS biophysical properties, and likely its interactions to the external environment (**Figure 8.3**).

8.4. Morphological characterization via scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) analysis of macromolecules represents a powerful tool to study surface morphology that can be correlated with microstructure of these materials. Many studies report the SEM morphological characterization of native *Flavobacterium* sp. Root935 EPS or other bacterial exopolysaccharides revealing shapes that include highly branched entangled porous structure, irregular highly porous spider web-like structure, morphologies with irregular lumps of different sizes, morphologies characterized by porous network structure with more uniform pore size distribution or compact, stiff and layer-like structure.^{16–20}

Compared with the morphologies described above, the surface morphology of *Flavobacterium* EPS obtained by SEM analysis (**Figure 8.4**) revealed a different shape, characterized by the presence of irregular flakes that reach lateral size up to 30–50 μm . This morphology can be due to presence of Y-like shape microstructure, in turn stabilized by *inter*-residual polar contacts and *inter*/*intra*-molecular hydrogen bonds that induce the formation of the large lamellae/flakes described above instead of filament networks. The morphology could be compatible with the high viscosity of EPS and its chemical nature, but rheological studies of these characteristics should be conducted.

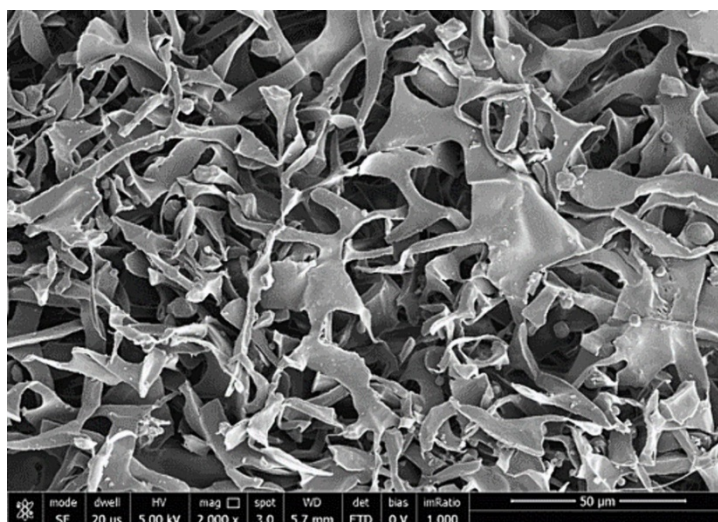


Figure 8.4. Scanning electron micrograph (SEM) images of *Flavobacterium* sp. Root935 EPS.

8.5. Microarray assays and immunological studies

With the aim of evaluating lectin recognition of *Flavobacterium* sp. Root935 EPS and EPS_{deO}, these were printed onto microarray slides at a range of concentrations and two sets of binding assays were performed.^{21,22} First, the binding of a panel of 41 model lectins with diverse carbohydrate-binding specificities was examined. As shown in **Figure 8.5**, no meaningful binding signals to EPS and EPS_{deO} were observed, which can be ascribed to the particular structure and extensive decoration of the glycan chain, while the lectin-binding profile for control glycoproteins was the expected one.²³ Furthermore, the binding of 16 human innate immune lectins was examined, where different lectin-dependent signals were observed (**Figure 8.5**). The lectin binding profile observed for *Flavobacterium* EPS was dominated by ficolins, innate immune receptors responsible for binding certain bacteria and opsonize them via the lectin pathway in mammals, in particular ficolin-1 (also called M-ficolin), followed by ficolin-2 (or L-ficolin).²⁴ Recognition of GlcNAc, GalNAc, and various acetylated structures, including non-carbohydrate acetylated compounds as CysNAc, is a

feature shared by all ficolins, although the architecture and even number of binding sites, and consequently their fine binding specificities, differ among the three lectins. Indeed, ficolin-1 is known to interact with sialic acid (in particular, 9-*O*-acetylated), ficolin-2 binds β -(1,3)-glucans and phosphocholine moieties of teichoic acids, and ficolin-3 recognizes Fuc.

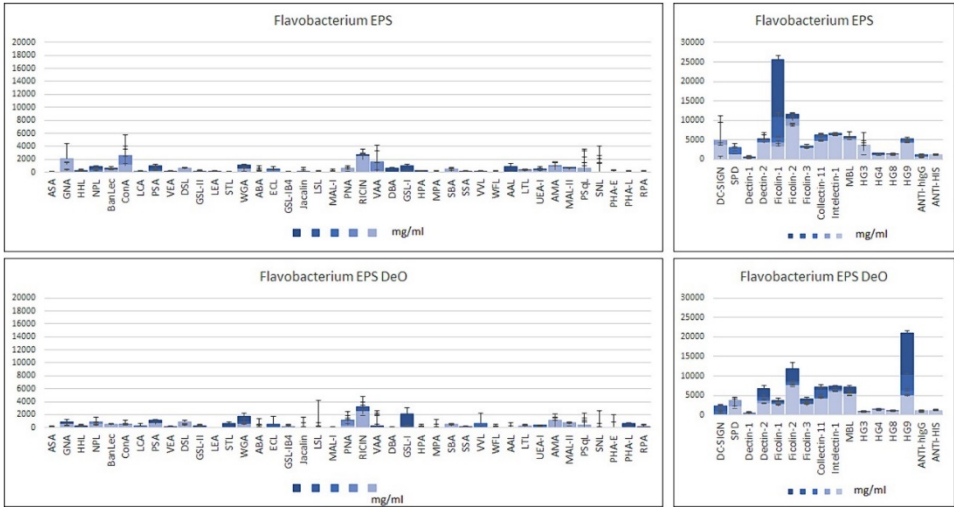


Figure 8.5. Lectin binding to microarray-printed *Flavobacterium* sp. Root935 EPS and EPS_{deO}. The samples were printed as triplicates at different concentrations (highlighted from light to dark blue colors) and the binding of two different collections of lectins was assayed, using AF647-streptavidin for final detection. Left panels: Binding of biotin-labelled model lectins with diverse carbohydrate-binding specificities. Right panels: Binding of Fc- or His-tagged innate immune lectins. Blank experiments, in the absence of lectins, with biotinylated goat anti-human IgG antibody and with mouse anti-His antibody pre-complexed with biotinylated goat anti-mouse IgG antibody (see Materials and Methods) were run in parallel. Data shown correspond to the mean of the fluorescence signals obtained for samples printed at 1 (■), 0.3 (■), 0.1 (■), 0.03 (■) and 0.01 (■) mg ml⁻¹ and error bars indicate the standard deviation of the mean.

Such differences in binding specificities may account, at least in part, for the different binding intensities here observed, and the extended *O*-acetylation pattern of *Flavobacterium* EPS might account for the high affinity toward Ficolin-1. In contrast, lectin binding profile observed for *Flavobacterium* EPS_{deO} depicted galectin-9 as dominant recognizing lectin.²⁵ Although all galectins share galactoside-binding ability, their fine carbohydrate-binding specificity may differ

significantly; this might explain why, among the tested, only galectin-9 recognized the epitope exposed after de-O-acylation in *Flavobacterium* EPS_{deO}.

To analyze the immunogenic potential of the natural EPS of *Flavobacterium* sp. Root935 and the impact of O-acylation, its ability to elicit early immune responses such as production of apoplastic reactive oxygen species (ROS) and cytosolic calcium influx in *Arabidopsis thaliana* was evaluated (**Figure 8.6A**). Whereas only low accumulation of ROS was observed with natural EPS, de-O-acylation resulted in significantly increased ROS accumulation in *Arabidopsis thaliana* leaves, indicating that O-acylation reduces the lectin recognition of the EPS. Previously, we showed that O-acylation also reduced perception of LPS isolated from another member of the *Arabidopsis* microbiome, *Herbaspirillum* Root189.²³ However, the ROS accumulation induced by de-O-acylated *Flavobacterium* sp. Root935 EPS was not as strong as with de-O-acylated *Herbaspirillum* Root189 LPS, suggesting a lower immunogenic potential for this carbohydrate structure. Furthermore, no cytosolic calcium influx was observed in *A. thaliana* leaves with either natural or de-O-acylated *Flavobacterium* sp. Root935 EPS (**Figure 8.6B**). In addition to EPS, lipooligosaccharide (LOS) isolated from *Flavobacterium* sp. Root935 was also tested, but did not induce ROS accumulation or calcium influx. The lack of cytosolic calcium influx in *A. thaliana* by *Flavobacterium* EPS indicates that it can sequester calcium ions and suppress calcium influx as was shown before for other EPS,¹⁴ thereby leading to a weaker ROS response as compared to flg22 which induces both calcium influx and ROS accumulation in *A. thaliana*; conversely a significant ROS accumulation was observed with *Flavobacterium* EPS_{deO}.

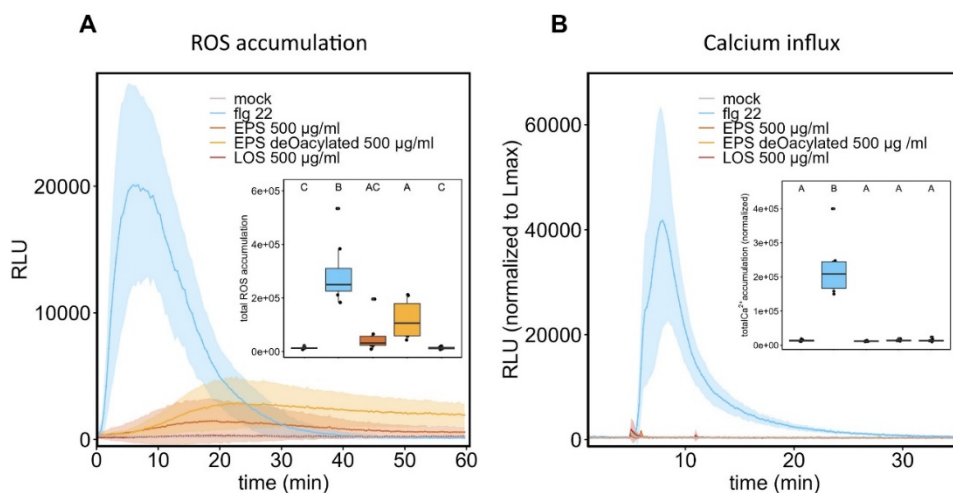


Figure 8.6. ROS accumulation and calcium influx in *Arabidopsis thaliana* in response to *Flavobacterium* sp. Root935 EPS, LOS and de-O EPS. ROS accumulation and calcium influx in *Arabidopsis thaliana* in response to *Flavobacterium* sp. Root935 EPS, LOS and de-O EPS. Apoplastic ROS accumulation **(A)** and cytosolic calcium influx **(B)** after treatment of *A. thaliana* Col-0^{AEQ} leaf discs from 28-days old plants or 12 days old seedlings, respectively, with 500 µg/ml of LOS, EPS and de-O-acylated EPS. 2.5 mM MES pH 5.6 and 250 nM flg22 were used as negative and positive control, respectively. ROS accumulation was monitored via a luminol-based chemiluminescence assay. Ca²⁺ influx was monitored via a coelenterazine-based chemiluminescence assay. Values are normalized to maximum total luminescence induced by addition of discharge solution, representing the aequorin expression, for each replicate. Values represent mean $\pm$ SD from eight replicates. Boxplots display total ROS accumulation or calcium influx over the measured period of time. Different letters represent statistically significant differences in expression based on a one-way ANOVA and Tukey's post hoc test (significance threshold: $P \leq 0.05$). ROS, reactive oxygen species. RLU, relative luminescence units.

8.6. Discussion and conclusions

Flavobacterium strains beneficially impact plant health and represent a significant fraction of root and leaf associated microbiomes in a broad range of plant species. Understanding how bacterial envelope glycans contribute to bacteria colonization, molecular features that mediate interactions with the host and allow bacteria to thrive in this competitive environment needs to be fully understood. Here, we revealed the chemical structure of the EPS produced by *Flavobacterium* sp. Root935, characterized by a branched, heptasaccharidic

repeating unit, with an extended hydrophobic interface and an elongated, left-handed helicoidal arrangement that expose acetyl and lactic groups outward the saccharide chain, therefore tuning its properties and masking *Flavobacterium* sp. Root935 EPS to the host. The morphology was characterized by irregular flakes, which could be compatible with the high viscosity of EPS and its chemical nature, but rheological studies of these characteristics should be conducted. Furthermore, the related high intrinsic viscosity of *Flavobacterium* EPS could represent an opportunity to produce homogeneous films by solvent casting or other deposition techniques, which could be a good starting point to study its potential in the food and pharmaceutical industries.^{26,27}

Microarray binding assays revealed that most lectins had weak or negligible binding to *Flavobacterium* EPS, likely due to its complex structure and its decoration with multiple acetyl groups. Ficolins are known to bind to specific carbohydrate structures, and their interactions can provide insight into the structural composition of bacterial surfaces, including the presence of acetyl groups. Indeed, significant binding signals were almost exclusively restricted to the innate immune lectins ficolins 1 and 2, indicating the dominance of acetyl groups in lectin recognition. This highlights the importance of acetylation in masking eukaryotic recognition. Indeed, fully acetylated *Flavobacterium* EPS was a poor inducer of plant immune responses, differently from EPS_{deO}. The findings suggest that *Flavobacterium* EPS exerts a significant impact by suppressing the cytosolic calcium influx in *A. thaliana*, thereby affecting the plant immune response. This suppression is achieved through apoplastic calcium sequestration and reduction of subsequent signaling. This confirms previous observations in other plant associated bacterial species, like *Herbaspirillum*, *Rhizobia*, that indeed EPS acetylation acts as a shielding strategy, to tune immune recognition of the bacterial envelope by the plant immune system and mediate interactions with the host environment.

In conclusion, the study takes a multidisciplinary approach aimed at analyzing structure, properties, and immune recognition of *Flavobacterium* sp. Root935 EPS, to dissect the structure-to-function relationships of the active glycan constituents of *Flavobacterium*. This offers valuable information to harness the potential of beneficial bacteria and to exploit the unique characteristics of *Flavobacterium* for sustainable and environmentally friendly practices in plant-microbe interactions.

8.7. References

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CHAPTER IX

Materials and methods

9.1. Bacterial strains, growth conditions and reagents

All bacteria analysed in this thesis were kindly provided by collaborators.

B. eggerthii 1_2_48FAA (**Chapter III**) was provided by Prof. Nathalie Juge team from Quadram Institute Bioscience and was grown under strict anaerobic conditions in BHI-YH (Brain heart infusion broth (Oxoid Ltd, UK) supplemented with 5 g/L Bacto™ yeast extract (Becton, Dickinson and company, Sparks, MD) and 5 mg/L hemin (Sigma-Aldrich, USA) for 18 h until reaching stationary phase and the cell pellet was collected from large-scale cultures (8 L) following centrifugation at 8000 x g for 15 min, before freeze drying. For *Akkermansia muciniphila* (**Chapter IV**) was provided by Prof. De Vos team from Wageningen University and was grown in anaerobic serum bottles sealed with butyl-rubber stoppers at 37 °C with N₂:CO₂ (80:20 ratio) in the headspace at 1.5 atm. The bacterial pre-cultures were prepared by overnight growth in: minimal media supplemented with type III hog gastric mucin (Sigma-Aldrich, St. Louis, USA).¹ Growth was measured by spectrophotometer as optical density at 600 nm (OD600) (OD600 DiluPhotometer™, IMPLÉN, Germany). *Pseudoruminococcus massili* E10-96H (**Chapter V**) was isolated from the feces of a healthy, pre-screened fecal donor. The use of the donor sample was approved by the Ethics Committee of Hospital District of Helsinki and Uusimaa Finland (DnroHUS124/13/03/01/11). The donor provided a written informed consent. The frozen fecal solution (saline-10% glycerol) was thawed anaerobically and serially diluted in PBS. The sample was treated with 70% ethanol (1:1) for four hours under aerobic conditions and grew most successfully on fastidious anaerobe agar (FAA) medium as well as in Gifu anaerobic medium (GAM) and reinforced clostridial medium (RCM) broths under anaerobic, but not under aerobic or microaerophilic atmosphere, indicating a strictly anaerobic lifestyle. Cultivation on semisolid GAM agar yielded the strongest growth of the strain. *P. massiliensis* E10-96H appeared in the Gram stain as a Gram-negative diplococcus. After 96

hours of anaerobic incubation, colonies were picked, re-streaked on new agar plates and purified.

9.2. Extraction and purification of R-LPS and S-LPS

Two main procedures were employed for the LPS extraction in this work. The PCP extraction that requires the preparation of a Phenol/Chloroform/Light Petroleum solution (2:5:8 v/v/v). In this solution the cells are solved at room temperature and stirred for 90 minutes. The mixture then is centrifuged for 30 minutes at $8,000 \times g$. After, the supernatant is collected in a round bottom flask, and the cells are subjected to two other extractions repeating the previous step. To isolate the phenol, the solution undergoes a rotavapor step where is concentrated and the chloroform and the petroleum are removed. The phenol solution is now transferred in a falcon where a few drops of water are added. Another centrifugation step is performed ($8,000 \times g$, 1 h, RT) to pellet any precipitate containing the LPS.² The other extraction method is the hot phenol/water procedure, following the PCP extraction or directly on the freeze-dried bacterial pellet. The initial material is suspended in warm water and the equal volume of warm phenol and is stirred for 120 min at 70 °C, then is centrifuged ($8,000 \times g$, 30 mins, 4 °C). The supernatant, formed by the water phase, is collected and the same volume of warm water is added to repeat the extraction two times more. Finally, the reunited water phases and the phenol are dialysed in separate containers against water (12-14 KDa). After 7 days the phases are collected and freeze-dried.³ Both the aqueous and the phenol phase are then subjected to enzymatic digestion with DNase (Sigma-Aldrich, Darmstadt, Germany) and RNase (Sigma-Aldrich, Darmstadt, Germany) followed by proteinase K (Sigma-Aldrich, Darmstadt, Germany) (310 K and 329 K).⁴

To check the nature of the material extracted, a 13,5% SDS-PAGE gel is prepared and stained via Alcian blue or silver nitrate.^{5,6} To evaluate possible protein contamination a staining using Coomassie Blue Solution (0.5% w/v Coomassie Brilliant Blue G-250 w/v in 40% methanol, 5% acetic acid) is used.⁷ Further purifications of the LPS material are also carried out. The methods will be summarised below for each project.

9.3. Isolation of the OS_{deAc} fractions of the LPS: *Bacteroides eggerthii* (Chapter III), *Akkermansia muciniphila* (Chapter IV) and *Fusobacterium gonidiaformans* (Chapter VI).

To determine the structure of the core OS, an aliquot of LOS (25 mg) was subjected to *O*-deacylation with anhydrous hydrazine (1 mL, at 37 °C for 90 min). The suspension was cooled and transferred into ice-cold acetone to enable the precipitation. The precipitate was then centrifuged (4 °C, 5000 × g, 30 min), rinsed with ice-cold acetone, dried, dissolved in water, and freeze-dried. Subsequently, the *O*-deacylated product underwent *N*-deacylation using 4 M KOH (120 °C, 16 h) obtaining the product OS_{deAc}. Salts were eliminated using gel-filtration chromatography on a Sephadex G-10 column (Pharmacia, 50 × 1.5 cm).

9.4. Chemical analysis

9.4.1. Acetylated *O*-methyl glycosides (AMG)

An aliquot (~ 0.5 mg) of the sample of interest (purified LPS, OS, O-antigen) was treated with 300 µL of 1.25 M HCl/CH₃OH (80 °C, 16 h). The solution was then neutralized via methanol washes and after was acetylated with acetic anhydride (25 µL) and pyridine (25 µl) (80 °C, 30 min). The obtained acetylated sugars were extracted using chloroform and water. Finally, the chloroform was

separated and dried, obtaining like this the purified acetylated sugars which were diluted in 100 μ L of acetone and analysed by GC-MS.⁸

9.4.2. Partially methylated alditol acetates (PMAA)

An aliquot of sample (~1 mg) was suspended in DMSO, then added powdered NaOH and stirred 1,5 h at room temperature. Afterwards, methylation was carried out with CH_3I , followed by hydrolysis with trifluoroacetic acid (4 M, 100 °C, 4 h) and dried under airflow, and neutralised with methanol washes. Then a carbonyl reduction with NaBD_4 stirring in ethanol (16 h, RT). The reduction was stopped by the addition of a few drops of HCL (2 M) and then neutralized with methanol. The acetylation was carried out with pyridine and acetic anhydride (80 °C, 30 minutes) followed by GC-MS study.

9.4.3. Acetylated alditols (AA)

An aliquot (~0.5 mg) was treated with TFA (2 M, 2 h, 120 °C) and then dried under airflow. The monosaccharides were reduced with NaBH_4 in ethanol in order to obtain alditols, and then acetylated with 25 μ L of acetic anhydride and 25 μ L of pyridine (80 °C, 30 minutes). The acetylated alditols were dried under airflow and diluted in 100 μ L of acetone and analysed by GC-MS.

9.4.4. Octyl-glycosides

To the aliquot (~1 mg) 100 μ L of enantiopure 2-(+ or -)-octanol were added. In a separate tube, 100 μ L of racemic 2-($\pm$)-octanol were added to another aliquot. In both samples, were added 15 μ L of acetyl chloride (60 °C, 16 h). Then, the samples were dried under airflow and acetylated with acetic anhydride in pyridine (80 °C, 30 minutes). The samples were injected into the GC-MS with known standards for comparison.⁹

9.4.5. Gas Chromatography Mass Spectrometry

All chemical analyses were conducted on a gas-chromatograph Agilent Technologies 6850A (Santa Clara, CA, USA) equipped with a mass selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., 0.25 µm as film thickness, flow rate 1 mL/min, He as carrier gas). The temperature program 413 K for 5 min, 413 K→553 K at 10 °C/min, and 553 K for 10 min was used for lipid analysis, and for sugar analysis the temperature program was 423 K for 5 min, 423 K→553 K at 3 °C/min, and 553 K for 5 min.

9.5. Isolation of lipid A

An aliquot of the extracted LPS was treated with acetic acid at 1%, 100 °C for 2 h to allow the separation between the lipid A and the core OS portion. To this aim, the solution was extracted three times with CHCl₃/MeOH/H₂O (100:30:30, v/v/v) and was subsequently centrifuged (4 °C, 8000 × g, 60 min). The lipid A-containing organic phases were then subjected to three washes with distilled water and then freeze-dried obtaining the isolated lipid A. The final product was analysed by MALD-TOF MS and MS² to define the chemical structure of the lipid A.

Dephosphorilation of lipid A and derivatization to alditol acetates

In **Chapter III**, *B. eggerthii*, to establish the nature of the hexosamines and Kdo decorating the *B. eggerthii* lipid A. A small aliquot (0.6 mg) of the LPS product was treated with 100 µL of 48% aqueous hydrofluoric acid (HF) (Sigma Aldrich®, St. Louis, MO, USA) at 4° C for 16 h to remove the phosphate groups. The sample was placed in an iced bath and then the HF was evaporated until the sample was dried. The resulting product was then dissolved in distilled water and freeze-dried.

9.6. Nuclear Magnetic Resonance (NMR)

All the structural assignments of the oligosaccharidic portion from *B. eggerthii* LOS (**Chapter III**), the oligosaccharide chain sequence from *A. muciniphila* (**Chapter IV**), the repeating unit from *P. massiliensis* CPS (**Chapter V**), and the LPS from *F. gonidiaformans* (**Chapter VI**) were studied as followed.

NMR spectra were recorded in D₂O at 298 K at pD 7 with a cryoprobe-equipped Bruker 600 AVANCE NEO. Total correlation spectroscopy (TOCSY) experiments were conducted with spinlock times of 100 ms using data sets (t1×t2) of 4096×512 points. Rotating-frame Overhauser enhancement spectroscopy (ROESY) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments were conducted with data sets (t1×t2) of 4096×512 points and mixing times ranging from 100 to 400 ms. Double-quantum-filtered phase-sensitive correlation spectroscopy (DQF-COSY) experiments were carried out using data sets of 4096×912 points. The data matrix in all homonuclear experiments underwent zero-filling in both dimensions to give a matrix of 4 K × 2 K points. Subsequently, resolution enhancement was applied in both dimensions using a cosinebell function prior to Fourier transformation. To determine coupling constants, 2D phase-sensitive DQF-COSY measurements were used. Heteronuclear single-quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments were performed in the ¹H-detection mode using single-quantum coherence with proton decoupling in the ¹³C domain employing data sets of 2048×400 points. For HSQC, sensitivity improvement and phase-sensitive mode was employed, along with echo/antiecho gradient selection and multiplicity editing during the selection step. In the HMBC experiments, optimization was carried out for long-range coupling constants, incorporating a low-pass J filter to eliminate one-bond correlations, applying gradient pulses for selection. Moreover, a 60 ms delay was implemented for the evolution of long-range correlations, with spectra optimization for 6–15 Hz coupling constants. To

enhance resolution, the data matrix in all the heteronuclear experiments was expanded to 2048×1024 points via forward linear prediction extrapolation.^{10,11}

9.7. MALDI-TOF Mass Spectrometry and MS²

The MS procedure was performed on ABSCIEX TOF/ TOF™ 5800 Applied Biosystems mass spectrometer equipped with an Nd:YAG laser ($\lambda = 349$ nm), with a 3 ns pulse width and a repetition rate of up to 1000 Hz. Each spectrum was the product of the accumulation of 1500 laser shots, whereas 6000–7000 shots were summed for the MS² data acquisitions. All the experiments were acquired in both negative and positive polarity, reflectron mode. For MS spectra accumulation of 2000 laser shots was required, whereas 5000–7000 shots were summed for the MS/MS spectra. Each experiment was performed in replication.

An aliquot of LPS was suspended in ethylenediaminetetraacetic acid (5 mM EDTA), shaken and left a few seconds in an ultrasonic bath. Then, LPS was desalted with cation exchange beads (Dowex 50X, NH_4^+) and then added to an equal volume of 2,5-dihydroxybenzoic acid (DHB) in 0.1 % citric acid. 1 μL of LOS/DHB mixture per well was transferred to the stainless-steel MALDI plate. MS spectrum of LPS was recorded in reflectron mode and negative ion polarity.¹² As for the analysis of OS_{deAc} fractions, sample was dissolved in water (1 mg/mL) and DHB in 0.1 % TFA/CH₃CN (80:20) was used as the matrix. 0.5 μL of sample and 0.5 μL of matrix solution were spotted on the plate and left to dry at room temperature. The spectra were recorded in negative polarity reflectron mode.

As for the lipid A fractions, this was dissolved in CHCl₃/CH₃OH (1:1, v/v). The matrix solution was 2,4,6-trihydroxyacetophenone in CH₃OH/0.1% trifluoroacetic acid/CH₃CN (7:2:1, v/v) at a concentration of 75 mg/mL.^{13,14} Also in this case, 0.5 μL of the sample and 0.5 μL of the matrix solution were deposited on the MALDI plate and left to dry at room temperature. For MS experiments each spectrum was a result of the accumulation of 2000 laser shots, whereas 5000–7000 shots were summed for the MS/MS spectra.

9.8. HEK-Blue™ hTLR4/CD14/MD-2 and hTLR2 cell culture, transfection and stimulation

HEK-Blue™ hTLR4 and HEK-Blue™ hTLR2 cells (InvivoGen, San Diego, CA, USA) were cultured at 310 K in 5% CO₂ using Dulbecco's minimal essential media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Microgem, Naples, Italy), 1% glutamine (Himedia Einhausen, Germany), 1% penicillin/streptomycin (Himedia, Germany), and 100 µg/mL Normocin (InvivoGen). Plasmid selection in HEK-Blue™ hTLR4 and hTLR2 cells needed the use of a mixture of selective antibiotics (HEK-Blue™ Selection) (InvivoGen), whereas HEK-Blue Null2™ cells required the use of 100 µg/mL Zeocin (InvivoGen).

For stimulation assays, HEK-Blue™ hTLR4 cells were seeded into 96-well plates (3×10^4 cells per well) and incubated with different concentrations of *B. eggerthii* 1_2_48FAA LOS (Chapter III), CPS from *P. massiliensis* (Chapter V) or LPS from *F. gonidiaforman* (Chapter VI) or *E. coli* 0111:B4 LPS (Invivogen, Ultrapure) (1, 10, 100 ng/mL) for 18 h to analyze NF-κB activation by evaluating NF-κB-dependent secreted alkaline phosphatase (SEAP) using QUANTI-Blue™ (InvivoGen). Likewise, HEK-Blue™ hTLR2 cell lines were treated for 18 h and analyzed as above. HEK-Blue™ hTLR2 cells were also stimulated with Pam3CSK4 (500 ng/mL) (InvivoGen) that was used as the positive control for this experiment.

9.9. moDCs stimulation test and flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from the blood bank of Sanquin Amsterdam, The Netherlands, from healthy adult volunteers (blood donors) following written informed consent in accordance with the Declaration of Helsinki. PBMCs were isolated through

density gradient centrifugation employing Lymphoprep (Serumwerk Bernburg AG). For dendritic cells, CD14⁺ monocytes were isolated using the MACS microbead system (Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytes were cultured for 5 days in RPMI 1640 (Gibco) supplemented with 10 % heat-inactivated FBS (Corning), 1000 U/mL penicillin and 1000 U/mL streptomycin (Thermo Fisher), 500 U/ml IL4 and 800 U/ml GM-CSF (both Miltenyi Biotec) and 2 mM glutamine, at concentration 1.25×10^6 in order to obtain monocyte-derived dendritic cells (moDCs). For stimulation, moDCs were seeded at day 6 in 96-well plates (5×10^5 cells/well) and exposed to 1, 10 and 100 ng/mL of *B. eggerthii* 1_2_48FAA LOS (**Chapter III**), CPS from *P. massiliensis* (**Chapter V**) or LPS from *F. gonidiaforman* (**Chapter VI**) or 10 ng/mL *E. coli* LPS 0111:B4 ultrapure (InvivoGen).

Treated moDCs were harvested and washed with PBS. Following incubation with anti-CD16/32 Fc block (1:500, Biolegend) and live-dead staining with eFluor780 (1:1000, Thermo Fisher), cells were stained with the following antibodies at the respective dilutions: anti-CD14 Alexa Fluor 700 1:100 (Biolegend), anti-HLA-DR BV510 1:200 (BD Biosciences), anti-CD80 FITC 1:100 (BD Biosciences), anti-CD40 PE-Cy7 1:200 (Biolegend) and anti-CD86 BV650 1:50 (Biolegend). After incubation for 30 min on ice in the dark, cells were washed and fixed with paraformaldehyde 2% (Electron Microscopy Sciences). Fluorescence intensities were measured using the Fortessa X-20 (BD Biosciences) and analyzed with FlowJo V.10.

Data are presented as mean $\pm$ S.D. of 3 independent donors. Experimental data were analyzed by one-way ANOVA corrected for multiple comparisons (Dunnett's multiple comparison test), with a statistical significance level of $\alpha=0.05$.

9.10. *In silico* analyses

For *B. eggerthii* 1_2_48FAA LOS and *Flavobacterium* sp. Root935 Molecular mechanics calculations were performed using the MM3* force field as included in MacroModel 8.0 from Maestro package. A di-electric constant of 80 was applied and the disaccharide structures were explored with an extended nonbonded cut-off distances (a van der Waals cut-off of 8.0 % and an electrostatic cut-off of 20.0 %). Each disaccharide structure underwent incremental variation of both Φ and Ψ , using a grid step of 18° . Every point (Φ, Ψ) on the map was then optimized using 2000 P.R. conjugate gradients.

MD simulations were carried out using AMBER 18 suite of programs.¹⁵ Parameters for non-standard residues, i.e. Kdo, GlcN and Galf, which are not included in the GLYCAM-web site, were parametrized and used as building blocks to construct the entire OS. Atom types and charges were designated in accordance with AMBER GLYCAM-06j-1 force field. By using the Leap module, the ligands were hydrated with truncated-octahedral boxes containing explicit TIP3P water molecules 15 Å away from any atom, also, counter ions were added to neutralize the system. After the preparation of the input files, an energy minimization process was performed to refine the initial structure. The SHAKE algorithm was applied to all hydrogen containing bonds, and a 1 fs integration step was used. Long-range electrostatic interactions were evaluated using periodic boundaries in conjunction with particle-mesh Ewald summation. The simulation was conducted under isothermal and isobaric conditions. Temperature was maintained at 300 K with the Langevin thermostat applying a collision frequency of 1.0 ps⁻¹ while pressure was kept at 1.0 atm. Energy minimization of the solvated systems was performed with solute restraints that were decreasingly gradually from 100 kcal/mol to no restraints. Temperature was increased from 0 K to 100 K at constant volume. Then, from 100 K to 300 K temperature in an isobaric ensemble. Thereafter, temperature was kept constant at 300 K during 50

ps with progressive energy minimizations and a solute restraint. After the restraints were removed, the systems progressed in an isothermal-isobaric ensemble during the production phase. Trajectory coordinates were sampled every ps to acquire 10000 structures of the progression of the dynamics. The stability of energy, pressure, temperature and other thermodynamic parameters were monitored along the trajectory and then, RMSD torsion values, cluster distances and hydrogen bonds were extracted by using the Cpptraj module. Trajectories were visualized with VMD molecular visualization program¹⁶ and analyzed with the ptraj module included in the AMBER18. Pymol 2.3 Software (<https://pymol.org/2/>) and Discovery Studio Software were used to visualize and draw the molecules.

9.11. References

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