Stockholm University

Bacteria-host cell interactions

Studies on initial colonization, antimicrobial peptides, and biofilms

Gabriela Wassing

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Abstract

The obligate human pathogen *Neisseria meningitidis* asymptomatically colonizes the upper respiratory tract, but crossing of the epithelial barrier can cause life-threatening meningitis and/or sepsis. *N. meningitidis* encounters numerous environmental challenges during colonization in the host, and has evolved different evasion strategies and virulence factors to ensure its survival. In contrast, *Lactobacillus* species are part of the human microbiota and their commensal colonization confers many benefits to the host, including the inhibition of pathogens.

The first cell type encountered by invading bacteria are epithelial cells and immune cells, which can effectively sense and respond to the presence of bacteria by alerting the immune system or by release of antimicrobial peptides. Antimicrobial peptides are small peptides that are able to directly kill bacteria, but also play a role in modulation of immune responses.

This thesis focuses on the interaction between the human host and bacteria. **Paper I** shows that epithelial colonization by different bacterial species induces the transcription factor early growth response protein 1 (EGR1). Induction of EGR1 is mediated primarily by signaling through EGFR and ERK1/2 pathway. In **paper II** the ability of *N. meningitidis* and *Lactobacillus* to modulate expression of antimicrobial peptide human beta-defensin 2 (hBD2) in epithelial cells is compared. Expression of hBD2 is upregulated by lactobacilli. In contrast, *N. meningitidis* dampens this effect, likely mediated by induction of the host molecule A20, a negative regulator of NF-κB. Since *N. meningitidis* is susceptible to hBD2-mediated killing, exploitation of A20 may be an immune evasion mechanism. In **paper III** we demonstrate that hBD2 is able to kill *N. meningitidis* without causing membrane permeabilization. *N. meningitidis* DNA can bind hBD2 and thereby inhibit hBD2-mediated killing, presenting a possible evasion mechanism. Finally, **paper IV** shows that the absence of D-lactate dehydrogenase LdhA in *N. meningitidis* promotes aggregation and biofilm formation through increased autolysis-mediated release of extracellular DNA.

Keywords: *Neisseria meningitidis, Lactobacillus, host responses, EGR1, antimicrobial peptides, hBD2, A20, biofilm.*

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Cover illustration by Gabriela Wassing. Taken in Lappis forest on my usual running loop, September 2019.

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To my parents

"'When someone is searching,' said Siddartha, 'then it might easily happen that the only thing his eyes see is that what he searches for, that he is unable to find anything, to let anything enter his mind, because he always thinks of nothing but the object of his search, because he has a goal, because he is obsessed by the goal. Searching means: having a goal. But finding means: being free, being open, having no goal.'"

Hermann Hesse, *Siddartha*

SUMMARY

The obligate human pathogen *Neisseria meningitidis* asymptomatically colonizes the upper respiratory tract, but crossing of the epithelial barrier can cause life-threatening meningitis and/or sepsis. *N. meningitidis* encounters numerous environmental challenges during colonization in the host and has evolved different evasion strategies and virulence factors to ensure its survival. In contrast, *Lactobacillus* species are part of the human microbiota and their commensal colonization confers many benefits to the host, including the inhibition of pathogens.

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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Neisseria meningitidis är en bakterie som kan orsaka allvarlig sjukdom hos människor. Dessa bakterier finns i svalget hos några procent av befolkningen. Vanligtvis orsakar de inga symtom, men i sällsynta fall kan de orsaka livshotande blodförgiftning och hjärnhinneinflammation. För att överleva i den mänskliga värden har *N. meningitidis* utvecklat speciella mekanismer. Andra bakterier som finns i människokroppen är lactobaciller, som ger fördelar för vår hälsa.

När bakterier invaderar människokroppen är en av de första celltyperna de möter epitelceller. Epitelcellerna bildar en fysisk barriär som skiljer "insidan" från "utsidan". När bakterier når epitelcellerna aktiveras immunsystemet och molekyler såsom antimikrobiella peptider börjar produceras. Antimikrobiella peptider kan avdöda bakterier och bidrar på detta sätt till att hålla oss friska.

I denna avhandling undersöker vi samspelet mellan bakterier och värd. I **artikel I** visar vi att epitelceller svarar på närvaron av ett brett spektrum av bakterier genom att stimulera produktionen av EGR1. EGR1 är ett protein som är inblandat i celltillväxt och andra viktiga processer. I **artikel II** visas att lactobaciller stimulerar produktionen av den antimikrobiella peptiden hBD2, medan *N. meningitidis* dämpar den. Vi fann att *N. meningitidis* är känslig för hBD2, och dämpning av hBD2 kan vara en mekanism för att bättre överleva i värden. I **artikel III** undersöker vi hur hBD2 avdödar *N. meningitidis*. Vi visar att *N. meningitidis* DNA kan binda till hBD2, vilket minskar förmågan hos hBD2 att avdöda *N. meningitidis*. Slutligen i **artikel IV** visar vi att bristen på ämnesomsättningsenzymet LdhA i *N. meningitidis* kan stimulera uppkomst av biofilmer. Biofilmer är kluster av bakterier täckta med ett slemlager som ger skydd mot närmiljön. Bakterier som saknade LdhA kunde bilda biofilmer genom att medvetet dö och gå sönder för att frigöra DNA som bildade slemlagret.

POPULAR SUMMARY IN ENGLISH

Neisseria meningitidis is a bacterium that can cause severe disease in humans. These bacteria are found in the back of the throat. Usually they do not cause any symptoms, but in rare cases they can cause life-threatening meningitis and sepsis. To survive in the human host, *N. meningitidis* has evolved special mechanisms. Other bacteria found in the human body are lactobacilli, which provide benefits to our health.

When bacteria invade the human body, one of the first cell types they encounter are epithelial cells. Epithelial cells form a physical barrier that separates the "inside" from the "outside". Detection of bacteria by epithelial cells will lead to activation of the immune system, and production of molecules such as antimicrobial peptides. Antimicrobial peptides can kill bacteria, and in this way contribute to keeping the host healthy.

In this thesis, we investigate the interaction between bacteria and the host. In **paper I** we show that epithelial cells respond to the presence of a broad range of bacteria by stimulating the production of EGR1. EGR1 is a protein that is involved in cellular growth and other important processes. **Paper II** shows that lactobacilli stimulate the production of the antimicrobial peptide hBD2, while *N. meningitidis* dampens it. We found that *N. meningitidis* is sensitive to the killing activity of hBD2, and the repression of hBD2 could represent an evasion mechanism to better survive in the host. In **paper III** we better examine how hBD2 kills *N. meningitidis*. We show that *N. meningitidis* DNA can bind to hBD2, which reduces the capacity of hBD2 to kill *N. meningitidis*. Finally, in **paper IV**, we demonstrate that the lack of metabolic enzyme LdhA in *N. meningitidis* can stimulate the bacteria to form biofilms. Biofilms are clusters of bacteria covered with a slimy layer that protects these communities against their local environment. Bacteria lacking LdhA were able to form biofilms by purposefully dying and breaking open to release DNA that formed the slimy layer.

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* These authors contributed equally

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ABBREVIATIONS

AMP	Antimicrobial peptide
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
eDNA	Extracellular DNA
EGR1	Early growth response protein 1
EPS	Extracellular polymeric substances
fHbp	Factor H binding protein
hBD	Human beta-defensin
HD	Human defensin
HNP	Human neutrophil peptide
IKK	IkB kinase
IL	Interleukin
KDO	2-keto-3-deoxy-manno-octulosonic acid
LBP	LPS binding protein
LbpB	Lactoferrin-binding protein B
LctP	Lactate permease
LDH	Lactate dehydrogenase
LdhA	Cytoplasmic D-lactate dehydrogenase
LdhD	Respiratory D-lactate dehydrogenase
LldA	Respiratory L-lactate dehydrogenase
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
NadA	*Neisseria* adhesin A
Neu5Ac	N-acetylneuraminic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NhbA	Neisserial heparin-binding antigen
OMV	Outer membrane vesicle
PAMP	Pathogen associated molecular pattern
PEA	Phosphoethanolamine
PRR	Pattern recognition receptor
ST	Sequence type
T4SS	Type IV secretion system
TbpB	Transferrin-binding protein B
TCS	Two-component system
Tfp	Type IV pili
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFAIP3	TNF-alpha induced protein 3

INTRODUCTION

Microorganisms are defined as those organisms too small to be seen clearly by the unaided eye. The first person to observe bacteria was the 17th century scientist Antoni van Leeuwenhoek, who referred to bacteria as "tiny animals". Today, many thousands of known species of bacteria are recognized that can grow in almost any environment.

The human body is host to a diverse community of bacteria. While some of these can cause disease, many are beneficial to us. The human immune system is well-equipped at keeping these bacteria at a safe distance. By sensing specific bacterial surface components, receptors on epithelial and immune cells can trigger a signaling cascade that ultimately results in clearance of the infection.

This thesis focuses on the interaction between the human host and bacteria. **Paper I** and **paper II** compare the effect of commensal and pathogenic bacterial stimulation on the expression of early growth response protein 1 and antimicrobial peptides. In addition, *Neisseria meningitidis* physiological responses to host and environmental factors are characterized. **Paper III** examines the killing effect of antimicrobial peptide human beta-defensin 2 on *N. meningitidis*. In **paper IV** the role of lactate metabolism in biofilm formation of *N. meningitidis* is determined.

Chapter 1: *Neisseria meningitidis*

The *Neisseria* genus belongs to the *Neisseriaceae* family and consists of gram-negative, aerobic diplococci that colonize humans and animals. Most species form part of the normal oral and nasopharyngeal microflora. Only *Neisseria gonorrhoeae* and *Neisseria meninigitidis* have been classified as pathogens [1]. These species are human obligates and share 90% genome identity, as well as several virulence factors [2,3]. Despite their similarity, *N. meningitidis* and *N. gonorrhoeae* differ in their colonization sites and disease outcome. *N. gonorrhoeae* (gonococci) colonize the urogenital tract and cause the sexually transmitted disease gonorrhea, while *N. meningitidis* (meningococci) colonize the upper respiratory tract and cause meningitis or sepsis [4]. This thesis will focus solely on *N. meningitidis*.

1.1 Classification

N. meningitidis species are divided into serogroups based on the structure of the polysaccharide capsule [5]. Serogroups can be further subdivided into serosubtype, serotype, and immunotype, based on differences in porins (PorA, PorB) and lipopolysaccharide (LPS) structure [6]. Currently, 13 meningococcal serogroups are recognized, of which serogroups A, B, C, W, X, and Y have been identified to cause invasive disease, although with considerable differences in geographical distribution and disease potential [7]. Traditional serogroup identification is performed by slide agglutination or polymerase chain reaction [8]. The more advanced method of multilocus sequence typing, where strains are discriminated by polymorphisms in multiple housekeeping genes, allows for more accurate and precise identification of clonal complexes [9].

1.2 Epidemiology

The incidence of meningococcal disease varies widely depending on geographical location. Worldwide the incidence rate is estimated to be 1.2 million disease cases per year, with 135,000 deaths. In developed areas such as the US, Europe, and Australia, incidence rates range from 0.3 to 3 cases per 100,000 inhabitants, while in developing countries the incidence rate lies much higher. The “meningitis belt”, a region of sub-Saharan Africa extending from

Senegal to Ethiopia, has the highest incidence rate in the world and during epidemics can reach as high as 10-1000 cases per 100,000 inhabitants [10].

Meningococcal epidemics in sub-Saharan Africa are mostly associated with serogroups A and X [11]. However, since the introduction of serogroup A vaccine programs in 2010 disease incidence rates have declined dramatically. Instead, an increase in cases caused by other meningococcal serogroups (such as serogroup C) has been observed [12]. In industrialized countries serogroups B and C are responsible for the majority of disease, although incidence rates have decreased since vaccination programs started in 2000 [11]. However, an increase in disease incidence caused by serogroups Y and X has been observed instead [11,13].

1.3 Transmission and carriage

Transmission of *N. meningitidis* occurs primarily through direct contact with respiratory droplets or saliva. Transmission from contaminated surfaces is rare, but cases have been reported [14]. Following meningococcal acquisition the bacteria can colonize the nasopharynx for several days, or many months. Colonization is often asymptomatic and has been described in approximately 10% of the healthy population [15]. Carriage rate is highly varied among the population and is increased during adolescence and in individuals living in close proximity, such as military recruits [16,17].

Only very rarely does colonization lead to invasive disease. It is unclear what predisposes an individual to develop disease. Multilocus sequence typing has revealed that most cases of disease are caused by a few clonal complexes (referred to as hypervirulent strains) and that isolates belonging to these strains are underrepresented in asymptomatic carriers [18]. Apart from clonal virulence of the bacteria, genetic and immune deficiencies of the host play an important role [7]. Other risk factors include respiratory co-infections, tobacco smoke exposure, season, and socio-economic status [19-22].

1.4 Meningococcal disease

Following meningococcal colonization, bacteria very occasionally spread to surrounding surfaces and cause local infections, such as pneumonia, sinusitis, or otitis media. Meningococcal disease is initiated by bacterial penetration of the mucosa whereupon the bacteria gain access to the bloodstream. In rare

cases, meningococci are cleared spontaneously, although in most cases disease develops [23].

The onset of meningococcal disease is characterized by flu-like symptoms including headache, fever, chills, lower back pain and general muscle ache [6]. In 20-30% of infected individuals, disease develops into septicaemia (or meningococcemia). Early signs of meningococcemia symptoms in children and young adults are lower limb pain, cold extremities, and pale skin. In infants, drowsiness, fast breathing and diarrhea can also be observed [24]. Late signs of meningococcemia are the classic appearance of purpuric rash, occurring in 40-80% of cases with meningococcal disease, as well as confusion and delirium [24,25]. Within a few hours after detection of the first symptoms, fulminant meningococcal sepsis may develop, characterized by high concentrations of endotoxin and cytokines in the blood. Progression of fulminant meningococcal sepsis depends on endotoxin responsiveness of the host, and endotoxin release of the bacterial strain [26]. Excessive activation of the immune system subsequently leads to microvascular endothelial damage, clot formation and haemorraging, organ failure, and ultimately death [24]. The mortality rate of patients with meningococcemia lies at 20-80%, and survivors may require amputation of limbs due to tissue necrosis [26].

Meningococci have a propensity to invade the meninges, and meningococcal disease most commonly manifests as meningitis, affecting 30-60% of infected individuals. In children and young adults, symptoms include fever, headache, vomiting, neck stiffness, drowsiness, photophobia, and in some cases a rash develops [24]. However, in infants symptoms are less pronounced and may be limited to irritability, lethargy and neck stiffness [27]. To cause meningitis, bacteria must pass the blood-brain barrier into the subarachnoid space, where they are detectable in high concentrations in the cerebrospinal fluid. In contrast to meningococcemia, patients with meningitis have very low bacterial presence in the bloodstream [26]. Recruitment of immune cells into the brain causes cerebral inflammation, leading to increased intracranial pressure, reduced cerebral perfusion, and death. The mortality rate of patients with meningitis is 5-20%. Survivors may develop long-lasting conditions such as hearing loss and other neurological defects [24].

1.5 Treatment

Mortality rates of meningococcal disease have drastically decreased with the use of antibiotics. Early treatment is imperative, and antibiotics should be administered immediately in patients with suspected *N. meningitidis* infection. However, the initial non-specific symptoms pose challenges in diagnosis,

especially in children where symptoms may be less present [7]. Initial treatment usually consists of a broad-spectrum combination of antibiotics. Individuals who have been in close contact with meningococcal-infected patients are at high risk of developing meningococcal disease, and will receive immediate antibiotic prophylaxis treatment [6].

The recognition of different pathophysiologies is important to determine treatment and management strategy. Antibiotics effectively dampen bacterial proliferation, however additional therapy can be focused on reducing intracranial pressure during meningitis, or fluid substitution during septic shock [7].

Meningococcal antibiotic resistance is relatively uncommon. Evolution of resistance in *N. meningitidis* has been shown to occur through horizontal gene transfer, and mixed colonization of *N. meningiditis* with other pathogens or commensals in the nasopharynx may result in the uptake of foreign DNA [28]. In addition, the widespread antibiotic resistance displayed by the related species *N. gonorrhoeae* may increase the likelihood of the emergence of meningococcal resistant clones [29]. Reduced susceptibility of meningococci to penicillin has been reported in several countries, thus it is important to carefully monitor antibiotic susceptibility of *N. meningitidis* isolates [30].

1.6 Prevention through vaccination

Diverse surface antigen expression among *N. meningitidis* strains, as well as host mimicry of meningococcal surface molecules, have caused difficulties in vaccine development against *N. meningitidis* [31]. The polysaccharide capsule is the most attractive surface structure as target antigen since it is shared by all members of each serogroup [32]. However, most polysaccharide capsule vaccines have proven poorly immunogenic especially in infants, with rapidly declining antibodies already after 6-12 months of immunization [28]. To stimulate vaccine efficacy, polysaccharide-protein conjugates have been developed with polysaccharides conjugated to tetanus or diphtheria toxoid, with improved immunological memory. Meningococcal conjugate vaccines are now available in monovalent form (against serogroup A or C), quadrivalent form (against serogroup A, C, Y, and W), or in combination with *H. influenzae* vaccines [33]. A nationwide vaccination program in the UK between 1999 and 2000 with MenC conjugate vaccine successfully reduced 85% of serogroup C disease cases, and the vaccine has now been introduced into routine immunization schedules in most developed countries [34]. In 2010 a mass vaccination campaign was initiated in sub-Saharan Africa using MenAfriVac, a serogroup A polysaccharide conjugate. The incidence of group A disease was

reduced by more than 99% and resulted in reduced carriage rates, indicating a herd immunity effect [12,35]. Development of a vaccine against serogroup B has been considerably more challenging. The capsule expressed by serogroup B strains closely resembles the human neural cell adhesion molecule NCAM-1, explaining the poor immunogenicity of this antigen, and additionally raises the concern of autoimmunity if used in vaccines [31]. Vaccines based on serogroup B outer membrane vesicles (OMV) demonstrated a reduced rate of disease, however, these vaccines lacked broad protection against the global diversity of serogroup B strains [33]. In 2006 the MenB-4C (Bexsero) vaccine was developed which is active against four surface-exposed antigens of *N. meningitidis* serogroup B: *Neisseria* adhesin A (NadA), Neisserial heparin-binding antigen (NhbA), factor H binding protein (fHbp), and an OMV derived from a MenB outbreak strain [36]. MenB-4C has been authorized in the EU in 2013 and in the US in 2015 and is currently recommended in immunization programs in several countries. Recent evidence of vaccine efficacy has been positive, showing an 80% decline in serogroup B disease cases. Cross-protection against serogroup W as well as *N. gonorrhoeae* has also been observed [36]. In 2018 a second MenB vaccine was developed, rLP2086 (Trumenba), containing two fHpb variants [37].

Chapter 2: *N. meningitidis* virulence and host cell interactions

2.1 Virulence factors

N. meningitidis has evolved a number of mechanisms in order to survive in the host. The expression of several virulence factors contributes to host colonization, evasion of immune responses, and pathogenesis. The main virulence factors include the polysaccharide capsule, lipopolysaccharide, type IV pili and opacity proteins [38] (**Figure 1**). In addition, *N. meningitidis* relies on the two-component signaling system to quickly sense and respond to changing environmental conditions [39]. These components will be discussed in more detail below.

Apart from its virulence factors, *N. meningitidis* can easily acquire new traits through its natural competence to take up foreign DNA from both related bacterial species (e.g. *N. gonorrhoeae*, *N. lactamica*) and unrelated bacterial species (e.g. *Haemophilus*) through horizontal gene transfer [40]. A striking feature of the meningococcal genome is the abundance of repetitive DNA sequences, including DNA uptake sequences and insertion sequences, which are involved in the recognition and uptake of DNA from the environment, and the insertion of DNA into the meningococcal genome, respectively [41]. *N. meningitidis* is thus able to create substantial genetic diversity.

In addition, expression of protein or carbohydrate surface antigens can be altered through antigenic variation or phase variation. Antigenic variation occurs due to genomic recombination events leading to changes in protein sequence [42]. Phase variation is a stochastic (random) process and occurs mainly through slipped strand mispairing at short nucleotide repeats during DNA replication. The nascent DNA strand can separate from the template strand and slip forward or backward along the template strand during re-annealing, resulting in a different number of repeats in the newly synthesized strand. When this happens within the coding sequence of a gene it can result in an out-of-frame mutation, leading to an ON or OFF expression state of the gene involved [43]. Slipped strand mispairing within a promoter sequence will influence the transcriptional activity of the gene involved, consequently resulting in a graded level of expression [44]. These strategies allow expression of a heterogeneous phenotype within a clonal population of bacteria.

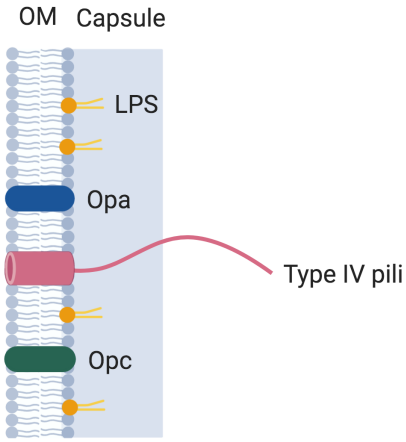

Figure 1. Schematic representation of selected major virulence factors in the outer membrane of *N. meningitidis*. OM, outer membrane; LPS, lipopolysaccharide; Opa and Opc, Opacity proteins. Adapted from [6]. The illustration is not drawn to scale.

2.1.1 Polysaccharide capsule

The polysaccharide capsule is one of the most important meningococcal virulence factors. The capsule envelops the bacterium and protects it from extracellular assaults: it prevents bactericidal activity of the complement system and antimicrobial peptides (AMPs) [45,46] and inhibits phagocytosis and intracellular killing [47,48]. It is thus essential for meningococcal survival in the bloodstream. Indeed, almost all invasive disease isolates express a capsule, whereas asymptomatically colonizing strains are found to be non-encapsulated in 50% of the cases [31].

The capsules of *N. meningitidis* serogroups B, C, W, and Y are composed of N-acetylneuraminic acid (NANA, or Neu5Ac), a derivative of sialic acid [49,50]. The capsule of serogroup A is composed of N-acetyl-mannosamine-1-phosphate [51]. Capsule-encoding genes are found in a single chromosomal cluster termed *cps*. The genes *siaA*, *siaB* and *siaC* are required for sialic acid synthesis, and *siaD*, a polysialyltransferase, is involved in sialylation of the capsule [52]. Capsule-deficient strains of *N. meningitidis* have been obtained by insertional inactivation of *siaD*, resulting in increased susceptibility to bacterial killing by the AMP LL-37 [46].

The capsule plays an important role in preventing immune recognition of the bacteria. Neu5Ac, the main structural component of the capsule, is a predominant sialic acid found in human cells where it dampens complement activation, among other functions. Incorporation of this molecule into the

capsule is an example of molecular mimicry [47,53]. In addition, the capsule effectively masks the presence of adhesin molecules on the bacterial surface that otherwise act as antigens. However, the masking of these adhesins also hinders meningococcal attachment to host cells [54]. Capsule expression must therefore be tightly regulated. Expression is affected by several mechanisms, for example by capsule switching between serogroups (mediated by horizontal gene transfer), phase variation, or transcriptionally by the MisR/MisS two-component system [55]. In addition, it was recently discovered that an increase in ambient temperature promotes capsule biosynthesis, as an elevated temperature indicates an activated host immune system [56].

2.1.2 Lipopolysaccharide

Endotoxin, also known as lipopolysaccharide (LPS), is a major constituent of the outer membrane of *N. meningitidis* and the main inflammatory mediator in invasive disease [57]. Meningoccoccal LPS consists of a hydrophobic membrane-bound lipid A portion connected to an inner oligosaccharide core composed of 2-keto-3-deoxy-manno-octulosonic acid (KDO) and a diheptose. Attached to the KDO oligosaccharide core are surface-exposed oligosaccharide chains [38]. In contrast to LPS found on many other bacterial species, neisserial LPS lacks repeating O-antigens and is therefore also known as lipooligosaccharide (LOS) [58].

The lipid A portion of LPS is responsible for meningococcal toxicity. LPS recognition by the host occurs through binding of LPS to the soluble LPS-binding protein (LBP). LPS is subsequently transferred to host cell membrane-bound cluster of differentiation (CD) 14 and toll-like receptor (TLR) 4, which results in a signaling cascade that ultimately causes the exaggerated pro-inflammatory immune response associated with septicaemia and meningitis [30]. Severity of the disease is directly correlated with LPS serum concentrations [57]. Curiously, an *lpxA* mutant deficient in LPS showed a pro-inflammatory response but signaled via TLR2 rather than TLR4 [59].

The outer oligosaccharide chains can vary in composition and this occurs by phase variation of *lgt* genes. *lgt* genes encode glycosyltransferases responsible for the biosynthesis of LPS oligosaccharide chains [60]. Differences in outer oligosaccharide chains result in a heterogeneous expression of LPS structures and can dramatically alter the virulence of the strain [61]. Similar to the capsule, LPS can be modulated through sialylation whereupon it can participate in molecular mimicry and enhance its resistance to complement-mediated killing [62]. Sialic acid can be synthesized endogenously or be scavenged exogeneously from host cell surfaces [63]. LPS is also involved in resistance to AMP-mediated killing, which occurs through steric or electrostatic hindrance [46], or by phophoethanolamine (PEA) decoration of lipid A [64].

LPS is released into the extracellular space during bacterial lysis but is also shed in the form of outer membrane vesicles, or blebs [65]. Outer membrane vesicles were shown to bind neutrophil extracellular traps, and to save *N. meningitidis* from neutrophil extracellular trap binding and subsequent killing [66].

2.1.3 Type IV pili

The ability of bacterial pathogens to cause disease greatly relies on their ability to attach to mucosal surfaces. The primary mechanism through which *N. meningitidis* binds to host surfaces is by using Type IV pili (Tfp). Tfp are long, filamentous structures expressed by gram-negative and gram-positive bacterial species [67]. Neisserial pili are approximately 6 nm in diameter and protrude several micrometers from the bacterial surface. Besides mediating contact with epithelial cells, they are also involved in bacterial twitching motility, DNA uptake, microcolony formation, and biofilm formation [68,69].

Pilus biogenesis requires fifteen genes, and the process can be divided into four distinct steps: assembly, functional maturation, emergence on the cell surface, and retraction. Pilin is the major pilus subunit and is encoded by the *pilE* gene [70]. Pilin is synthesized as a pre-protein and requires cleavage by PilD peptidase, which occurs in the periplasmic space [71]. In addition, pilin can undergo several post-translational modifications, including glycosylation, although this does not play a major role in pilus assembly and subsequent adhesion to host cells [72]. Multiple pilin subunits are packed together to form a fibre, which occurs through hydrophobic interactions of the conserved hydrophobic N-terminus [73]. Pilin assembly occurs at the inner membrane and involves a multitude of proteins, including PilQ, which forms the outer membrane secretin pore and PilF, an inner membrane ATPase that pushes the pilin through the outer membrane pore (pilus elongation) [70,74,75]. Another inner-membrane ATPase is PilT, which is involved in pilus retraction. Unlike PilF, PilT is not required for pilus biogenesis; however, PilT deficient strains show hyper-piliation and hyper-aggregation [76]. PilT is a key component in DNA uptake and twitching motility and plays a major role in mediating intimate adhesion to host cells [76,77]. Adherence to host cells is mediated by PilC protein. The corresponding *pilC* gene is present in two copies, *pilC1* and *pilC2*, however, only *pilC1* is involved in meningococcal-host cell adhesion [78]. PilC is located at the pilus tip and the bacterial surface [78,79]. In the latter location, PilC is unable to confer host cell adherence but is thought to be involved in pilus generation [78].

Tfp are one of the most variably expressed meningococcal virulence factors. Pilus expression is affected by antigenic variation and phase variation of several genes involved in pilus biogenesis. Antigenic variation occurs

through recombination events between *pilE* and *pilS*, mediated by RecA-dependent recombination, resulting in different versions of the pilin, or a defect pilin leading to non-piliated bacteria [42,80]. *pilS* are truncated copies of *pilE* that are not expressed but serve as a source of variable pilin gene sequences [70]. Additionally, phase variation occurs in *pilC* [81,82]. However, disease isolates are often piliated, indicating the importance of pili in neisserial pathogenesis [83].

2.1.4 Opacity proteins

Besides Tfp *N. meningitidis* also expresses other surface molecules, of which the opacity proteins are best characterized. Together with Tfp the opacity proteins are considered the major adhesins. Their name is derived from the opaque colour that gonococcal strains exhibit when expressing Opa proteins, a characteristic that is only visible for meningococcal strains that are unencapsulated [70]. Opacity proteins mediate intimate adhesion between meningococci and host cells, which plays an important role in the internalization and invasion of host cells [69].

N. meningitidis expresses two types of opacity proteins, Opa and Opc. Opa proteins are transmembrane structures consisting of a conserved β-barrel with four surface-exposed loops. Two of these surface-exposed loops are hypervariable, regulated by antigenic and phase variation. *N. meningitidis* genome contains three/four complete copies of *opa* genes, each expressed independently. Like Opa proteins, Opc proteins consist of a conserved β-barrel, however, there are five surface-exposed loops. Opc proteins are encoded by only one gene, *opcA*, which undergoes phase variation. Expression of Opc has not been demonstrated in *N. gonorrhoeae* or certain clonal complexes of *N. meningitidis*. It is thought that Opc expression is required for invasion of the meninges [70,84].

Opacity proteins interact with a number of host cell receptors, but strains must be unencapsulated to expose the opacity proteins [85]. Opa interacts with multiple members of the CEACAM (carcinoembryonic antigen-related cell-adhesion molecule) family, found on endothelial and epithelial cells, and some immune cells [84]. The affinity for CEACAMs varies depending on the specific sequence of the hypervariable loops, and the level and repertoire of CEACAM expression on host cells [86,87]. Both Opa and Opc can interact with heparan sulphate proteoglycans, found at the cell surface and extracellular matrix of most host cells [88,89]. In addition, Opc can bind to endothelial cells through interactions with fibronectin and integrin [90,91].

2.1.5 Two-component system

The ability of bacteria to survive within the human host requires adaptation to new environments. Meningococci rely on the two-component system (TCS) to sense and respond to environmental changes. TCSs are mostly found in gram-negative bacteria and consist of a membrane-bound sensor kinase that senses a specific environmental stimulus, and a cytoplasmic DNA-binding regulatory protein that subsequently mediates a cellular response through gene modifications [92]. *N. meningitidis* contains only four TCSs, in contrast to *Escherichia coli* which possesses thirty TCSs [93,94]. The best-characterized TCS in meningococci is the MisR/MisS system. MisR/MisS (also known as PhoP/PhoQ) is involved in remodeling of virulence factors, colonization and invasion of epithelial cells, and the PEA decoration of LPS [39,95-97]. Inactivation of MisR/MisS resulted in the perturbation of a number of genes involved in protein folding, transcriptional regulation, metabolism and iron acquisition [98]. The MisR/MisS system is therefore involved in a broad range of biological functions.

2.2 Interactions with host cells

2.2.1 Host cell colonization and invasion

Key events in the pathogenesis of *N. meningitidis* are the initial attachment to host cells, followed by a more intimate adhesion that can lead to host cell invasion (**Figure 2**). After meningococcal acquisition bacteria must quickly adhere to host cells to avoid being flushed out by the flow of mucus [99]. Initial adhesion of capsulated meningococci is mediated by pili that extend through the capsule. Pili interaction with host cells has been reported to occur through the surface-expressed CD46 receptor and by CD46-independent attachment [100-105].

After initial adherence meningococci proliferate and form aggregates on the host cell surface, termed microcolonies. A key protein in the formation of microcolonies is PilX, which mediates interbacterial interactions [106]. Meningococcal adhesion induces remodeling of the host cell surface by recruitment of multiple host cell receptors, including ezrin/radixin/moesin-binding transmembrane proteins, CD44, and intracellular adhesion molecule-1 [107]. The accumulation of these components results in cortical plaque formation underneath microcolonies and produces numerous cellular protrusions (microvilli) that protect the microcolony against shear stresses, such as those encountered in the nasopharynx or bloodstream [108]. Meningococcal adhesion to host cells through PilC1 results in the transient

release of intracellular Ca^{2+} stores [109]. Modulation of calcium signaling has also been shown for other bacterial species including *Salmonella enterica* Typhimurium, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae*, and facilitates bacterial adherence and invasion to host cells [110-112].

After several rounds of replication, meningococci start to detach from microcolonies in a process termed dispersal. Meningococcal dispersal has been shown to be a highly synchronized event induced by host-cell derived lactate [113]. Microcolony dispersal in gonococci can be induced in a cell-independent manner by depletion of oxygen [114]. Detachment of bacteria from microcolonies occurs by pilT-dependent pilus retraction and allows bacteria to form more intimate adhesion with host cells, as well as re-localize to new colonization sites [76]. While the capsule is essential for meningococcal survival in the host, close contact to host cells requires downregulation of both pili and capsule. This occurs mainly through phase variation, but contact regulated gene A (CrgA) regulatory protein may also be involved by downregulating expression of *pilC1*, *pilE*, and capsule synthesis *sia* genes [115]. Exposure of outer membrane adhesins (such as opacity proteins) in unencapsulated bacteria allows interaction with host cells via a number of receptors that mediate a tight adhesion. However, encapsulated bacteria have been shown to interact through Opa proteins during high expression of their target receptor CEACAM, which can occur during inflammation from a prior infection [116].

Following intimate adhesion between meningococci and host cells, bacteria may become internalized. Invasion of the submucosa is facilitated by epithelial damage caused by excessive cytokine release and immune cell activation [117]. However, meningococcal passage through cells can also occur without disrupting the epithelial cell layer integrity and requires expression of capsule and pili [118]. The invasive ability of meningococcal strains is related to their capacity to cause disease, but it can also represent an immune evasion mechanism to allow bacteria to enter epithelial cells, as was shown by the presence of subepithelial microcolonies in healthy individuals [119].

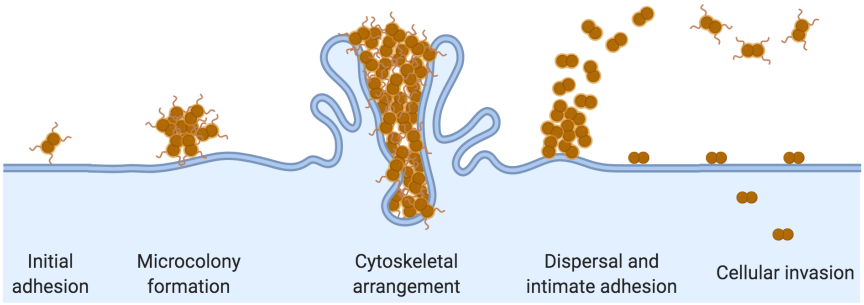

Figure 2. *N. meningitidis* colonization of host cells. Initial adhesion of capsulated meningococci relies on pili. Subsequent to adhesion, bacteria proliferate and form microcolonies, and induce cytoskeletal arrangement of the host cells underneath. Pilus retraction promotes microcolony dispersal, allowing re-localization to new colonization sites. Downregulation of capsule allows intimate adhesion to host cells and facilitates cellular invasion. Adapted from [113]. The illustration is not drawn to scale.

2.2.2 Meningococcal biofilm

Bacteria can exist as planktonic (single-cell bacteria) or as a biofilm. Biofilms consist of aggregated cells embedded in a network of self-produced extracellular polymers such as polysaccharides, proteins, and DNA [120]. Bacteria within a biofilm show enhanced resistance against harsh environments, mechanical forces, host immunity, and antimicrobial compounds. These protective benefits cause major complications in the clinic, where chronic infections are caused by biofilm formation on abiotic surfaces such as catheters and implants. Biofilms can consist of a single bacterial species or of a multispecies community, and often show remarkable phenotypic heterogeneity in their expression of surface molecules, nutrient utilization, and antibiotic resistance [121].

Evidence of meningococcal biofilms is mostly limited to *in vitro* studies [122-127]. Biofilm architecture is diverse and is strain-dependent. Biofilms of clonal complex sequence type (ST)-32 form well-defined channels that allow exchange of nutrients and waste products; biofilms of clonal complex ST-11 form intracellular spaces that do not resemble channel-like structures; and biofilms of clonal complex ST-53 form patchy structures without obvious channel formation or connections between the biomass [68].

The life cycle of a biofilm can be divided into distinct events. The first step is surface attachment, usually mediated by pili, flagella, or fimbriae. Bacteria proliferate and start to develop microcolonies that produce extracellular polymeric substances (EPS). EPS is responsible for cell-to-cell

adhesion and forms the three-dimensional biofilm structure. Furthermore, it protects the bacteria against various stresses and retains quorum-sensing molecules within the biofilm, which allows cellular communication. Finally, environmental changes can induce bacteria to disperse from a mature biofilm and re-enter a planktonic lifestyle to explore other niches and attach to a new surface [128].

For most bacteria the EPS consists of polysaccharides, however, this is not the case for *N. meningitidis*. Rather, the main constituent of meningococcal biofilms is extracellular DNA (eDNA). Initially, eDNA is important for bacterial attachment; in late biofilms, eDNA plays a role in mechanically stabilizing the biofilm structure. DNA is likely released during a lytic process known as autolysis, which can be driven by expression of autolysins [124]. *N. gonorrhoeae* can actively secrete single-stranded DNA via a type IV secretion system (T4SS) which plays an important role in gonococcal biofilm formation [129,130]. It is unlikely that this mechanism is utilized in *N. meningitidis*: while some meningococcal strains contain genes for the T4SS, these genes are disrupted by insertions and deletions [131]. Remarkably, highly purified DNA or proteinase-treated DNA did not induce biofilm formation, indicating that DNA alone is not sufficient but requires proteins that are also released during lysis or that are actively secreted into the extracellular milieu, for example OMVs [124]. Meningococcal biofilms that form independently of eDNA also exist, and this has been shown for strains of clonal complexes ST-8 and ST-11. It is currently unknown how initial attachment occurs for these strains. eDNA-independent biofilms were shown to be more sensitive to mechanical forces. Interestingly, ST-8 and ST-11 are strains usually isolated from patients with invasive meningococcal disease. It was thus hypothesized that *N. meningitidis* strains associated with low carriage but high invasion rates form fragile biofilms that promote cellular invasion ("spreader" phenotype), while *N. meningitidis* strains associated with high carriage but low invasion rates form sticky biofilms that enable long-term colonization of the nasopharynx ("settler" phenotype) [124].

Capsule and Tfp play an important role in host cell adhesion and likely affect meningococcal biofilm formation. Several studies show that the presence of capsule inhibits biofilm formation on abiotic surfaces and that disease isolates with higher capsule expression produce less biofilm [125,127]. However, capsule expression did not affect biofilm formation on epithelial strains, probably due to pili-mediated adhesion [68,126]. Tfp are not required for biofilm formation on abiotic surfaces but do affect biofilm architecture. It was shown that strains deficient in *pilE* or *pilX* formed flat biofilms that were not as thick as biofilms formed by wild-type strains [125,127].

Factors that play a role in biofilm formation include several secreted and cell-surface exposed proteins, collectively known as the meningococcal

secretome [132]. Many of these proteins are autotransporters, which are characterized by the ability to cross the outer-membrane autonomously [133]. NalP is an autotransporter that releases itself from the bacterial surface through autoproteolytic cleavage [134]. However, it remains temporarily anchored to the bacterial surface where it can cleave other proteins, such as NhbA and IgA protease, into the extracellular milieu. NhbA is a surface-exposed lipoprotein consisting of a positively charged C-terminus responsible for binding heparin [135]. Through this C-terminal domain, NhbA can also bind eDNA. Cleavage of NhbA by NalP releases the C-terminal domain from the cell surface [123]. IgA protease, an autotransporter protein that cleaves IgA molecules, is composed of two domains, a protease domain and an α-peptide [136]. The α-peptide has been shown to bind heparin and DNA [123,137]. Similar to NhbA, the release of IgA protease from the bacterial membrane requires NalP cleavage, however in the absence of NalP IgA protease can undergo autocatalytic processing, resulting in the release of only the protease domain while the α-peptide remains anchored in the bacterial membrane [134]. Thus, the remaining α-peptide can contribute to biofilm formation by binding eDNA. NalP undergoes frequent phase variation and can thereby play a key role in the regulation of meningococcal biofilm formation: the absence of NalP results in DNA-binding proteins to remain in the outer membrane and bind eDNA, thereby promoting biofilm formation; on the other hand, the presence of NalP releases DNA-binding proteins into the extracellular milieu, consequently leading to biofilm dispersal [68,123,134].

Other components of the meningococcal secretome are implicated in biofilm formation. Expression of autotransporter AutA at the bacterial surface induces autoaggregation and in addition can bind DNA [122]. Autotransporter TspB has been shown to bind eDNA in the presence of serum [138]. Finally, the autotransporters App, NhhA and NadA mediate adhesion to host cells [139-141].

2.3 Subversion of host defences

The capacity of *N. meningitidis* to colonize the human host and cause invasive disease is dependent on its ability to evade host defences. Most of these host defence evasion strategies have briefly been mentioned in the previous sections.

The expression and structure of the outer membrane components of *N. meningitidis*, including the capsule, pili and opacity proteins, vary at high frequency through antigenic and phase variation mechanisms (see Chapter 1). Antigenic variation occurs due to genomic recombination events [42]. An

example of this is variation within the *pilE* sequence through RecA-mediated recombination between *pilE* and *pilS* genes [42,80]. Phase variation involves stochastic (random) genetic switches that occur through slipped strand mispairing. Slipped strand mispairing within the coding sequence of a gene will result in either an ON/OFF expression state of the gene involved [43]; within a promoter sequence it will influence the transcriptional activity, consequently resulting in a graded level of expression [44]. Antigenic and phase variation produce a heterogenous bacterial population and allows for selection of the best-adapted phenotype under selective environmental pressure, for example that imposed by the immune system.

The natural ability of *N. meningitidis* to take up foreign DNA from its environment greatly enhances its genetic diversity. It is hypothesized that the *cps* locus encoding the capsule was acquired from *Pasteurella* through horizontal DNA exchange [142]. Horizontal DNA exchange may also be responsible for the acquisition of the antigenic variation in *porA* [143]. Horizontal DNA exchange is promoted by the natural autolysis of meningococci that occurs during stationary phase [38]. The ubiquitous presence of DNA uptake sequences and insertion sequences in the meningococcal genome are involved in the recognition and uptake of DNA from the environment, and the insertion of DNA into the meningococcal genome, respectively [41].

Prolonged carriage of *N. meningitidis* relies on resistance against epithelial defences. Iron, an essential cofactor for many bacterial biological processes, is withheld from bacteria by host glycoproteins human transferrin and human lactoferrin that bind iron tightly but reversibly. *N. meningitidis* expresses transferrin-binding protein B (TbpB) and lactoferrin-binding protein B (LbpB), specialized receptors with high affinity against these host glycoproteins in order to acquire iron [144,145]. Receptors that scavenge zinc have also been described [146]. Furthermore, *N. meningitidis* shows intrinsic resistance to the bactericidal action of many AMPs, including cathelicidins and defensins. These molecules play an important role as the first line of defence at the epithelial barrier. Meningococcal resistance against AMPs is conferred by the presence of capsule, LPS and efflux pumps [46,47,64].

After invasion into the bloodstream, meningococci will encounter a different range of host defences provided by antibodies, the complement system, and phagocytes. The capsule is an important protection against these defences by concealing various antigen structures on the outer membrane, thereby preventing antibody recognition and opsonization [55]. Antibody recognition is further dampened by release of IgA protease, an autotransporter protein that cleaves IgA antibody [136]. The complement system plays an essential role in innate immunity by attracting phagocytic cells, opsonization of bacteria for more efficient phagocytosis, and direct killing of bacteria [147]. A negative regulator of the complement system is sialic acid, a molecule found on

almost all human cells. Molecular mimicry by incorporation of sialic acid in the meningococcal capsule and LPS effectively dampens complement activation [47,53]. The binding of several meningococcal factors to negative complement regulators can further inhibit complement activation: NspA and fHbp bind to factor H, and PorA binds to C4-binding protein [148-150]. Furthermore, the presence of NhhA on the meningococcal surface protects against the deposition of the membrane attack complex [151]. Phagocytosis is prevented by the capsule, sialylated LPS, fHbp and NhhA [117,151]. NhhA also promotes macrophage apoptosis [152]. Shedding of OMVs (small structures consisting of a phospholipid bilayer containing mainly outer membrane proteins, lipoproteins, and LPS) from the bacterial outer membrane surface can act as a decoy for antibodies and other antibacterial molecules, thereby contributing to immune evasion [153,154].

2.4 *N. meningitidis* lactate utilization

A crucial step in the ability of *N. meningitidis* to colonize the nasopharynx and to survive in the bloodstream is its capacity to successfully adapt to nutrient limitation. Growth of *N. meningitidis* is limited to glucose, pyruvate, or lactate as carbon sources [155]. These carbon sources are found at several locations in the human body, albeit at variable concentrations. Glucose is the predominant carbon source in the blood and cerebrospinal fluid, while lactate is present in saliva and at mucosal surfaces that are colonized by lactic acid bacteria, such as the nasopharynx [156]. Glucose is oxidized to pyruvate via the Entner-Doudoroff pathway and can be further catabolized by the citric acid cycle [157]. In the human body, lactate can be found in two stereoisomer forms: L-lactate and D-lactate. Humans can only produce L-lactate, whereas lactic acid bacteria that colonize mucosal surfaces produce D-lactate. *N. meningitidis* is able to grow well with either L-lactate or D-lactate [158]. The enzyme lactate permease (LctP), localized in the bacterial membrane, is responsible for the uptake and output of lactate [159]. Once taken up, lactate is oxidized to pyruvate. This conversion is catalyzed by three lactate dehydrogenases (LDHs): a cytoplasmic LDH catalyzes the NAD^+-dependent, reversible conversion of D-lactate into pyruvate (LdhA), and two membrane-bound LDHs catalyze the conversion of L-lactate (LldA) or D-lactate (LdhD) into pyruvate [158,160,161]. After lactate is oxidized into pyruvate it can fulfill two functions: i) enter gluconeogenesis to produce glycerol and ethanolamine moieties for lipid membrane and LPS synthesis, ii) formation of acetyl-CoA which is the precursor for fatty acid synthesis (for lipid membrane and LPS synthesis) and the citric acid cycle. Energy (in the form of NADH) is released during the conversion of lactate to

pyruvate, pyruvate to acetyl-CoA, and during the citric acid cycle. In contrast, glucose oxidation in the Entner-Doudoroff pathway provides relatively little energy [162]. The fate of lactate utilization is dependent on the presence of glucose: when glucose is absent lactate will be utilized for both gluconeogenesis and acetyl-CoA formation; however, when glucose is present gluconeogenesis is inhibited and instead lactate provides an extra source of energy [163-165].

The benefits of lactate utilization towards *Neisseria* pathogenicity have been revealed in several studies. *N. meningitidis* in a medium of glucose and lactate exhibited increased bacterial growth rate compared to growth in glucose alone, while *N. meningitidis* deficient in lactate permease failed to respond to the added lactate [166]. In addition, the lactate permease-deficient strain showed reduced resistance to human serum [166], which may have been caused by reduced sialylation of LPS [163,167]. *N. gonorrhoeae* is able to utilize host-derived L-lactate to increase its metabolism and thus increase oxygen consumption rates in order to evade oxygen-dependent neutrophil killing [168]. Furthermore, meningococcal microcolony dispersal is stimulated by host-cell derived lactate, indicating that lactate may play a role in promoting cellular invasion. Interestingly, this mechanism is independent of lactate metabolic utilization [113]. **Paper IV** showed that meningococcal biofilm formation was induced by deletion of the cytoplasmic lactate dehydrogenase LdhA. This occurred in an eDNA-dependent manner through increased autolysis [169].

Chapter 3: *Lactobacillus*

The *Lactobacillus* genus is a group of gram-positive, facultative anaerobic bacteria that at present (September 2019) includes 240 species [170]. They are characterized by the production of lactic acid as the major metabolic end-product of carbohydrate fermentation, and for this reason are commonly exploited in food microbiology, for example in the production of cheese, yoghurt, sourdough bread, and beer. Lactobacilli are found ubiquitously in the human body as an important component of the microbiota. They are present in the oral cavity, respiratory tract, gastrointestinal tract, and genital tracts, especially dominating in the female genital tract [171]. Lactobacilli have been designated as "generally recognized as safe" and are used as probiotics to confer health benefits to the host. Examples of probiotic properties attributed to *Lactobacillus* include reduction in antibiotic-associated diarrhea, modulation of innate immune responses, reduced gastritis, and alleviation of allergies [171].

The mechanisms through which lactobacilli confer health-promoting advantages to the human host include i) enhancement of epithelial barrier function, ii) modulation of immune responses, iii) pathogen inhibition [172] (**Figure 4**). These mechanisms will be discussed in more detail below.

Recent *Lactobacillus* phylogenetic analysis has shown that there exists a significant heterogeneity within individual *Lactobacillus* species. One should therefore keep in mind that it is difficult to extrapolate phenotypic properties from one strain to another [171,173].

3.1 *Lactobacillus* interactions with host cells

Although lactobacilli do not represent the main constituent of the gut microbiota, the substantial interest in ingesting lactobacilli as probiotic therapy has led to the vast majority of work on host interactions to focus on the gastrointestinal tract [171].

The probiotic potential of lactobacilli mostly depends on their ability to colonize epithelial surfaces. Lactobacilli are able to bind the mucus layer, which covers the epithelial surface, through specialized mucin-binding proteins. Lactobacilli adhesion to host extracellular matrix components (such as collagen, fibronectin, and laminin) is mediated by the lactobacilli surface layer (S-layer). The S-layer is composed of identical (glyco)proteins that form into a structured lattice that is attached to the underlying cell wall. Furthermore, lactobacilli

elongation factor Tu and lipoteichoic acid (LTA) are involved in binding to mucins and intestinal epithelial cells [174]. Certain exopolysaccharide molecules have been shown to play a role in the *in vitro* formation of microcolonies and biofilm [175].

Exposure to lactobacilli can enhance epithelial barrier function, for example by inducing mucin secretion [176], strengthening tight junctions [177,178], and inhibiting apoptosis of epithelial cells [179]. These activities can be mediated by direct bacteria-host cell contact or by bacterially secreted factors [176,178,179]. An additional antibacterial barrier in the gut is provided by the expression of AMPs, which can be induced by the presence of different lactobacilli strains. In this manner, lactobacilli play a role in maintaining homeostasis in the intestinal tract [180,181]. In addition, *Lactobacillus* DNA can reduce inflammation by regulating different host cell signaling pathways, including mitogen activated protein kinase (MAPK), p38, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [182,183].

Lactobacilli can modulate host immune responses by acting on different cells. Macrophages exposed to lactobacilli strains *L. rhamnosus* and *L. reuteri* are able to suppress the pro-inflammatory cytokine tumor necrosis factor (TNF)-α [184,185]. Lactobacilli can also enhance the phagocytic activity of monocytes [186]. The effect of lactobacilli on dendritic cells appears to be more strain-dependent. Mouse bone marrow dendritic cells stimulated with various *Lactobacillus* species showed strong induction of the pro-inflammatory cytokines TNF-α and interleukin (IL)-12 for some species, while other species were less potent inducers. The lactobacilli species tested also exhibited different capacities to induce IL-6 and IL-10 [187]. By inducing a certain cytokine profile, lactobacilli are thus able to skew T-cell polarization toward either a T helper 1 or T helper 2 response [188]. However, a different study showed that dendritic cells were practically unresponsive when stimulated with *L. plantarum* [189]. These observations show that lactobacilli can modulate the human immune system, promoting either an immune-stimulatory or immune-inhibitory response, but that the precise response is dependent on many factors.

3.2 *Lactobacillus* interactions with pathogens

Lactobacilli can protect the human host against pathogens by several mechanisms. Lactobacilli can effectively prevent the initial colonization of pathogenic species by competing for nutrients [190]. In addition, lactobacilli produce a number of antimicrobial compounds including lactic acid, bacteriocins, and hydrogen peroxide (H_2O_2). For example, lactic acid produced

by *L. rhamnosus* exhibits strong antibacterial activity against *S.* Typhimurium [191]. The precise mechanism of killing by lactic acid is not known, but it has been shown that lactic acid can directly permeabilize the outer membrane of gram-negative bacteria [192]. Furthermore, lactic acid has chelating properties and can remove iron and other elements necessary for bacterial growth [193,194]. Lactobacilli can secrete small antimicrobial peptides called bacteriocins that can induce membrane permeabilization and killing of target bacteria [195]. *L. salivarius* has been shown to produce a bacteriocin that can significantly protect mice against infection with *Listeria monocytogenes,* whereas an *L. salivarius* mutant deficient in bacteriocin production was unable to protect mice [196]. H2O2 is primarily produced by *Lactobacillus* species isolated from the vagina, where it plays an important role in the non-specific defence mechanism of the normal vaginal ecosystem [197]. However, H_2O_2 has also been shown to have bactericidal activity against *S.* Typhimurium *in vitro* [198].

Another mechanism by which lactobacilli prevent pathogen growth is through competitive exclusion, where lactobacilli and pathogenic bacteria compete for attachment sites on the host mucosal surfaces. However, this requires lactobacilli to be present on the host surface beforehand (for example through preventive administration of probiotics), since displacement of pathogens by lactobacilli has rarely been observed [172]. Competitive exclusion can occur indirectly by *Lactobacillus*-induced upregulation of mucin [176]. In addition, lactobacilli can co-aggregate with pathogens, thereby facilitating clearance by mucus flow or phagocytic cells of the immune system [199,200]. Finally, lactobacilli can reduce expression of toxins and virulence factors. This has been shown for *Helicobacter pylori* and *E. coli* virulence genes and *Clostridium difficile* and *Staphylococcus aureus* toxins [201-207].

Since lactobacilli are an important component of the vaginal microbiota, most studies regarding pathogenic *Neisseria* have focused on *N. gonorrhoeae*. Individuals with a reduced number of colonizing lactobacilli in the vaginal tract show an increased risk to acquire *N. gonorrhoeae* infection [208,209]. The protective effect of lactobacilli against *N. gonorrhoeae* occurs through inhibition of gonococcal adherence to host cells, as well as the low pH and H_2O_2 release caused by lactobacilli colonization [210-213]. A protective effect of lactobacilli against *N. meningitidis* has also been observed. *L. plantarum* co-incubation with *N. meningitidis* showed inhibition of meningococcal adhesion and invasion of nasopharyngeal epithelial cells. In the same study, *L. plantarum* could attenuate inflammatory and cytotoxic effects, mediated by a secreted factor that modulates TLR2 signaling [214]. A recent study using a mouse model infected sequentially with influenza A virus and *N. meningitidis* showed that mice treated with *Lactobacillus paracasei* experienced reduced weight loss, improved survival and lower meningococcal load, compared to mice that did

not receive *L. paracasei* treatment. This effect was associated with higher recruitment of inflammatory cells, including monocytes and dendritic cells [215]. However, the presence of lactate can induce faster growth and microcolony dispersal of meningococci [113,166], which is associated with invasion and disease progression. A clear role for lactobacilli in meningococcal infections is therefore not available.

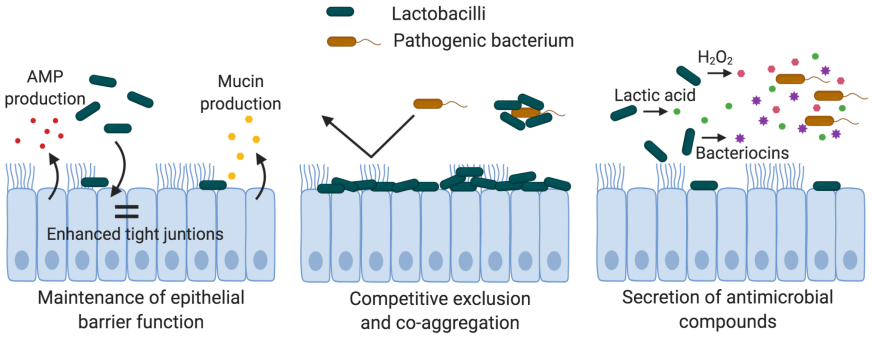

Figure 3. Overview of *Lactobacillus* interactions with the host and pathogens. Adapted from [216]. The illustration is not drawn to scale.

Chapter 4: The host and host responses

4.1 Epithelial defences

Humans are constantly exposed to microbial organisms via ingestion, inhalation, or contact with contaminated surfaces. The first and most important barrier against foreign agents is the epithelial surface. The epithelium is a layer of cells that covers the surface of the body exposed to the external environment, creating a physical barrier that separates the inside from the outside. The type of epithelium depends on the body site and function. For instance, the epithelium of the upper respiratory tract is mostly made up of pseudostratified columnar cells that are ciliated, which propel mucus (and entrapped particles) towards the nose or mouth to be expelled by coughing or swallowing. On the other hand, alveolar epithelium is made up of squamous cells that are thin and flat, to enable passive diffusion of gasses [217]. Cell junctions (such as tight junctions and adherens junctions) mediate tight contact between epithelial cells, resulting in an uninterrupted, sealed sheath [218]. The epithelial surface is often lined with a viscous layer of mucus that provides lubrication as well as an additional barrier against microorganisms and harmful particles. The major components of mucus are mucins, which are glycoproteins secreted by submucosal glands and goblet cells that reside within the epithelium [219]. Other components of the mucus layer are antimicrobial factors, such as lysozyme, lactoferrin and AMPs, and immunoglobulins. These components of the mucosal layer are expressed constitutively to replace the mucus that is constantly degraded by bacteria, or lost through continuous flow [220]. Several studies have shown the importance of the mucus layer in limiting bacterial infection. Bacterial attachment induces enhanced secretion and shedding of mucins [221,222]. In addition, a mouse model deficient in MUC1 (a type of mucin) showed an increased susceptibility to develop infection with the gastric pathogens *H. pylori* and *Campylobacter jejuni*, as well as more severe pathology [223-225].

4.2 Innate immune defences

If bacteria breach the mucus layer they encounter epithelial and innate immune cells (such as neutrophils and macrophages), which can detect the presence of

invaders. This results in the activation of a signaling network that recruits innate, and later adaptive, immune responses to clear the infection. The detection event occurs when specific pathogen associated molecular patterns (PAMPs) are recognized by receptors on the host cell, known as pattern recognition receptors (PRRs). Examples of PAMPs are typically bacterial cell surface components, such as LPS, LTA, peptidoglycan, porins, and flagella. PRRs are evolutionary conserved and are located on the cell surface, cytoplasm, and in endosomal compartments, and are thus able to detect both extracellular and intracellular pathogens [226]. TLRs are the most widely studied of the PRRs. They are expressed in various cell types including epithelial cells, endothelial cells, and immune cells [227]. In humans 10 TLRs have been identified: TLR1, 2, 4, 5 and 6 are primarily expressed on the host cell surface and detect bacteria, fungi, and protozoa, while TLR3, 7, 8 and 9 are expressed in endosomal compartments and recognize bacterial- or viral-derived nucleic acids [228]. Other families of PRRs include NOD-like receptors, RIG-I-like receptors, and C-type lectin-like receptors. These different PRRs respond to different PAMPs, but share many similarities in the downstream signal transduction pathways and commonly activate the NF-κB pathway [229]. The activation and regulation of NF-κB will be discussed in more detail below.

TLR activation induces the expression of pro-inflammatory cytokines and chemokines that recruit immune cells such as monocytes, macrophages, neutrophils and dendritic cells to the site of infection. Neutrophils can eliminate bacteria by granular release of AMPs and other antibacterial components, or by undergoing netosis [230]. In addition, neutrophils and macrophages can internalize bacteria through phagocytosis, whereupon the bacteria are killed by reactive oxygen and nitrogen species and proteolytic cleavage by proteases and antimicrobial peptides [230,231]. Dendritic cells are essential for the recruitment of adaptive immune responses [231].

The complement system is an important component of innate immunity. It is the primary defence mechanism in the bloodstream but it is also activated by epithelial surfaces. The complement system is activated by bacteria, bacterial components or antibody-pathogen complexes and results in attracting phagocytic cells, opsonization of bacteria for more efficient phagocytosis, and direct killing of bacteria [232].

4.2.1 NF-κB

NF-κB represents a family of inducible transcription factors that are involved in the regulation of a large array of genes that mediate immune and inflammatory responses, as well as cell survival processes. Under normal conditions, NF-κB is bound by IκB inhibitory protein, which keeps NF-κB sequestered in the cytoplasm. Activation of NF-κB is initiated by the degradation of IκB. This

occurs by a signaling cascade induced by various ligand-PRR interactions, for example LPS signaling with TLR4 [229] (**Figure 4**).

LPS is an important component of gram-negative bacteria and signals via TLR4. The first step by which LPS interacts with host cells is through the binding of host LPS binding protein (LBP) to the lipid A portion of LPS. This LBP:LPS complex interacts with CD14 receptor (either soluble or membrane-bound), which in turn interacts with TLR4/MD2 heterodimer. TLR4 undergoes oligomerization and recruits downstream adaptors that signal through MyD88-dependent or –independent pathways. The MyD88-dependent pathway signals through IRAK1, which subsequently associates with TRAF6. TRAF6, in turn, interacts with TAK1, which activates the IκB kinase (IKK) complex. The MyD88-independent pathway signals through TRIF, RIP-1, and TRAF6, which also leads to the activation of IKK complex. IKK ubiquitinates IκB protein which targets it for proteasomal degradation. Upon degradation of IκB NF-κB is released and rapidly translocates into the nucleus, where it induces expression of a large array of genes involved in immune and inflammatory processes. These include induction of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which can then recruit and further amplify the immune response [233].

All TLRs, except for TLR3, activate the MyD88-dependent pathway to induce NF-κB. MyD88-dependent signaling by TLR 7, 8 and 9 can also induce the expression of type I interferons. TLR3 and 4 activate the MyD88-independent pathway and induce expression of type I interferons [234].

4.2.2 A20

The immune response generated by bacterial recognition, such as the one induced by TLR4 described above, is crucial for the containment and eradication of microbial infections. However, excessive immune activation can be harmful to the host, leading to the development of autoimmune or inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, or septic shock. The activation of inflammatory responses therefore needs to be tightly controlled. TLR-mediated immune responses can be controlled by endogenous molecules that negatively regulate components in the signaling cascade, particularly targeting the NF-κB pathway [226]. A key protein responsible for the regulation of NF-κB is TNF alpha-induced protein 3 (TNFAIP3, also known as A20). A20 is a cytoplasmic zinc-finger protein and is encoded by the *TNFAIP3* gene. A20 was first identified in endothelial cells treated with TNF and was characterized as a protein protecting against TNF-induced cytotoxicity [235,236]. Further studies showed that A20 can terminate NF-κB activation from TNF-independent signals also [237]. One of the main mechanisms in which A20 can inhibit NF-κB activation is through its deubiquitinating/ubiquitinating properties. Deubiquitination of signal

molecules upstream of NF-κB can prevent further protein interactions, halting the signaling cascade. Subsequent ubiquitination then targets them for proteolytic degradation. A20 has been shown to restrict NF-κB activity triggered by TNF receptor, TLR4, and NOD2 by deubiquitination of RIP1, TRAF6, and RIP2, respectively [237-239]. A20 also dampens TLR3-induced NF-κB activation by targeting TRIF, although this does not require deubiquitination of TRIF [240] (**Figure 4**). Most cell types express low A20 expression levels under basal conditions, but expression is rapidly induced upon NF-κB activation due to the presence of two NF-κB binding sites in the A20 promoter, thus creating a negative feedback loop [241].

The importance of A20 is demonstrated in various disorders that show dysregulation of A20, including type-1 diabetes, inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus [241]. A20-deficient mice develop spontaneous inflammation and die prematurely due to multiorgan failure and cachexia. Treatment with broad-spectrum antibiotics abolished this outcome, suggesting that commensal bacteria are responsible for the constitutive TLR signaling [242]. This was verified by the observation that *Lactobacillus jensenii* induced anti-inflammatory responses in antigen presenting cells by upregulating expression of A20 and two other TLR regulators, SIGIRR and IRAK-M [243]. More recently it was found that another *Lactobacillus* strain, *Lactobacillus helveticus*, induces A20 expression in macrophages through interaction of cell wall fragments with TLR2 [244]. The skin commensal *Staphylococcus epidermidis* induces expression of A20 in keratinocytes resulting in dampened IL-1β and AMP levels, which may contribute to *S. epidermidis* persistence on the skin [245]. These findings suggest an important role for A20 in maintaining homeostasis during chronic microbial exposure, such as with commensal colonization [241].

It has been speculated that A20 induction could be a general principle exploited by bacteria to downregulate immune defences [245]. Indeed, the gastric pathogens *H. pylori* and *C. jejuni* induce A20 expression in gastric epithelial cells in order to impede apoptotic host cell death, which improves their survival [246]. Additionally, hepatitis C virus induced expression of A20 to support its replication [247]. A role for A20 as an immune evasion mechanism has been suggested in **paper II**, where it was demonstrated that the pathogen *N. meningitidis* induced expression of A20 in parallel with a downregulation in hBD2, an antimicrobial peptide with bactericidal effects against meningococci. In contrast, the commensal *L. reuteri* induced lower levels of A20 and was associated with higher hBD2 expression.

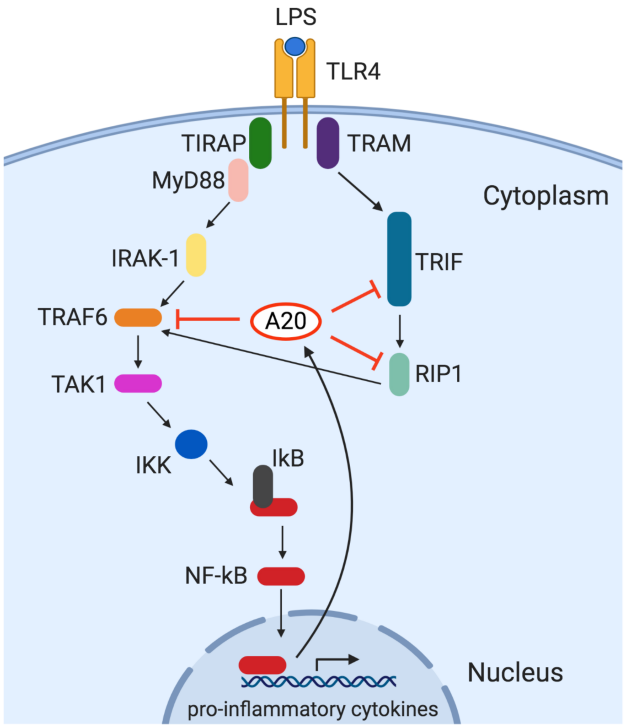

Figure 4. Simplified schematic representation of NF-κB signaling in response to TLR4 activation by LPS. LPS recognition by TLR4 induces a signaling cascade that ultimately results in the translocation of NF-κB into the nucleus where it induces expression of pro-inflammatory cytokines. To prevent excessive inflammation A20 downregulates NF-κB activity in a negative feedback manner. Adapted from [233]. The illustration is not drawn to scale.

4.3 Early growth response protein 1

Early growth response protein 1 (EGR1) is a zinc finger protein that functions as a transcription factor. It belongs to the Early Growth Response family and is found ubiquitously in human tissue. EGR1 expression is constitutively low but can be rapidly and transiently induced by numerous stimuli, such as growth factors, cytokines, oxidative stress and mechanical stress [248-251]. Various bacterial species have also been shown to induce EGR1 expression, including *Chlamydia pneumonia*, *H. pylori*, *N. meningitidis*, *N. gonorrhoeae*, *Porphyromonas gingivalis* and *Streptococcus intermedius* [252-257]. EGR1 activation is mediated by various signaling pathways, including PKA and the MAP kinases ERK1/2, JNK and p38, but also integrins on the cell surface can induce EGR1 expression by activating PI3 or ERK [258-260]. In **paper I** we show that

upregulation of EGR1 in epithelial cells is a common response to host-bacteria interactions, with signaling occurring mostly via EGFR-ERK1/2 and β1-integrin [261].

EGR1 plays an important role in regulating numerous cell physiology processes and also inflammatory responses such as wound healing and tissue repair [260]. EGR1 contains a highly conserved DNA-binding domain composed of three zinc-fingers, with which it can bind to the consensus target sequence GCG(G/T)GGGCG that is present in the promoter region of many genes [262]. EGR1 can thereby regulate the transcription of genes involved in cellular growth, differentiation, and apoptosis, as well as genes encoding cytokines, extracellular matrix proteins, and growth factors [263-265]. EGR1 expression is self-limiting by binding to the proteins NGFA-I binding proteins 1 and 2, which inhibit EGR1 activation, thus creating a negative feedback loop to prevent disproportionate EGR1 levels [260].

Abnormal EGR1 expression has been implicated in carcinogenesis, atherosclerosis, and diabetes [266-268]. EGR1 involvement in bacterial infections has also been shown, although its exact role is unclear. A link between EGR1 and bacterial infection is suggested by the presence of a functional NF-κB binding site within the EGR1 promoter. This allows NF-κB to induce EGR1 expression, although this has only been shown to occur by UV radiation [269]. TNF-α expression induced by peptidoglycan in macrophages is mediated by EGR1 signaling [270]. An anti-inflammatory role for EGR1 has also been reported, where EGR1 mediated tolerance to LPS in macrophages by upregulating SOCS-1 expression, a negative regulator of cytokine signaling [271]. Furthermore, EGR1 has recently been shown to impede macrophage clearance of *P. aeruginosa*. Downregulation of EGR1 expression in *P. aeruginosa*-infected macrophages by the AMPs human beta-defensin 2 and human beta-defensin 3 resulted in improved macrophage phagocytosis and intracellular killing of *P. aeruginosa*, caused by inhibition of EGR1-induced macrophage autophagy [272]. It is tempting to speculate that *P. aeruginosa* actively induces EGR1 expression as a bacterial evasion strategy, however, this was not tested.

Chapter 5: Antimicrobial peptides

The success of the immune system to keep the human host free from infections is greatly enhanced by the presence of antimicrobial peptides (AMPs). These small peptides are highly conserved and are found in both animals and plants, indicating they serve a fundamental role [273]. Their importance is further highlighted in studies that show that deficiencies in AMPs correlate with increased susceptibility to infections [274-277]. AMPs show antimicrobial activities that extend to bacteria, fungi, and viruses; in humans they additionally play an important role in modulating immune functions [278]. AMPs are also commonly known as 'host defence peptides' to emphasize their multi-faceted nature [279].

Discovery of the first AMP occurred in 1981 when Hans Boman et al. described the isolation and characterization of two small peptides with potent antibacterial activity in the cecropia moth [280]. Since then, the AMP repertoire has expanded to contain more than 3000 peptides, of which 130 are found in humans [281]. Classification of AMPs is based on their structure. AMPs are short peptides (10-50 amino acids) and are generally cationic and amphipathic, containing both hydrophobic and hydrophilic side chains [273]. The two major classes of AMPs expressed in humans are the cathelicidins and the defensins, which will be discussed in more detail in the sections below.

5.1 AMP expression and regulation

5.1.1 Cathelicidins

The cathelicidin family is a heterogeneous group of molecules. They are characterized by a highly conserved N-terminal domain and a variable C-terminal domain that contains the antimicrobial activity. Multiple cathelicidin homologues have been identified in cow, pig, rabbit, chicken, and fish. Humans, mice, and rats however express only a single cathelicidin [282].

In humans the sole cathelicidin is hCAP18, which is encoded by the *CAMP* gene. The peptide is synthesized as a pre-pro-peptide that requires proteolytic cleavage to generate the biologically active peptide, termed LL-37. The *CAMP* gene is composed of four exons, of which the first three code the pre-pro-region, and the last codes for the antimicrobial domain [283]. LL-37 is highly expressed in myeloid cells such as neutrophils, where they are stored in secondary granules in their unprocessed form [284,285]. Upon neutrophil

activation, the granule contents are released and the peptide is cleaved by proteinase 3 to release the fully active peptide [286]. LL-37 is also expressed by epithelial cells of the skin, lung, gut, mammary glands and epididymis [287-291]. In keratinocytes proteolytic cleavage is performed by the serine proteases kallikrein 5 and 7 [292]. LL-37 secreted into sweat by the eccrine gland and sweat ductal epithelial cells is further degraded to yield fragments of various sizes that show increased antimicrobial activity against several skin pathogens [293].

LL-37 exhibits a disordered structure in aqueous environments but adopts an alpha-helical conformation in the presence of membrane bilayers or ionic environments such as plasma and interstitial fluid [294,295]. It displays potent *in vitro* antibacterial activity against both gram-positive and gram-negative bacteria [287,288,296], however, its killing effects are inhibited by the presence of serum proteins and salts [287,294]. Despite this, cathelicidin plays an important role in immune defences as demonstrated by cathelicidin-deficient mice that show increased susceptibility to bacterial infection with the pathogens *Streptococcus pyogenes* and *S. aureus* [287,297]. Concomitantly, exogenous cathelicidin treatment showed reduced pneumonia symptoms in methicillin-resistant *S. aureus*-infected mice, as well as improved survival in a rat sepsis model [298,299].

The regulation of cathelicidin expression is variable and depends on the cellular source. Physiological concentrations of LL-37 range between 0.03 μg/ml (6.5 nM) in human plasma to 8 μg/ml (1.8 μM) in bronchoalveolar lavage fluid [300,301]. During inflammatory conditions cathelicidin levels can be dramatically increased, for example in psoriatic lesions where concentrations can reach up to 7 mg/ml (1.5 mM) [276]. Cathelicidin expression is stimulated by the presence of bacteria or bacterial structures such as LPS or LTA, potentially mediated by NF-κB signaling [302-304]. In addition, LL-37 can be induced by various compounds. The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3, can induce LL-37 expression in bronchial epithelial cells, keratinocytes, and myeloid cells by binding to a vitamin D response element located in the promoter region of *CAMP* [305-307]. Histone deacetylase inhibitors such as butyrate, a short-chain fatty acid produced in the colon by bacterial fermentation of dietary fibers, can also induce expression of cathelicidin, mediated by direct chromatin remodeling of the *CAMP* promoter, and also by several signaling pathways including MEK-ERK and MAPK signaling [308-310].

5.1.2 Defensins

The defensins are a group of AMPs found in a number of mammals, including humans. These peptides adopt a beta-sheet structure stabilized by three

characteristic intramolecular disulphide bridges that form between six conserved cysteine residues. These disulphide bonds provide structural support and protect the peptide against enzymatic degradation [311]. Based on the topology of the disulphide bridges, defensins are organized into alpha-, beta- and theta-defensins [312]. Theta-defensins (cyclic defensins) have so far only been isolated from non-human primate leukocytes [313].

To date, six alpha-defensins have been identified in humans that are further subdivided into four human neutrophil peptides (HNP1-4) and two human defensins (HD5 and 6). HNPs are expressed in the primary granules of neutrophils [314], while HD peptides are expressed by paneth cells of the small intestine, and epithelial cells of the female genital tract [315-317]. Like cathelicidins, defensins require one or more proteolytic steps to produce the fully active peptide. However, after HNP synthesis in neutrophil precursor cells in the bone marrow, the peptides are processed into their mature form before storage in the primary granules of neutrophils [318]. As a consequence, mature neutrophils contain large amount of HNPs, but no longer express HNP-encoding genes [319]. HD peptides are stored in paneth cell granules in their inactive form and undergo processing by trypsin cleavage upon release into the extracellular space [320,321]. Curiously, trypsin is co-packaged with HD5 and HD6, but it is unclear how trypsin remains inactivate inside the paneth cell granules until the release of granule contents is triggered. It is hypothesized that trypsin is stored in its precursor form of trypsinogen, or alternatively is kept inactivated by serine proteases that are abundantly expressed in paneth cells [321]. In some cases, the expression of alpha-defensins is constitutive [317,322] where it contributes to the mucosal antimicrobial barrier by exhibiting broad killing activity against enteric pathogens but also by shaping the microbiota [180,323,324]. In other cases, the expression can be induced during infection or inflammation [325,326]. The concentration of alpha-defensins varies per location and ranges between 10 ng/ml in bronchoalveolar lavage fluid and 320 ng/ml in plasma, and has been estimated to reach even as high as 10 mg/ml at paneth cell crypts [325,326].

A distinct subgroup of the defensin family is the beta-defensins. In humans six beta-defensins are found, termed hBD1-6. Beta-defensin genes contain only two exons: the first encodes the pro-piece, the second encodes the mature peptide. Similar to cathelicidins and alpha-defensins, proteolytic cleavage is required to release the mature peptide [327], although it is unclear which molecule is responsible for this. Beta-defensins are expressed in the skin and by epithelial cells of the urinary, genital, respiratory, and gastrointestinal tract [328-332], and they are also found in monocytes, macrophages and dendritic cells [333]. hBD1 is expressed constitutively, while expression of hBD2 is readily induced by stimuli such as bacteria, bacterial factors and cytokines [329,332,334,335]. Sometimes AMP induction relies on a dual

stimulation, as was shown for hBD2. Direct stimulation of epidermal cultures with LPS did not induce hBD2 expression, however, when monocytes acted as intermediaries between LPS and the epidermis hBD2 expression was greatly enhanced, mediated by IL-1 release by LPS-stimulated monocytes. This type of indirect activation allows for a more controlled response [336]. hBD2 is also induced by vitamin D_3, which occurs by binding to a vitamin D response element located in the promoter region of the hBD2 gene [306]. Induction of beta-defensins is often mediated by NF-κB activation and involves multiple signaling pathways, including MAPK, PI3K and PKC [337].

hBD2 is encoded by the *DEFB4A* gene. It consists of 41 amino acids with a molecular weight of 4.5 kDa. The peptide exhibits a net charge of +6 [337]. Concentration of hBD2 in unstimulated conditions is usually low but can reach higher levels during inflammation or infection. A basal level of 0.5 μM hBD2 was detected in bronco-alveolar lavage fluid, which was increased to 1.6 μM during infection [301]. The concentration of hBD2 in psoriatic skin lesions was reported as 25 μM and even reached as high as 150 μM [276].

5.2 Killing activity

One of the main activities of AMPs is their ability to kill a broad range of microorganisms. Initial interaction occurs through electrostatic attraction, where positively charged AMPs interact with negatively charged bacterial surfaces (formed by the presence of phospholipids, LPS or teichoic acids). In contrast, the eukaryotic outer membrane is mainly composed of lipids with no net charge, explaining the low cytotoxicity of AMPs towards host cells [273]. Subsequent to initial AMP-bacterial interaction, the attached peptides aggregate and insert into the bacterial membrane bilayer in such a way as to align the hydrophobic and hydrophilic portions of the peptide with those of the bacterial lipid membrane. This results in pore formation, destruction of the transmembrane potential and pH gradient, and consequently respiration is inhibited, leading to bacterial lysis [338]. This mechanism is known as membrane disruptive killing. However, it is widely accepted that membrane depolarization is not the only mechanism by which AMPs affect bacterial viability. This was exemplified by polymyxin B which does not cause significant membrane depolarization in *P. aeruginosa*, yet rapidly kills it [339]. It is therefore likely that bacterial membrane disruption is not essential for killing and that AMP activity is also mediated by nonmembrane disruptive mechanisms, for example by targeting intracellular bacterial components. Examples of these intracellular targets include inhibition of processes such as DNA and protein synthesis, chaperone-assisted protein folding, enzyme

activity, and cytoplasmic membrane and cell wall formation (**Figure 5**) [340]. To reach these targets AMPs must enter the cell, and it is proposed that this occurs through transient pore formation without complete breach of bacterial membrane integrity [341,342]. Hancock et al. describe the uptake of AMPs across the gram-negative bacterial outer membrane to occur in a "self-promoted" fashion. This occurs by initial binding of the AMP to LPS, which results in the displacement of divalent cations that bridge LPS, leading to transient cracks that permit the passage of molecules [343]. Alternatively, AMPs can be taken up by membrane transporter proteins [344,345].

The antimicrobial killing mechanism employed by cathelicidin and defensins varies. Membrane disruptive killing is exhibited by LL-37 [296,346] and HNP1-3 [347]. However, HNP1 has also demonstrated bacterial killing with limited membrane permeabilization by binding to the peptidoglycan precursor molecule lipid II and thereby disrupting cell wall synthesis in *S. aureus* [348]. A similar lipid II-binding mechanism was shown for hBD3 [349]. Alpha-defensin HD5 also exhibits a nonmembrane disruptive mechanism and accumulates in the cytoplasm where it may exert its antibacterial activity by binding DNA [350,351]. In contrast, another study showed that HD5 permeabilized the inner membrane of *E. coli* [352]. Rapid membrane permeabilization in *E. coli* has been shown to occur within 6 minutes in response to hBD2, 3 and 4 [353]. However, in **paper III** we demonstrate that hBD2 is able to reduce meningococcal viability without permeabilizing the outer membrane of *N. meningitidis*, suggesting a nonmembrane disruptive killing mechanism in this microorganism. Recently an interesting discovery was made on a novel antimicrobial mechanism exhibited by HD6. Despite having relatively low antibacterial activity compared to other alpha-defensins, HD6 was able to protect the host from *S.* Typhimurium through formation of self-assembled oligomer structures termed nanonets, that surround and entangle bacteria and thereby prevent bacterial invasion and dissemination [354]. A similar finding was made involving hBD1, which also forms a net-like structure around bacteria, which remains stable even in the presence of duodenal proteases [355].

Several AMP characteristics have been described to affect antibacterial killing. These include peptide size, sequence, charge, and hydrophobicity [338]. However, it is difficult to predict the optimal AMP structure. For example, increasing the charge of the frog AMP magainin from +3 to +5 increased its antibacterial activity, but a further increase to +6 or +7 resulted in loss of antibacterial activity and an increase in haemolytic activity instead [356]. An additional factor that can affect AMP activity is the redox state of the peptide. hBD1, while ubiquitously expressed in human tissue shows only minor bacterial killing activity. This seemingly paradoxical finding was explained when the group of Wehkamp et al. discovered that reduction of the disulphide

bonds in hBD1 greatly improves its antibacterial activities [357]. This is especially relevant in anaerobic environments, such as in the gut or within glands, wounds or infection sites. This finding implies that other defensin peptides could also show altered bactericidal potency upon reduction of their disulphide bridges.

In addition to bacterial killing activity, AMPs also demonstrate antibiofilm activity, as was shown for LL-37 against *P. aeruginosa* biofilm [358]. However, antibiofilm activity is not correlated to killing activity [359], indicating that the mechanism employed to prevent biofilm formation is different from the killing mechanisms described above. This has led Haney et al. to suggest that antibiofilm peptides are distinct from AMPs [279].

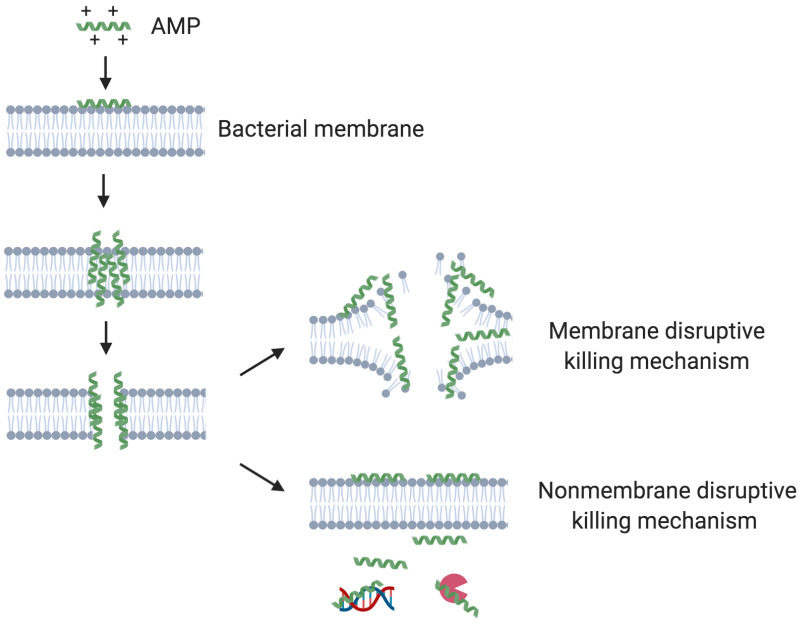

Figure 5. Membrane and nonmembrane disruptive killing mechanisms by AMPs. AMPs interact with the bacterial membrane through electrostatic attractions. The peptides aggregate and insert into the phospholipid bilayer, resulting in pore formation and destruction of the transmembrane potential, leading to membrane disruption. Additionally, transient pore formation can allow AMPs to enter into the bacterial cytoplasm where they can target intracellular components. This type of killing does not necessarily disrupt the membrane. Adapted from [273]. The illustration is not drawn to scale.

5.3 Immunomodulatory activity

Despite their broad antibacterial activity, some AMPs are readily inactivated by physiological salt concentrations (e.g. 150 mM NaCl) [328,360]. Furthermore, AMP concentrations required for bacterial killing are usually much higher than those found in physiological conditions [278]. For this reason it is hypothesized that their fundamental role in *in vivo* antimicrobial defence is through the stimulation and modulation of the immune system. These functions are often exhibited at lower concentrations than those required for killing activity and importantly are not inhibited by physiological salt and serum conditions [361].

Examples of immunomodulatory functions are the recruitment of different immune cells (including neutrophils, monocytes, dendritic cells, and T-cells) [362-364], induction of cytokine and chemokine expression [365,366], suppression of neutrophil apoptosis [367], enhancement of phagocytosis [368], enhancement of mast cell degranulation [369], and the promotion of wound healing and angiogenesis [370,371]. Both alpha- and beta-defensins are chemotactic for monocytes, macrophages, mast cells, T-cells, and dendritic cells [372,373]. Recruitment of these cells can occur in a direct manner, but also indirectly by induction of cytokines. For example, LL-37 and hBD2, 3 and 4 can induce expression of cytokines IL-6 and IL-10 and chemokine MCP-1, in monocytes and keratinocytes, respectively [374,375], which occurred through MAPK signaling [374,376]. Thus, AMPs can act as a bridge between innate and adaptive immunity [278].

In addition, an important role of AMPs in host defence is their ability to prevent LPS-mediated inflammatory responses that can lead to sepsis. The first step by which LPS activates the immune system is the transfer of LPS to CD14 receptor by LPS binding protein (LBP). Polymyxin B and a synthetic peptide termed CP29 have been shown to prevent LPS-mediated sepsis by blocking the binding of LPS to LBP and thereby prevent macrophage activation [377]. LL-37 showed similar protective properties by preventing release of TNF-α from LPS-stimulated monocytes and macrophages by acting on NF-κB translocation [378]. AMPs can also scavenge and bind to LPS in order to prevent the signaling cascade triggered by TLR4 activation [379,380].

AMPs can influence the inflammatory state in different ways. For example, HNP1 released by neutrophils can drive anti-inflammatory responses by entering macrophages and inhibiting protein translation, resulting in prevention of macrophage-driven inflammation without compromising pathogen clearance [381]. On the other hand, it has been shown that LL-37 can selectively permeabilize apoptotic leukocytes such as macrophages, NK cells, and neutrophils, resulting in the cells undergoing lysis rather than apotosis,

with the subsequent release of inflammatory factors that skew towards a pro-inflammatory state [382].

Another immunomodulatory role of AMPs is in the regulation of commensal species. Distribution of several enteric AMPs was found to vary along the gut and was most concentrated in intestinal crypts, decreasing through the mucus layer towards the lumen, thus controlling commensal contact with the host [180]. By skewing the immune response towards an anti-inflammatory state, HD5 has been shown to preserve homeostasis between the host and the gut microbiota [323].

5.4 Bacterial resistance to AMPs

Despite their ubiquitous nature surprisingly little bacterial resistance to AMPs has developed, unlike the bacterial resistance seen to pharmaceutical antibiotics. Several theories for this exist, including their multifunctionality in the host, high local concentrations reached *in vivo*, and the large diversity of AMPs [278]. However, several bacterial species have evolved mechanisms to evade the actions of AMPs.

Various AMPs can be degraded by proteases and proteinases secreted by human pathogens. The simple linear structure of LL-37 is particularly susceptible to this mechanism [383-385], although enzymatic degradation of beta-defensins has also been shown [386]. In addition, AMPs can be inactivated by binding with bacterially-derived DNA. This was demonstrated for hBD3, which binds extracellular DNA released by *Haemophilus influenzae* and subsequently shows diminished activity against *H. influenzae* biofilms [387]. In **paper III** it was demonstrated that hBD2 can bind to meningococcal DNA, which reduces its antibacterial efficacy against *N. meningitidis*.

Since initial contact between bacteria and AMPs is made through electrostatic interactions, bacteria can modulate the expression of their anionic surface molecules as a way to subvert AMP binding. For example, the bacteria *N. gonorrhoeae* and *N. meningitidis* achieve elevated resistance to polymyxin B by modulating the expression of *lptA*, a gene encoding the PEA transferase involved in decorating lipid A with phosphoethanolamine, resulting in a more positive charge of LPS [64,388]. Also, addition of a positive charge to teichoic acid by esterification with D-alanine lowered the attraction of alpha-defensins and other AMPs to the gram-positive bacterium *S. aureus* [389].

Removal of intracellular AMPs can be achieved by efflux pumps. In *N. meningitidis* and *N. gonorrhoeae* this involves an efflux system termed MtrCDE that increases the resistance against LL-37 [64,390]. Efflux systems in other

bacteria also reduce sensitivity against AMPs, including HNP1 and hBD1 and 2 [391].

Bacteria in biofilm show a significantly increased resistance against antimicrobial agents [121]. Upon sensing the presence of AMPs some bacterial species can induce biofilm formation. For instance, in response to LL-37 *S. epidermidis* increases the expression of the polysaccharide intracellular adhesin, which is involved in biofilm formation [392].

Active downregulation of AMP-encoding genes is an escape mechanism used by several bacteria [304,393-395]. The expression of LL-37 in cervical epithelial cells was reduced in response to *N. gonorrhoeae* [393]. Furthermore, in *Shigella flexneri*-infected intestinal tissue the reduced level of AMPs was mediated by bacterial plasmid DNA, and this was coupled with deeper progression of bacteria towards intestinal crypts, indicating enhanced virulence [396].

5.5 Therapeutic applications of AMPs

The alarming rise in resistance to antibiotics displayed by many bacterial species has resulted in the search for alternative antimicrobial therapies. AMPs present an interesting option due to the multiplicity of their actions and the low levels of resistance developed against them. One strategy in which the beneficial properties of AMPs can be exploited is by increasing their expression levels. Expression of LL-37 and hBD2 can be induced by butyrate (and its analogs) [309,397] and vitamin D_3 [306]. *Shigella*-infected rabbits that are treated with sodium butyrate show improved clinical outcome [398], and diseases such as cystic fibrosis and tuberculosis also show indications in benefiting from vitamin D_3 treatment by reducing inflammation [399,400]. However, it is important to note that LL-37 and other peptides influence many other cellular processes in the human body, including pro-inflammatory responses. It is therefore necessary to investigate to what extent AMPs can be elevated without inducing adverse effects [401].

Although AMPs are often not considered to be effective antibacterial agents compared to conventional antibiotics, their antibacterial activity can be enhanced in combination with other AMPs or antibiotics. In this way, AMPs that permeabilize the bacterial membrane allow facilitated entry for antibiotics to reach their intracellular targets [402]. This approach has shown successful *in vitro* results in the eradication of bacteria growing within biofilms [403].

Another promising concept is the development of synthetic peptides. These peptides are based on naturally occurring AMPs but have been optimized to display increased killing activity or altered inflammatory responses. Several

of these synthetic peptides have entered clinical trials, but development has been slow due to difficulties with predicting outcome of biological activity, and cost of manufacture [404]. A promising candidate is innate defence regulator-1018, a synthetic peptide that was derived from the bovine bactenecin. It shows selective enhancement of chemokine production while preventing excessive pro-inflammatory responses and in addition targets biofilms of several antibiotic-resistant bacterial species [405].

PRESENT INVESTIGATION

Aims

The overall aim of the studies presented in this thesis was to gain a better understanding of host-bacteria interactions, as well as to characterize *N. meningitidis* physiological responses to host and environmental factors.

Specific aims:

- To determine the effect of bacterial colonization on EGR1 expression in epithelial cells (**paper I**)
- To compare the modulation of hBD2 in epithelial cells in response to commensal species, such as *Lactobacillus*, and pathogenic species, such as *N. meningitidis* (**paper II**)
- To study the antibacterial effect of hBD2 peptide on *N. meningitidis* (**paper III**)
- To understand the role of deficiencies in lactate metabolism on the ability of *N. meningitidis* to form biofilms (**paper IV**)

Results and discussion

Paper I

EGR1 is an early response transcriptional regulator that is rapidly induced by various external stimuli. It is involved in many cellular processes such as cell growth, differentiation, and survival, and is implicated in diseases such as carcinogenesis, diabetes, and atherosclerosis [266-268]. Several bacteria are able to modulate EGR1 expression, but the underlying mechanisms in this process are mostly unknown. The aim of this study was therefore to explore whether EGR1 induction by bacterial stimulation is a general host response, and by what mechanism this occurs.

We stimulated different epithelial cells with a variety of bacterial strains corresponding to their natural site of colonization, i.e. pharyngeal epithelial cells were stimulated with upper-respiratory tract bacteria, gastric epithelial cells were stimulated with gastric isolates, etc. Twenty-five strains were included and consisted of gram-negative and gram-positive strains, as well as pathogens and commensal species. EGR1 expression was analyzed by qPCR and western blot.

We found that EGR1 expression was induced by almost all bacteria tested, irrespective of gram staining or pathogenicity. This induction was seen to occur at 1 h post-infection and peaking at 2 h post-infection, after which expression dropped. Exceptions include non-pathogenic *L. rhamnosus*, *S. enterica* serovar Typhimurium, and *E. coli*, which did not upregulate EGR1 expression in gastric and intestinal epithelial cells, respectively. In addition, *N. gonorrhoeae* and non-pathogenic *L. crispatus* showed different time kinetics and induced EGR1 in cervical epithelial cells only at 4 h post-infection. Induction of EGR1 was confirmed at protein level. Expression of genes either upstream (β1-integrin, epidermal growth factor (EGFR)) or downstream (fibronectin, amphiregulin) of EGR1 was also examined. There was no upregulation of β1-integrin or fibronectin. EGFR expression was induced by *H. pylori*, and amphiregulin expression was induced by several bacteria, which did not necessarily induce EGR1 expression. Interestingly, the upregulation of amphiregulin and EGFR occurred at later time points than EGR1 induction, indicating that EGR1 and EGFR/amphiregulin regulation are independent of each other.

We speculated that the strength of EGR1 induction is correlated to the adhesion level of bacteria to host cells. However, we found that the induction in

EGR1 expression did not correlate to the level of bacterial adherence to epithelial cells, indicating that bacterial adhesion is not important for EGR1 induction.

Next, we investigated whether EGR1 induction is specific for cell type. Different epithelial cells were stimulated with representative bacteria for different body sites. Here we found that the strongest induction in EGR1 expression in epithelial cells was seen for bacteria that naturally colonize this site, whereas induction was low (or absent) for bacteria that did not represent the natural colonization site. Exceptions include the intestinal pathogen *S. enterica* serovar Enteriditis, which induced very high expression of EGR1 in gastric epithelial cells, as well as the nasopharyngeal pathogen *N. meningitidis*, which induced EGR1 expression in gastric and cervical epithelial cells albeit it at a later time point. Interestingly, the induction pattern displayed by *N. meningitidis* matched that of *N. gonorrhoeae.* This finding may be unsurprising since *N. meningitidis* and *N. gonorrhoeae* are closely related strains that have been found to colonize both the nasopharynx and genital tract [406,407] and are thus likely to induce a similar host response. Here again, no correlation was found with EGR1 expression and level of bacterial adherence.

To investigate the importance of bacterial viability we heat-killed bacteria. Subsequent stimulation of epithelial cells saw upregulation in EGR1 expression only in response to *S.* Enteriditis. This indicates that for *N. meningitidis*, *H. pylori*, *S. pyogenes*, and *N. gonorrhoeae* live bacteria are necessary, while *S.* Enteriditis may signal via a surface component molecule that is released upon bacterial lysis. By using a 0.4 μm filter bacteria were separated from host cells, while still allowing passage of secreted molecules. This resulted in inhibition of EGR1 expression in response to all bacteria, which suggests that although adhesion to host cells does not affect EGR1 response, direct contact with host cells is important to elicit an induction in EGR1 expression.

To determine by which signaling pathway EGR1 expression is induced, we blocked specific pathways with chemical inhibitors. Stimulation of epithelial cells with bacteria subsequently showed that the blocking of EGFR and ERK1/2 led to a loss in EGR1 upregulation, indicating that these signaling pathways are commonly used by bacteria. Other pathways were used as well, but these were species-specific.

It has previously been shown that integrin and EGFR crosstalk can be involved in the modulation of EGR1 expression [408]. We therefore investigated the involvement of β1-integrin and integrin-linked kinase (ILK) in the modulation of EGR1 during bacterial stimulation. siRNA was used to silence and inhibit these signaling molecules. We found that silencing of β1-integrin signaling inhibited EGR1 upregulation after infection. Silencing of ILK did not affect EGR1 expression (with the exception of *S.* Enteriditis). These findings

suggest that EGR1 upregulation involves signaling by β1-integrin, and that ILK is not essential in this process.

In conclusion, in this project, we show that EGR1 expression is upregulated in host cells by diverse bacterial species independent of bacterial adhesion. Cell type specificity is important, as well as bacterial viability and direct contact between host cells and bacteria. Finally, the common pathways used by bacteria to induce EGR1 expression are through EGFR-ERK1/2 and β1-integrin signaling.

Paper II

In this study, we investigated bacteria-host interactions with regard to AMP modulation during bacterial stimulation. AMPs are a component of the innate immune response and play an important role in eliminating bacteria by direct killing activity as well as through immuno-modulatory properties [278]. In some cases, AMPs are induced in response to bacterial infection, while in other cases bacteria are able to downregulate their expression in order to evade immune responses [304]. In this study, we examined the expression of two members of the defensin family, hBD1 and hBD2, during incubation of human pharyngeal epithelial cells with commensal lactobacilli and pathogenic *N. meningitidis*.

We stimulated epithelial cells with lactobacilli strains *L. reuteri* and *L. salivarius*, isolated from human saliva. Both strains induced hBD2 expression, while the expression of hBD1 remained unchanged. This expression pattern is in agreement with the constitutive and inducible nature of hBD1 and hBD2, respectively, as shown previously [334]. Interestingly, meningococcal infection of epithelial cells did not induce hBD1 or hBD2 expression, which indicates that epithelial cells may discriminate the presence of commensal from pathogenic bacteria by differential hBD2 induction. Since infection of epithelial surfaces rarely occurs in isolation of other bacterial species, we co-infected epithelial cells with *L. reuteri* and *N. meningitidis* simultaneously. The presence of *N. meningitidis* dampened *L. reuteri*-induced hBD2 expression, and also reduced *L. reuteri* adhesion to host cells. We therefore hypothesized that the mechanism by which *N. meningitidis* dampens hBD2 expression is by reducing *L. reuteri* adhesion to host cells. Further investigation discouraged this theory, as stimulation of epithelial cells with increasing doses of *L. reuteri* did not increase hBD2 induction, even though higher *L. reuteri* dosage correlated with increased adhesion. This indicates that in order for *L. reuteri* to induce hBD2 expression bacteria must adhere to host cells over a certain threshold value, though further attachment does not induce increased expression.

To determine whether induction of hBD2 expression requires an active interaction between *L. reuteri* and the host, or if a secreted factor mediates the effect, we incubated epithelial cells with heat-killed bacteria, as well as with bacteria that were physically separated from host cells. By using a 0.4 μm filter *L. reuteri* were separated from host cells while still allowing passage of secreted molecules. We found that epithelial cells stimulated with heat-killed bacteria did not induce hBD2 expression, indicating that viability is important. Similarly, bacteria separated from host cells also did not induce hBD2 expression, which suggests that hBD2 induction does not involve a

secreted factor. This was confirmed by stimulating host cells with *L. reuteri* conditioned medium, which also did not induce hBD2 expression.

Since induction of hBD2 expression has previously been shown to occur through NF-κB signaling, we measured activation of NF-κB in response to *L. reuteri* and *N. meningitidis* stimulation by using an NF-κB -inducible reporter plasmid. NF-κB activation followed the same pattern as hBD2 induction, indicating that *L. reuteri* induces hBD2 expression through the NF-κB signaling pathway.

To further elucidate the mechanism by which *N. meningitidis* dampens *L. reuteri*-induced hBD2 expression, we speculated that meningococci may act directly on the host. A recent finding implicated the host molecule A20 in the dampening of hBD2 by *S. epidermidis*. A20 is a negative regulator of NF-κB, and the authors suggest that by inducing expression of A20, *S. epidermidis* can downregulate hBD2 expression, thereby evading immune responses in order to facilitate permanent colonization of the skin [245]. We measured A20 expression in epithelial cells and observed that *N. meningitidis* induced A20 expression in epithelial cells, while *L. reuteri* does not. Protein levels of A20 were verified by western blot and showed similar results. These observations suggest that *N. meningitidis* may exploit A20 signaling to dampen hBD2 expression.

In order to understand why *N. meningitidis* maintains low expression levels of hBD2, we speculated that *N. meningitidis* is susceptible to its killing activity. We compared the susceptibility of *N. meningitidis* and *L. reuteri* to synthetic hBD2 peptide, and found that at 1 μM hBD2 the survival of *N. meningitidis* was significantly decreased, while *L. reuteri* viability was not affected. The dampening of hBD2 expression by *N. meningitidis* therefore seems to correlate with a higher susceptibility to killing by the peptide.

To summarize, hBD2 expression is upregulated by commensal *Lactobacillus* species by direct interaction with host cells. In contrast, pathogenic *N. meningitidis* dampens *Lactobacillus*-induced hBD2 expression, likely mediated by negative regulation of NF-κB by inducing the host molecule A20. Since *N. meningitidis* is susceptible to hBD2-mediated killing, exploitation of A20 may be an immune evasion mechanism.

Paper III

In **paper II** we explored the effect of *N. meningitidis* on hBD2 expression. In **paper III** we investigate their interaction from a different perspective, rather, what is the effect of hBD2 on *N. meningitidis* viability?

We incubated exogenous hBD2 peptide with *N. meningitidis*, and demonstrated that hBD2 is able to kill actively growing bacteria, starting at a concentration of 1 μM hBD2. Lower concentrations than 1 μM hBD2 were not lethal to *N. meningitidis*. We observed that hBD2 killing coincided with binding of hBD2 to meningococci, suggesting that binding to the bacterial membrane is an important step for hBD2 to exert their bactericidal effect.

Interestingly, we observed killing by hBD2 only after 2 h incubation, but not at earlier time points. It is known that bacterial killing by AMPs can proceed in two distinct manners, namely, by membrane disruptive and nonmembrane disruptive mechanisms. In the latter, the peptide traverses the bacterial membrane without affecting membrane integrity, whereupon it targets intracellular components [338]. Since we observed that killing of *N. meningitidis* by hBD2 is a delayed process, we speculated that hBD2 kills by a nonmembrane disruptive mechanism. Indeed, we found that incubation with lethal concentrations of hBD2 does not induce outer membrane permeabilization of *N. meningitidis*. Intracellular processes that hBD2 might target instead include inhibition of the cell cycle. Using flow cytometry we therefore measured meningococcal DNA content during killing with hBD2. Treatment with lethal concentrations of hBD2 resulted in a large portion of the bacterial population to display an increased number of chromosomes. This shift resembled that caused by the antibiotic cephalexin, which is known to kill bacteria by inhibiting cell division. To further investigate the effect of hBD2 on *N. meningitidis* cell cycle, we visualized the bacteria by fluorescence microscopy. Cephalexin caused a perceptible increase in bacterial cell size that was not seen in the presence of hBD2, suggesting that hBD2 does not affect bacterial viability by inhibition of cell division. However, it is possible that hBD2 inhibits cell division in a different manner than cephalexin and this option remains to be investigated. Interestingly, we observed a shift in the ratio of bacteria found in diplococci compared to aggregates. While the number of aggregates remained unchanged regardless of hBD2 treatment, the number of bacteria found in diplococci was decreased during treatment with hBD2. From this, we hypothesized that bacteria in aggregates are better protected against killing by hBD2, possibly in a similar manner as bacteria in biofilms are more resistant to antimicrobial agents. The increased chromosome size measured with flow cytometry likely corresponded to aggregated bacteria.

To further investigate whether bacterial aggregation contributes to protection against hBD2, we used a meningococcal mutant deficient in *pilE.* These bacteria do not express type IV pili and are therefore unable to aggregate. Non-piliated bacteria were more sensitive to hBD2 killing, which further supports the theory that aggregated meningococci are less susceptible to hBD2.

Previous research has shown that *H. influenzae* extracellular DNA can bind hBD3, resulting in reduced killing activity of the peptide. Since hBD2 and hBD3 are structurally similar, we explored the possibility that hBD2 can bind meningococcal DNA. We incubated meningococcal DNA with increasing concentrations of hBD2 and subjected it to agarose gel electrophoresis. We found that in the presence of 1 μM hBD2 DNA only partially migrated through the gel, while at 5 μM hBD2 DNA was no longer able to migrate into the gel, indicating that meningococcal DNA is able to bind hBD2. Furthermore, we showed that this interaction resulted in a significant reduction in hBD2 killing activity. Thus, DNA release by *N. meningitidis* presents a possible evasion mechanism of host defences.

In summary, we have demonstrated that hBD2 exerts its antibacterial effect on *N. meningitidis* in a nonmembrane disruptive manner. The intracellular components targeted by hBD2 remain to be found. Meningococcal aggregates showed reduced susceptibility to hBD2 killing, however, the presence of hBD2 did not induce the formation of aggregates. Furthermore, meningococcal DNA can bind hBD2 and thereby inhibit hBD2-mediated killing of *N. meningitidis*, presenting a possible evasion mechanism for *N. meningitidis* against the bactericidal activity of hBD2.

Paper IV

Biofilms are bacterial communities embedded in a network of extracellular polymers such as polysaccharides, proteins, and DNA, which protects bacteria against environmental stress and antimicrobial agents [120]. Bacterial aggregation is an important first step in the formation of meningococcal biofilm [128]. Previously it has been shown that lactate induces dispersal of meningococcal microcolonies [113]. It is also known that lactate utilization influences *N. meningitidis* pathogenesis [166]. However, it is not clear what the role of lactate metabolism is in meningococcal biofilm formation. In this study, we used several meningococcal mutant strains deficient in lactate metabolism enzymes and examined their ability to form biofilms.

We found that mutants lacking the lactate dehydrogenase *ldhA* gene formed more biofilm than the wild-type. Deletion of the other lactate dehydrogenase genes (*lldA* and *ldhD*) or of lactate permease (*lctP*) however did not affect the ability to form biofilm. Since the meningococcal capsule prevents biofilm formation, we created an *ldhA/cap* double mutant. This mutant showed increased biofilm formation compared to the *cap* mutant. *ldhA* complementation restored the level of biofilm formed to that of the wild-type. The increased formation of biofilm by the *ldhA* mutant was verified by microscopy.

Meningococcal biofilm architecture is influenced by the ability of the bacteria to form aggregates [125,127]. We hypothesized that *ldhA* deficient mutants may show altered aggregation compared to the wild-type. Indeed, *ldhA* mutants were more aggregative than wild-type bacteria, however, this was not due to increased expression in *pilE*. In addition, we did not see an increase in adhesion of *ldhA* mutants to host cells either. This was a surprising finding since bacterial aggregation and adhesion to host cells are usually correlated [106,409,410]. We measured the bacterial surface hydrophobicity of *ldhA* mutants and found that it was increased compared to wild-type bacteria. This increase was not associated with altered expression of capsule between the strains. These data suggest that the increased formation of biofilm exhibited by *ldhA* mutant bacteria is due to their enhanced surface hydrophobicity.

Extracellular DNA (eDNA) is a major component in meningococcal biofilms [124]. The presence of eDNA in biofilms of other bacterial species has been shown to influence bacterial surface hydrophobicity [411]. We therefore speculated that eDNA release may be altered in *ldhA* mutants. Addition of DNase I at the beginning of biofilm formation resulted in a significant reduction in biofilm formation for *ldhA* mutants, indicating that eDNA plays a role in *ldhA* biofilm formation. Biofilm formation of wild-type bacteria or *cap* mutants was not affected by DNase I treatment. This was as expected since it is known that

bacteria belonging to clonal complex ST-11 (such as the FAM20 strain used in this study) form biofilm independently of eDNA [124]. Since meningococci release eDNA during bacterial lysis, we measured the rate of autolysis of the *ldhA* mutant. We found that autolysis in the *ldhA* mutant was increased compared to that of wild-type. This elevated rate of autolysis correlated with increased concentrations of DNA in the bacterial culture supernatant as well as in biofilm. The role of eDNA in the enhanced aggregation displayed by *ldhA* mutant bacteria was also confirmed by DNase I treatment. These data indicate that the enhanced aggregation and elevated formation of biofilm in *ldhA* mutants occurs through increased eDNA release mediated by autolysis.

To explore whether the increased eDNA release occurs due to differential expression of autolysin genes and other genes previously linked to biofilm formation, we performed qPCR analysis on *ldhA* mutant and wild-type bacteria, at log-phase and during biofilm growth. *ldhA* mutant bacteria showed an increase in expression of *norB* during both log-phase and biofilm growth, and *narP* expression was upregulated during log-phase growth. These genes are involved in respiration in a microaerobic environment. The gene *aniA* that is also involved in this process was not affected. In addition, several genes in *ldhA* mutant bacteria were downregulated during biofilm growth. Expression of *pilE* and *cap* was suppressed, as well as the expression of autotransporter *nalP*, and autolysins *mltA* and *ampD*. A downregulation in *pilE* expression was surprising since pili mediate initial attachment necessary for biofilm formation. However, since *pilE* expression during log-phase growth was unaffected, it is possible that bacterial attachment was established during initial phases of biofilm formation and that subsequent *pilE* expression was not required. NalP cleaves bacterial cell-surface eDNA-binding molecules. Absence of NalP can therefore promote biofilm formation [123], which corresponded to our findings. It is surprising that expression of *mltA* and *ampD* is downregulated, and their role in biofilm formation of *ldhA* mutant bacteria remains to be investigated.

Acetate production is a byproduct of *N. meningitidis* glucose metabolism. Acetate and acetate intermediates are known to influence biofilm formation of several pathogens by expression of genes that encode proteins that are important in bacterial autolysis [412-414]. We therefore speculated whether acetate may play a role in biofilm formation of the *ldhA* mutant. We found decreased levels of acetate in biofilm formed by *ldhA* mutant, which suggests that the presence of acetate is inversely correlated with biofilm formation in *ldhA* mutant bacteria. However, addition of different concentrations of sodium acetate to *ldhA* mutant bacteria did not influence biofilm formation, indicating that acetate does not play a role in biofilm formation.

In conclusion, we report that a lack of *ldhA* in meningococci leads to increased autolysis and eDNA release, which promotes bacterial aggregation and biofilm formation.

Future perspectives

Answering questions will inevitably lead to new ones.

In **paper I**, we observed that EGR1 induction in the host appears to be a general response by bacterial stimulation. However, it is not known what the role is of bacterially-induced EGR1 expression during infection. Do bacteria induce EGR1 to their advantage? Or does EGR1 expression rather contribute to host defence by modulating the immune system? It was previously shown that downregulation of EGR1 expression by the AMPs hBD2 and hBD3 inhibits macrophage autophagy, which resulted in improved phagocytosis and intracellular killing of *P. aeruginosa* [272]. Thus, EGR1 has the potential to be exploited by bacteria as an immune evasion strategy. This is especially interesting considering that *N. meningitidis* dampens the expression of hBD2, as shown in **paper II**, and warrants further investigation. Silencing of EGR1 would provide a useful tool to investigate the role of EGR1 during infection. By comparing parameters such as cytokine release, cell proliferation, and apoptosis, we could determine whether EGR1 is involved in mounting an immune response during bacterial infection. Furthermore, since EGR1 is involved in regulating a vast number of genes, performing a microarray or RNA sequencing analysis may provide information on a wider range of roles for EGR1 during host-bacterial interactions. The relevance of these findings could be further explored in an animal model deficient in EGR1.

A different aspect that would be interesting to investigate is the bacterial component responsible for eliciting the induction in EGR1 expression. Since we observed cell type specificity and variation in the signaling pathways used leading to EGR1 expression, it is unlikely that this component is a shared factor conserved among the bacteria tested.

In **paper II** we showed that expression of hBD2 in epithelial cells is induced in response to *L. reuteri*. To increase the relevance of our findings it would be interesting to repeat these experiments in a primary epithelial cell line, or in an *in vivo* model. We attempted to confirm hBD2 expression on protein level, but protein detection of hBD2 using Western Blot has unfortunately proven unsuccessful. We speculate that hBD2 is not released at sufficiently high concentrations for detection. Concentration of hBD2 in the supernatant by filtration or dialysis, or intracellular retention of hBD2 by treatment with brefeldin A, may result in hBD2 reaching detectable concentrations. Possibly a primary cell line is more responsive to bacterial stimulation and induces higher levels of hBD2.

It is important to confirm the involvement of NF-κB in upregulating hBD2 expression during stimulation with *L. reuteri.* This can be performed by treating epithelial cells with an NF-κB inhibitor. In addition, the involvement of A20 in mediating a reduction in hBD2 expression during *N. meningitidis* stimulation needs to be verified, for instance by silencing A20 expression in epithelial cells using A20-targeting siRNA.

The high induction of A20 expression in response to co-incubated *L. reuteri* and *N. meningitidis* was an unexpected result. Since we hypothesized that A20 and hBD2 expression are inversely correlated, we expected the co-incubated bacteria to induce A20 expression in between that of *L. reuteri* and *N. meningitidis* alone. This discrepancy could be caused by epithelial cells responding synergistically to the higher bacterial load. However, A20 protein levels corresponded better to the hBD2 expression profile.

The mechanism by which *N. meningitidis* dampens *L. reuteri*-induced hBD2 expression was initially attributed to the reduction of *L. reuteri* adhesion to host cells. This theory was abandoned when we observed that *L. reuteri* adhesion is not involved in hBD2 induction. However, in these experiments, adhesion levels of lactobacilli to host cells did not fall below the hypothesized threshold level that is necessary to elicit induction of hBD2. It would be useful to test hBD2 expression during lower *L. reuteri* adhesion levels to confirm whether *N. meningitidis* dampens hBD2 expression by inhibiting *L. reuteri* adhesion. Furthermore, *N. meningitidis* may affect *L. reuteri* in a different manner than adherence to host cells only. To get an overview of the processes affected by *N. meningitidis* in *L. reuteri*, it would be interesting to perform RNA sequencing analysis.

Continuation of this project could focus on determining the bacterial factors responsible for modulation of the host responses demonstrated in this paper. We have shown that direct contact between *L. reuteri* and the host is necessary to induce hBD2 expression, indicating that the factor involved is likely a surface molecule. In addition, *L. reuteri* conditioned medium or *L. reuteri* separated from host cells was unable to induce hBD2 expression, ruling out the involvement of any secreted components. It is possible that a heat-sensitive surface molecule that is released during heat-killing is involved. In addition, construction of meningococcal mutants deficient in surface molecules such as pili, LPS, or outer membrane proteins could provide information on the molecular mechanism responsible for A20 induction seen in response to *N. meningitidis*.

It is important to keep in mind that hBD2 is often regarded to have a more important role in immunomodulation than in bacterial killing, especially *in vivo* where physiological salt concentrations may affect its killing potential. *N. meningitidis* may therefore dampen hBD2 expression for other reasons than solely for its killing activity. Comparing cytokine release and other immune

responses during *N. meningitidis* and *L. reuteri* stimulation of host cells would provide a new perspective.

In **paper III** we further explored the killing effect of hBD2 on *N. meningitidis*. We showed that hBD2 kills bacteria but only starting after 2 h, suggesting a slower process of killing. We believe this time delay can be explained by hBD2 attacking *N. meningitidis* through a nonmembrane disruptive killing mechanism, which requires time for hBD2 to traverse the bacterial membrane and to affect intracellular processes that will ultimately kill the bacteria. This theory was strengthened by the lack of outer membrane permeabilization shown during hBD2 treatment. However, more substantial evidence could be provided by performing single-cell imaging [415]. Using such a technique the localization of hBD2 to the cytoplasm can be verified, and additionally would deliver spatial information into key mechanistic events of hBD2 killing.

The finding that hBD2-treated bacteria are less likely to be in planktonic/diplococci form prompted the hypothesis that bacteria in aggregates are better protected against hBD2-mediated killing. This was verified by using *pilE* mutant bacteria that do not aggregate, which showed increased susceptibility to hBD2. It would be interesting to visualize *pilE* mutant bacteria with microscopy to ascertain their cell morphology during hBD2 treatment. If these bacteria exhibit similar aggregation as the wild-type, this would indicate that meningococcal aggregation is not involved in protecting bacteria against hBD2, and would compel us to reconsider the involvement of DNA damage.

An exciting finding in this study was the observation that hBD2 is bound by meningococcal DNA. This experiment used a 1786 bp non-coding DNA fragment. We speculate that the interaction between the positively charged hBD2 peptide and the negatively charged DNA molecule relies on a simple electrostatic attraction, as was suggested previously [416]. To demonstrate that this interaction is indeed unspecific, hBD2 can be incubated with DNA from other bacterial species or eukaryotic DNA, using both coding and non-coding DNA regions. If the interaction between DNA and hBD2 is general and driven by electrostatic attraction, intracellular components targeted by hBD2 can be further broadened to include mRNA and proteins and would indicate that hBD2 can kill *N. meningitidis* by affecting a range of processes. AMP inhibition of ribosome activity, protein translation, and enzymatic activity has been shown previously [417,418], although these effects were not necessarily due to charge interactions.

Since hBD2 bound by meningococcal DNA exhibits a reduced capacity to kill *N. meningitidis*, it is tempting to speculate that the bacteria release DNA as an evasion mechanism to avoid killing by hBD2. This can be demonstrated by pre-treating *N. meningitidis* with DNase I prior to incubation with hBD2. If

indeed bacteria release extracellular DNA in response to hBD2, degradation of released DNA by DNase I would result in increased susceptibility to hBD2-mediated killing. To explore if *N. meningitidis* actively respond to the presence of hBD2 by inducing autolysis, expression profiles of autolysin genes (such as the lytic transglycosylases MltA and MltB), could be assessed during hBD2 exposure.

Paper IV showed that deletion of dehydrogenase *ldhA* resulted in increased aggregation and biofilm formation. Formation of biofilm was performed in a microtiter plate by incubating bacteria for 24 h under static conditions. Biofilm mass was then quantified by staining with crystal violet. Crystal violet is a dye that binds to negatively charged molecules, including polysaccharide or DNA in the extracellular matrix, and can thereby quantify biofilm mass in the entire well. However, this method cannot discriminate living from dead cells, therefore the viability of bacteria within biofilm is difficult to assess. Since we show that *ldhA* mutant bacteria enhance biofilm formation by enhanced autolysis, it is possible that the biofilm consists of only dead cells, rather than an increase in live bacteria also. Bacterial viability in the biofilm can be assessed by performing a live/dead stain.

A limitation in using static conditions to assess biofilm formation is that these circumstances do not accurately reflect the physiological conditions *N. meningitidis* biofilm may encounter in the human body. Biofilm formation in a microfluidics chamber with constant fluid flow and nutrient supplementation would for example provide a better representation of a blood flow-like environment. Another method by which a more physiologically relevant environment can be assessed is by evaluating biofilm formation on host cells. This would additionally provide information on the role of eDNA in host cell adhesion and could explain why *ldhA* mutant aggregation is increased, while adhesion to host cells is not affected.

The absence of capsule greatly enhances the surface hydrophobicity of *N. meningitidis*. Interestingly, *ldhA* mutant bacteria also showed enhanced surface hydrophobicity, although *ldhA* mutant bacteria are not affected in their capsule content or expression of the *siaD* gene compared to wild-type bacteria. It is possible that the increase in eDNA release affects hydrophobicity of *ldhA* mutant bacteria, perhaps by binding to Tfp since these structures are the only components that remain exposed in encapsulated bacteria. Several other surface components are able to induce biofilm formation by binding to DNA, including NhbA and the autotransporters IgA protease and AutA [68]. Their capacity to bind DNA is affected by NalP, which cleaves and removes these structures from the bacterial surface. Therefore, the absence of NalP can induce biofilm formation, while its presence can induce biofilm dispersal. We observed a downregulation in *nalP* expression in *ldhA* mutant bacteria during biofilm

growth, indicating that NhbA and IgA protease may play a role in biofilm formation of *ldhA* mutant bacteria. It would be interesting to further characterize their involvement.

This thesis provides new knowledge on host responses during bacterial colonization of both commensal and pathogenic species. The interplay between host and bacteria is a complex process that is dependent on many factors. In addition, characterization of *N. meningitidis* responses to host and environmental factors has increased our understanding of meningococcal virulence.

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