Glycan-dependent *Helicobacter* spp. and *Streptococcus oralis* binding to mucins in the gastric and oral mucosal niche

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Cover illustration: Gastric *O*-glycan diversity by Gurdeep Chahal

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You are only bound by your duty, not to the results thereof

Bhagwad Gita 2:47

To my family, friends and my teachers

ABSTRACT

Helicobacter pylori infects the stomach of half of the world's population, while *Helicobacter suis* colonizes pigs and is the most common non-*H. pylori* *Helicobacter* species that also infects humans stomach. Infection with *Helicobacter* spp. is associated with chronic gastritis, peptic ulcer disease, and gastric cancer. *Streptococcus oralis* colonizes human oral cavity and can cause infective endocarditis (IE). First barrier pathogens encounter is the mucus layer constituted by highly glycosylated glycoproteins, the mucins. Mucins glycans provide an extensive surface of interaction for bacteria. Here, we show the interactions of *Helicobacter* spp. and *S. oralis* with glycans in the gastric and oral mucosal niche.

In paper I, the glycans from *H. pylori* infected and non-infected human stomachs were characterized by mass spectrometry. An enormous diversity of glycosylation exists in the human stomach. Infection with *Helicobacter* spp. is associated with large inter- and intra-individual diversity. The differences in glycosylation between mucins from infected and non-infected individuals are reflected by differences in binding of *H. pylori* to the mucins. **In paper II,** the binding of different *H. pylori* strains J99, P12, 26695 and G27 was analyzed. We show that these strains differ in their binding preferences and that mucins from infected or non-infected human stomachs affect the adhesion of different strains differently. Further, we show that infection, rather than inflammation, determines these effects. **In paper III,** we show that experimental *H. suis* infection alters the composition of mucins and their glycosylation in a manner that reduces the amount of *H. suis* binding glycan structures, decreases *H. suis* binding ability, and changes mucin phenotype towards more *Helicobacter* spp. growth promoting. Thus, *Helicobacter* spp. infections impair the mucus barrier to create a stable niche in the stomach.

In the fourth study, the carbohydrate binding of IE isolates of *S. oralis* subspecies was investigated. Mucins were isolated from the saliva from blood group A and B positive individuals. Salivary mucins were characterized by antibody binding, lectin binding, mass spectrometry. We show that *S. oralis* adhesion occurs to salivary mucins and the binding differs between strains. *S. oralis* binding differs between mucins and individuals. Further, we show that *S. oralis* subsp. *oralis* binding to oral mucins is mediated by a cell wall

anchored surface protein(s) and Leb, SLex and LNT like glycans present on the mucins.

We demonstrate that mucin glycans are highly diverse and differ between individuals and with infection status. The glycan repertoire governs the ability of the mucins to bind to pathogens. *Helicobacter* spp. infection increases the diversity of glycosylation in the host and changes the host mucin composition. Understanding the adhesion mechanisms of *H. pylori, H. suis* and *S. oralis* could help develop preventive strategies against these pathogens.

Keywords: *Helicobacter*, diversity, glycosylation, adhesion, *Streptococcus*

SAMMANFATTNING PÅ SVENSKA

Helicobacter pylori (*H. pylori*) är en bakterie som koloniserar magen på halva jordens befolkning. *H. pylori* kan förekomma i magsäckens slemhinna och trivs i magsäcken. En nära släkting till *H. pylori* är *Helicobacter suis* (*H. suis*) som koloniserar grisens magsäck och samtidigt är den vanligaste icke-*H. pylori Helicobacter* arten i människans magsäck. Infektion med *H. pylori* orsakar kronisk inflammation i magen som kan leda till farlig situation och är den viktigaste orsaken till magsår och magcancer. Det är svårt att få bort dessa bakterier från slemhinnan i magsäcken och behandling av dessa infektioner med antibiotika är problematisk för att *H. pylori* kan utveckla antibiotikaresistens. *S. oralis* förekommer normalt i munhålan, och är en opportunistisk patogen som kan orsaka infektiös endokardit (IE), vilket är en infektion lokaliserad till hjärtklaffar. Bakterier kommer in i kroppen via slemhinnan (även kallad mukosan). Denna yta täcker många hålrum bland annat andningsvägarna och magtarmsystemet. Denna yta är täckt av ett kontinuerligt utsöndrat slem (mucus) som tvättar bort bundna partiklar. Slemmet består av glykoproteiner (muciner). I munhålen byggs detta slemlager upp av mucinerna MUC5B, MUC7 och Salivary agglutinin och i magen av MUC5AC, MUC6 och en mindre mängd av MUC1. Längst ut på dessa mucinerna sitter kolhydrat strukturer som Leb, Ley, sialyl-Lex och sialyl-Ley samt även andra epitoper inehållande fukos, sialinsyra och galaktos. Dessa muciner bär ett stort antal kolhydrastrukturer, vilket ger många potentiella ställen för bakterier att binda till.

I denna avhandling har vi visat att det finns en enorm mångfald av glykanstrukturer i människans mage. Infektion med både *H. pylori* och *H. suis* orsakar kvalitativa och kvantitativa förändringar i kolhydratstrukturerna som sitter på mucinerna. Vi visade att infektion med *Helicobacter* ökar glykan mångfald i magen samtidigt minskar mängden av strukturer som binder till *Helicobacter*. Detta kan främja tillväxt av bakterie i magen att skapa en lämplig nisch för bakterien i magen. Vidare visade vi att IE-framkallande *S. oralis* binder till saliv muciner och den bindning förmedlas av bakteriernas ytprotein och Leb, SLex och LNT glykaner som sitter på saliv muciner. Resultaten som beskrivs här ger insikt i interaktionerna av *Helicobacter* med glykanerna i magslemhinnor samt till bindningen av IE orsakande *S. oralis* till salivslemhinnor. Vi tror att förståelse av interaktionerna samt bindnings mekanismerna för *H. pylori*, *H. suis* och *S. oralis* kan hjälpa till att utveckla förebyggande strategier mot dessa patogener som kan leda till ett alternativ till antibiotika i framtiden.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **A complex connection between the diversity of human gastric mucin *O*-glycans, *Helicobacter pylori* binding, *Helicobacter* infection and fucosylation.**

Gurdeep Chahal, Médea Padra, Mattias Erhardsson, Chunsheng Jin, Vignesh Venkatakrishnan, János Tamás Padra, Helen Stenbäck, Anders Thorell, Niclas G Karlsson, Sara K Lindén. Under Review for publication in MCP, 2021.

II. **Effects of *Helicobacter* spp. infection on the pig and human gastric mucin *O*-glycome and mucin-*Helicobacter pylori* interactions.**

Gurdeep Chahal, Médea Padra, Mattias Erhardsson, A Thorell, NG Karlsson, Sara K Linden. Manuscript.

III. ***Helicobacter suis* infection alters glycosylation and decreases the pathogen growth inhibiting effect and binding avidity of gastric mucins.**

Médea Padra, Barbara Adamczyk, Bram Flahou, Mattias Erhardsson, **Gurdeep Chahal**, Annemieke Smet, Chunsheng Jin, Anders Thorell, Richard Ducatelle, Freddy Haesebrouck, Niclas G. Karlsson, Sara K. Lindén.

Mucosal Immunology **12**, 784–794 (2019)

IV. **Binding of *Streptococcus oralis* to human salivary mucins is inhibited by Lewis b and sialyl-Lewis x.**

Gurdeep Chahal, John Benktander, Meztlli O. Gaytán Samantha J. King, Sara K. Lindén. Manuscript.

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ABBREVIATIONS

Ser/Thr	Serine/Threonine
Le	Lewis
IgA	Immunoglobulin A
HCL	Hydrochloric acid
H. pylori	*Helicobacter pylori*
H. suis	*Helicobacter suis*
S. oralis	*Streptococcus oralis*
BabA	Blood group antigen binding adhesin
SabA	Sialic acid binding adhesin
LabA	LacdiNAc specific adhesin
kDa	Kilo dalton
ECM	Extracellular matrix
AlpA	Adherence-associated lipoprotein A
LPS	Lipopolysaccharide
SP-D	Surfactant binding protein D
NHPH	Non-*H. pylori* Helicobacter
IE	Infective endocarditis
SRRP	Serine rich repeat protein
ATP	Adenosine triphosphate
SPR	Surface plasmon resonance

1 INTRODUCTION

1.1 Mucosal surface, mucus and mucins

Mucosal surfaces are an enormous interface between the internal organs of the body and the external environment and provide protection against invading pathogens or infection through innate immunity and adaptive immunity systems. The mucosa, or mucous membrane, lines the various cavities in the body, mainly respiratory, digestive and urogenital tracts. The mucosa of organs are composed of one or more layers of epithelial cells over a deeper layer of lamina propria of loose connective tissue. Generally, the epithelial layer of the membrane is composed of either stratified squamous epithelium or simple columnar epithelium. These epithelia are tough – able to bear injury and other wear associated with external influences (e.g. food particles). In humans, the mucosal surfaces together comprise about 400 m^2 [1]. Mucus is secreted by the epithelial surfaces across the entire gastrointestinal tract (GI tract) - stomach to the colon. The thickness of mucus layer differs considerably among organs, it is thinnest, 70-110 µm, in the oral cavity [2], approximately 240 µm in stomach, 160-400 µm in the small intestine and thickest 800-900 µm in the colon [3]. In the human GI tract, peristaltic movements continuously clear the mucus and the constitutive pathway continually secretes plenty mucins to keep up the baseline mucus layer [4]. The mucus layer is highly versatile and may be regulated in response to various external stimuli such as foods, cytokines and microbes. The mucus layer guards the mucosal tissue against invading pathogens by providing epitopes that can bind to bacterial adhesins and thereby inhibiting further interactions [5]. Glycosylation is the major post-translational modifications (PTM) of mucin glycoproteins and lipids and alteration in glycosylation influences many biological functions including bacterial adhesion, receptor activation, cell differentiation, and cell signaling.

The term *mucous membrane* originates from the primary substance secreted from the membranes that is mucus. Mucus is a complex aqueous fluid that consists of approximately 90-95% water, at the same time it also contains electrolytes, lipids and smaller defensive proteins such as defensins, lysozyme, lactoferrins, immunoglobulins, trefoil factors and epithelial repair growth factors [6]. The principal constituent of mucus is a mucopolysaccharide called mucin.

1.2 Mucins (Oral & Gastric)

Mucins are a heterogeneous family of heavily glycosylated proteins expressed on all epithelial surfaces. They are major constituents of mucus layer and represent the first line of defense of our innate immune system.

Mucin domains consist of a protein core of tandem repeat sequences rich in proline, threonine and serine enabling post-translational *O*-glycosylation [7-9]. The highly glycosylated characteristics of mucins render them resistant to proteolysis and ability to retain water gives them the gel-like properties found in the mucus layer covering the mucosal epithelial cells. Mucins may also be attached to cell membranes and can act as ligands for sugar-binding molecules. Transmembrane (TM) mucins are localized to apical surfaces of mucosal epithelial cells and differ in length and composition. In healthy tissue, the transmembrane mucin family includes: MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC16, and MUC17 [10]. TM mucins play major roles in maintaining mucosal barrier function and they restrict the invading pathogens at mucosal surfaces by translating external stimuli to cellular responses [11]. Secreted/gel-forming mucins constitute the extracellular mucus. They are large, heavily *O*-glycosylated with high molecular weight, which form gel and provide mucus its viscous properties. There are five gel-forming secreted mucins (MUC2, MUC5AC, MUC5B, MUC6 and MUC19) and one non gel-forming secreted mucin (MUC7). The gel-forming mucins oligomerize via inter-molecular disulfide bonds that occur between the cysteine-rich domains [12]. In the oral cavity, MUC5B is the predominant gel-forming mucin [13], but transcripts and glycoproteins of MUC19, another gel-forming salivary mucin, have also been identified [14-16]. MUC7 is another secreted mucin that lacks gel-forming properties. In healthy human gastric mucosa, major gel-forming mucins are MUC5AC (found in the surface epithelial region), MUC6 (located in the gland region) [17, 18].

In the oral cavity, the secretory mucins are produced by mucous cells in the salivary gland [13, 19]. In the GI tract, specialized epithelial cells produce and secrete mucins continuously into the lumen [20]. Mucins are secreted by two separate processes: compound exocytosis/regulated secretion and basal/constitutive secretion. The constitutive pathway secretes continuously to maintain the baseline mucus layer whereas regulated secretion occurs when goblet cells rapidly release a massive discharge of mucus when exposed to stimuli, including mucin secretagogues or other agents such as inflammatory cytokines, hormones, prostaglandins and intracellular messengers (Ca^{2+} and cAMP) [21-23]. Mucin production and secretion is essential to maintain the

mucus barrier. A host of factors including microbes, cytokines, toxins and microbial products regulate these processes, thus affecting the mucus barrier [24, 25].

1.3 Mucin glycosylation

Mucins are large glycoproteins, which contain complex multi-domain structures. Various posttranslational modifications and their large polypeptide chains provide the structural complexity to the mucins. Glycosylation is the primary post-translational modifications of mucins. Mucins are by definition highly *O*-glycosylated but can also carry *N*-glycans. *N*-glycosylation of mucins is initiated in the endoplasmic reticulum [26]. Notably, *O*-glycosylation is the principal type of glycosylation. The primary sites of *O*-glycosylation are the characteristic tandem repeat domains of mucins rich in proline, threonine and serine (PTS) amino acids [31]. *O*-glycosylation occurs in the Golgi apparatus and is initiated by *N*-acetylgalactosaminyltransferase (GalNAc-Ts) family of enzymes [27] by adding α-N-acetylgalactosamine (GalNAc) to Ser/Thr residues in the PTS region of protein backbone [28].

Tn (GalNAc-α1-*O*-Ser/Thr) antigen is the first *O*-glycan formed [29]. After addition of the first sugar, GalNAc (Tn) extension of sugar chains is then processed in a stepwise manner by the sequential action of large number of different glycosyltransferases that add specific monosaccharides yielding high order glycan structures. For example, GalNAc is extended by specific glycosyltransferases that add various monosaccharides, generating different core structures (1-8), followed by the backbone region (type-1 and type-2 chains) and a peripheral region. The chains in peripheral region are terminated by GalNAc, galactose, fucose, or sialic acid forming histo-blood group antigens such as A, B, H Lewis b (Leb), Lewis y (Ley), Lewis a (Lea), Lewis x (Lex), as well as sialyl Lewis a (sLea) and sialyl Lex structures (sLex) (Figure 1). This creates linear or branched structures. The glycans structures are highly complex and diversified and can be further diversified by the addition of sulfation on Gal and N-acetylglucosamine (GlcNAc) residues.

Mucin *O*-glycans account for nearly 70-80% of the mass of the proteins [30]. The terminal structures and sequence of mucin oligosaccharides chains are highly heterogeneous and show intra- and inter-species variation [31] and vary even with tissue location and site-specific glycosylation within a single individual [32, 33].

For example, in the oral cavity, the glycosylation of salivary mucins MUC5B and MUC7 differs and it also varies between individuals of same blood group [19, 34, 35]. In human gastric mucosa, type1 blood group antigens are found

in the surface epithelial region compared to type 2 antigens which are expressed mainly in the cells of glandular regions [36]. The majority of normal gastric *O*-glycans are neutral and fucosylated [37] and salivary mucins contain a high degree of sialylation [38].

Possibly, this structural diversity of glycans helps us withstand the infection by diverse and constantly evolving pathogens, as people with different histo-blood groups are differently susceptible to specific pathogens [39]. In addition, infection/inflammation can lead to host glycosylation alterations as seen in infection with the nematode *Nippostrongylus brasiliensis* in gastric epithelial cells in rats [40], in individuals with Cystic fibrosis (CF) [41] and alterations in glycosylation promote chronic *Pseudomonas aeruginosa* lung infections in CF patients [42]. Infection with *H. pylori* appears to increase expression of sialyl Lewis x (SLe^x) [43-45] and *H. pylori*-infected individuals contain higher levels of Sialyl-Le^a than non-infected [46]. *H. pylori*-induced gastritis decreased both the diversity as well as the amount of *O*-linked mucin glycans in the rhesus stomach [47]. In human gastric cell lines, *H. pylori* induces the increased expression of SabA-ligand sialyl-Le^x [48]. In humans, gastric glycosylation changes with inflammation [43, 46] and a global increase in sialylation is associated with cancer [49, 50].

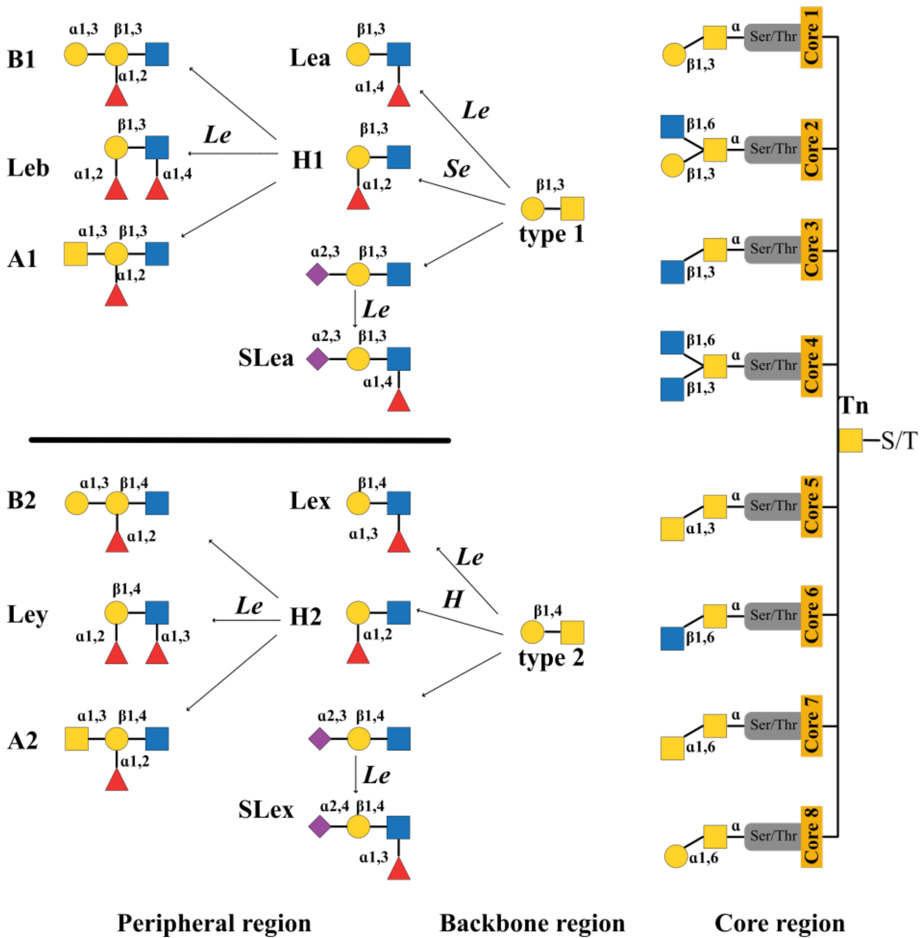

Figure 1. Simplified overview of carbohydrate structures present on mucins. ABH and Lewis antigens are synthesized by enzymatic addition of monosaccharide residues to specific precursor substrates with Galβ1,3-GlcNAcβ- (type1) and Galβ1,4-GlcNAcβ- (type 2) linkages.
Histo-blood group type O individuals express H type 1 and 2. H antigens are further diversified to blood type A, type B or type AB by addition of GalNAc, Gal, or either carbohydrate , respectively, to the galactose in the H antigen [51]. Non-secretor individuals lack a functional FUT2 enzyme and, therefore, produce very little amounts or no Leb antigens on their epithelial surfaces, as the Leb precursor (*i.e.* the H type 1 sequence) is not formed [52]. ● galactose; ■ N-acetylglucosamine(GlcNAc); ▲ Fucose; ■ Nacetylgalactosamine (GalNAc); ◆ Neu5AC.

Human salivary mucins

The oral cavity is covered by mucous membrane consisting of a stratified squamous epithelium. Saliva in the oral cavity represents the link between the internal environment and the pathogens. It is produced by the three major salivary glands: the parotid, the sublingual and the submandibular [53]. Mucins are the major components of the secretion, along with, innate defense related proteins. The human salivary proteome is found to carry over one thousand proteins and peptides [54] and these components of saliva exert antimicrobial, antifungal and antiviral activities. For example, lysozyme has bactericidal properties [55], lactoferrin has antimicrobial activity [56], salivary peroxidase is antimicrobial [55] and histatins, cystatins and defensins form complexes with mucins [57, 58].

In human saliva, the predominant mucins are the high molecular weight MUC5B and low molecular weight MUC7. MUC5B is a gel-forming mucin [13] and MUC7 is a salivary soluble mucin [59]. MUC7 exists as a monomer since it lacks a terminal cysteine rich domain in its structure, therefore, MUC7 mucins are unable to form polymers [60]. Recently, MUC19 another gel forming large, high molecular weight mucin in human saliva has been reported [14]. Saliva also contains agglutinins such as salivary agglutinin (SAG) [61]. Both MUC5B and MUC7 are involved in bacterial interaction (oral streptococci) [62, 63] and MUC7 has antifungal (candidacidal) activity [64, 65]. They also form complexes with other antibacterial salivary proteins such as statherins and histatin 1 [57]. SAG interacts with a large number of pathogens, including tooth-decaying streptococci and *H. pylori*, but also forms complexes with mucosal defense proteins, such as surfactant proteins, IgA, and MUC5B.

Many oral bacteria, including *Streptococcus. oralis* (*S. oralis*), possess adhesins that recognize specific glycan motifs on salivary mucins. *H. pylori* binds to salivary MUC5B and SAG via interaction of Leb glycan [66, 67]. Different salivary mucins interact with pathogens differently. For example, *S. sanguinis*, *S. oralis* and *S. sobrinius* bind to MUC7 but not MUC5B [68]. Another study shows that MUC7 aggregates *S. gordonii* but MUC5B has no effect [35], indicating that MUC5B and MUC7 use different mechanisms to bind. The difference in MUC5B and MUC7 structure influence bacterial binding (through adhesins) by the type of glycan, its linkage and its chain length of the glycan [69-71].

Human gastric mucins

Histologically, the stomach mucosa of humans consists of 4 different parts: the cardiac, fundus, body and pylorus. The mucosal layer of stomach, comprised of gastric glands, is covered by epithelial tissue. The foveolar cells in the cardia region produce mucus that adheres firmly to the gastric mucosal surface as a protective layer shielding it from being self-digested by the aggressive pepsin (from chief cells) and HCl (from parietal cells).

Mucins constitute the major components of the viscous gels lubricating and protecting epithelial cell lining of the GI tracts (7). Mucins are heavily glycosylated high molecular weight glycoproteins produced either as membrane-bound or secreted products. Mucins contain PTS (Pro/Thr/Ser) domains comprised of tandemly repeated sequences of amino acids rich in PTS residues. The PTS domains are extensively glycosylated at the threonine and serine residues through GalNAc *O*-linkages (7).

In the healthy human stomach, both membrane-bound (MUC1) and secreted mucins (MUC5AC and MUC6) are produced. In humans, mucin genes encode mucin proteins and these genes are expressed in a manner that is cell- and tissue-specific. MUC1 is expressed in foveolar cells of the surface epithelium and neck region of the gastric antrum, and to a lesser extent, in mucous glands. MUC1 is highly expressed in the stomach and its expression is upregulated in infection [72] and acts as a decoy to inhibit *H. pylori* adhesion to the cell surface [73]. The secreted MUC5AC mucin is a key component of the surface mucus and is expressed in the foveolar epithelium, whereas the secreted MUC6 is expressed primarily in the glands [74-76]. This distinct distribution of mucins determines the pattern of gastric glycosylation given that expression of MUC5AC is correlated with expression of type 1 Lea and Leb blood group antigens, while MUC6 expression is associated with the type 2 Lex and Ley antigens [18].

1.4 Host-pathogen interactions in the mucus niche

Humans are host to trillions of microbes that reside in immediate proximity to our epithelia, and we are generally able to prevent their colonization. The mucus layer lubricating the host epithelial surfaces in the oral and gastrointestinal tract is the first barrier between invading pathogens and host cells. Mucus, therefore, also provides an initiation surface for host-pathogen interactions. Microbes interact with the mucins and their *O*-glycan structures as well as with the host glycocalyx to colonize the mucosal surfaces [77]. Mucins, the post translationally modified polypeptides with complex glycan structures, exert their beneficial effects in a myriad of ways. A large diversity in glycosylation has been found on the mucins isolated from mucus [37]. This provides an extensive repertoire of attachment sites for bacteria [78]. Microbes use several adhesins to bind to the mucin glycans with different oligosaccharide specificities [79-81] and the differential expression of mucin glycans present in different tissues can lead to tissue-specific colonization by bacteria [82]. Pathogen binding to mucins on the cell surface supports the barrier function of mucus both by providing a disadhesive protective barrier in glycocalyx and by acting as releasable decoy [73, 83-85].

Mucus can house tremendous number of microbes, [86] and be used as matrix for bacterial growth and colonization [87]. These microbes rarely cause infections in healthy mucus [86], suggesting that mucus layer employs mechanisms that regulate virulence [88]. Mucus also provide an abundant source of nutrients for bacterial growth and to host microbial communities [89]. For example, oral streptococcus species including *S. oralis* can utilize pig gastric mucins as nutritional substrate by using exoglycosidase and fucosidase activity to degrade the oligosaccharides [90]. Several bacteria produce diverse mucinases, which include sialidases, sulphatases, glycosidases that degrade mucins and use the released glycans as source of energy [91-93]. The microbiota residing in the mucus layer is able to modulate the mucus niche in a manner that favors the bacteria. For example, enzymes in the outer membrane in the human gut symbiont *B. thetaiotaomicron* degrade host mucus *O*-glycans [94-96] and metabolize human milk oligosaccharides [97]. A study using germ-free mice showed that *B. thetaiotaomicron* forages on host mucin *O*-glycans [96]. Mucin-glycans have also been shown to regulate the bacterial phenotype and act in a manner that "tames" microbes, rendering them less harmful to the host. *In vitro* studies showed that mucin glycans attenuate the virulence of *P. aeruginosa* in infection [88]. Host mucins influence *H. pylori* behavior by regulating gene expression, growth and virulence of pathogens [101]. Some pathogens can also alter the rheological properties (via elevating

pH) of the mucus in their microenvironment to decrease its viscoelasticity, which can facilitate bacterial motility [98].

1.5 *Helicobacter pylori*

History

In the 1930s and 1940s, spiral organisms were observed in the human stomach [99, 100], but no attention was paid to gastric bacteria. In the 1980s, Warren and Marshall performed self-ingestion experiments and isolated a previously unknown bacterium that caused gastritis and peptic ulcer disease [101-105]. The organism was initially named "*Campylobacter*-like organism," "gastric *Campylobacter*-like organism," and "*Campylobacter pylori*", but was later changed to its present name *Helicobacter pylori* [106].

Morphology

H. pylori is a microaerophilic, gram-negative, spiral-shaped bacterium found in the human stomach, which chronically infects almost half of the world's population. Prevalence of *H. pylori* infection is high in the developing countries worldwide and in northern Europe and North America, one third of adults are infected [107]. *H. pylori* infection is acquired early in childhood and becomes chronic [108]. Although most infected individuals remain asymptomatic, infection with *H. pylori* can lead to gastric ulcers and persistent infection may cause intestinal metaplasia (IM), dysplasia and gastric carcinoma [109]. Gastric adenocarcinoma is a leading cause of cancer mortalities in the world. In 1994, based on epidemiologic evidences, the International Agency for Research on Cancer classified *H. pylori* as a class I carcinogenic agent [110]. The clinical outcomes associated with infection of *H. pylori* can be determined by intricate interplay of several factors involving the pathogen (*i.e. H. pylori)*, host related and environmental factors as addressed in [111].

H. pylori **adhesion to human gastric mucins**

The majority of *H. pylori* bacteria reside in the gastric mucus and adhesion to membrane bound mucins protects it from being sloughed off during luminal clearance [112], which is essential for bacteria to maintain a stable niche in the mucus layer. *H. pylori* adhesion to epithelial cells allows the bacteria to gain access to nutrients from the host [113, 114] and triggers host inflammatory

responses [115-117]. To adapt to the dynamic microenvironment in the stomach, *H. pylori* encodes for several outer membrane proteins [118]. *H. pylori* carries carbohydrate-binding adhesins that bind both glycolipids and glycoproteins (mucins). Glycolipids provide more direct adherence to the host gastric epithelial cells and mucins serve as decoys and component of host defence system [73, 81, 119].

The blood group binding adhesin (BabA) represents the best-characterized *H. pylori* adhesion protein; it is 78-kDa protein and is encoded by the *babA* gene [120]. BabA recognizes the mono (H-type)- or di (Leb)-fucosylated structures and mediates a high affinity binding of *H. pylori* to these structures on human gastric epithelial cells and the overlying mucin glycoproteins [121, 122]. *H. pylori* strains expressing BabA appear more virulent than strains lacking this adhesin, since patient infected with BabA positive strains are more likely to develop peptic ulcer disease or gastric cancer [123-125].

The sialic acid binding adhesin (SabA) is a 66 kDa protein encoded by the *sabA* gene (JHP662/HP0725) [43]. SabA mediates binding to alpha 2,3 sialylated structures such as the sialyl Lex and sialyl diLex structures [43]. Sialylated glycans are found in low concentrations in normal gastric mucosa [126]. In a healthy human stomach, sialyl Lex antigen is scarcely expressed, whereas stomach tissue expresses high levels of sialylated structures after *H. pylori* infection and infection associated inflammation [43, 127]. Regulation of SabA expression is complex and is regulated by the external pH, through the ArsRS two component signal transduction system [128]. At high pH, SabA expression is upregulated and is repressed at low pH (<5.0). SabA expression can also be regulated by mucins and their type present in the gastric mucosa [129].

More recently, the **LacdiNAc** binding adhesin (LabA), a protein with a molecular weight of 77 kDa was described [130]. LabA specifically recognizes the lacdiNAc structure (GalNAcb1-GlcNAc) on the gastric mucins [130]. LacdiNAc is a unique terminal structure in the outer chains of *N*- and *O*-glycans [131, 132]. LacdiNAc is expressed on the gastric epithelial surface and has been identified on MUC5AC mucin in the gastric mucosal epithelia surface [130, 132]. In human gastric tissues, LacdiNAc was found expressed more deeply in pyloric glands and absent in cardiac glands [130].

However, not every *H. pylori* strain expresses functional BabA, SabA, LabA adhesins, implying that other bacterial proteins accomplish the binding function or are involved in adhesion.

H. pylori binds to both glycolipids and glycoproteins. Binding to lactotetraosylceramide (Galb3GlcNAcb3Galb4Glcb1Cer) from human and pigs gastric mucosa has been identified both in *H. pylori* and *Helicobacter suis* (see below for more information on *H. suis*) [133, 134]. *H. pylori* proteins such as adherence-associated lipoprotein A and B (AlpA and Alp B) contribute to binding of *H. pylori* to host laminin an ECM molecule and causes severe gastric inflammation in mongolian gerbils [135, 136]. HpaA has been characterized as an *N*-acetylneuraminyllactose-binding hemagglutinin which is critical for mouse colonization [137]. The *H. pylori* outer inflammatory protein A (OipA) is an outer membrane protein that mediates binding of *H. pylori* to gastric epithelial cells, contributes to gastric inflammation [138] and induces apoptosis in gastric cell lines [139]. The neutrophil activating protein A (NapA) of *H. pylori* acts as an adhesin that binds specifically to sulfated oligosaccharide structures such as sulfo-Lewis a, and Lewis x blood group antigen structures on mucins [140] and to sulfated neutrophil glycosphingolipids as sulfatide and sulfated gangliotetraosyl ceramide [141].

Lipopolysaccharide (LPS) on the cell wall of *H. pylori* strains expresses carbohydrate structures that are structurally similar to the lewis blood group antigens expressed by human cells [142, 143]. This antigenic mimicry, where fucose residues on *H. pylori* imitate human lewis blood group antigens helps *H. pylori* avoid recognition by immune cells of the host [144]. This imitation suppresses its elimination from the gastric mucosa and contributes to prolonged chronic infection [144]. Most likely, *H. pylori* LPS binds targets in the host cells via the galectin-3, β-galactoside-binding lectin [145] and the trefoil factor family (TFF) protein TFF1 [146], the leukocyte endothelium adhesion molecule E- and L-Selectin [147]. *H. pylori* LPS also binds to SP-D, a C-type lectin that has specifically been shown to be involved in antibody-independent pathogen recognition and clearance [148] in innate immunity at mucosal surfaces. Upregulated expression of SP-D in human patients with gastritis has been shown with *H. pylori* infection and binding of *H. pylori* LPS to SP-D results in agglutination of *H. pylori* cells [149].

Helicobacter suis

The discovery of *H. pylori* increased curiosity about other spiral shaped bacteria that had been observed in other animal species. Most of these bacteria belong to the genus *Helicobacter* [150]. These non-pylori *Helicobacters* are increasingly found in human clinical specimens. *H. suis* is a gram-negative bacterium with a typical spiral-shaped morphology, that requires highly biphasic medium at pH 5 to grow in the laboratory and grows *in vitro* under microaerobic conditions [151]. The main host of *H. suis* is the pig, in which it colonizes mainly the antrum and fundic zone of the stomach [152]. In pigs, *H. suis* infection causes gastritis, reduced daily weight gain and other gastric pathological changes such as ulceration in the *pars oesophagea* [153, 154].

H. suis can also colonize the stomach of humans [155, 156]. *H. suis* is considered to be the most common gastric non-*H. pylori Helicobacter* (NHPH) in humans [156-158] and humans can get infected with *H. suis* by coming in close contact with infected pigs or possibly by consuming raw pork taken from infected pigs, as *H. suis* remain viable for up to 48 hours in fresh raw pork [159]. In humans, infection with *H. suis* has been associated with gastritis, gastric ulceration, as well as gastric mucosa-associated lymphoid tissue (MALT) lymphoma and sporadically gastric adenocarcinoma [160-162].

In addition to pigs and humans, *H. suis* has also been reported to colonize rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (crab-eating macaques; *Macaca fascicularis*) [163-165]. *H. suis* infection in macaques in general seems asymptomatic [166], although mild gastritis has been described occasionally [167].

1.6 *Streptococcus oralis*

Streptococcus derives from the Greek word strepto (twisted) and coccus (spherical). *S. oralis* is a member of *Streptococcus mitis* family and belongs to the *Viridans* group. Viridans streptococci is a broad term used for oral streptococci based on green coloration surrounding colonies developed due to the partial hemolysis on blood agar (viridans in latin means green) [168].

Streptococcus species such as *S. oralis* and *S. mitis* colonize the human oral cavity [169]. Most often, they constitute the commensal flora of the human oral cavity, but they can also cause diseases including meningitis [170], bacteremia and infective endocarditis (IE) [169]. IE is an infection of the endocardium (of heart) caused by systemic bacteria that settle on the surface of heart valves. IE remains a disease with considerable morbidity and mortality and the clinical effects include valvular incompetence and congestive heart failure [171, 172]. The viridans group streptococci can enter the bloodstream through lesions in the oral epithelia or after dental procedures and normal brushing activities [173]. *S. oralis* causes meningitis [170], dental plaque formation [174] and is a common cause of IE [175]. *S. oralis* is composed of three subspecies: *S. oralis* subsp. *oralis*, *S. oralis* subsp. *Tigurinis* and *S. oralis* subsp. *Dentisani* [176, 177].

In order to attach to oral epithelial surfaces, salivary components and platelets, members of the *Streptococcus* genera express a family of large, glycosylated serine-rich repeat proteins (SRRPs), which allow them to recognize a broad range of adhesion substrates, including salivary glycoproteins present in the human saliva [178]. SRRPs are characterized by the presence of (i) a N-terminal signal peptide, (ii) two, short and long, serine-rich repeat regions (SRR), the long domain is highly glycosylated, (iii) one non-repeat binding region (BR), and (iv) a C-terminal cell wall anchoring domain carrying a LPXTG motif (Figure 2).

Figure 2. Domain Organization of streptococcal SRRPs.

To display proteins to the cell surface, streptococci use anchoring mechanism, "sorting", where sortase family of enzymes covalently anchor streptococcal surface proteins to the peptidoglycan structure of the bacterial cell wall [179]. Sortases are classified into four subfamilies SrtA, SrtB, SrtC and SrtBD [180]. The sortase A (SrtA) subgroup, often referred to as the "housekeeping" sortase,

anchors the majority of surface proteins to the cell wall of the bacteria with a C-terminal motif, typically LPXTG. SrtA specifically cleaves the LPXTG sequence (where X represents any amino acid) [181, 182]. A study of surface proteins from streptococci, staphylococci, lactococci, listeriae, and enterococci identified more than 60 proteins having the LPXTG sequence [183]. Studies have demonstrated that isogenic SrtA knockout strains display reduced ability to anchor surface proteins, and are less lethal than wild-type strains in many animal models of infection [184].

For several streptococcal species, SRRPs mediate binding to host surfaces, including platelets, saliva, and oral epithelial cells, via sialic acid, keratin, fibrinogen or so far unidentified molecules on bacteria depending on the strain- or species [69, 185-187]. In *S. oralis*, SRRP Fap1 is required to bind to both sialic acid and β-1, 4 galactose [188]. Some *S. oralis* species encode multiple SRRPs while others lack SRRPs entirely indicating that *S. oralis* subspecies use distinct SRRPs or adhere to host surfaces including saliva or oral epithelia using SRRP-independent adhesion mechanism. Recently, in *S. oralis* IE-isolates lacking SRRPs, a novel binding adhesin, Asa (associated with sialic acid adhesion A) was identified that binds platelets via sialic acid [189]. However, no *S. oralis* IE-isolate adhesin and/or host carbohydrate structure of importance for binding has been identified as critical for binding to saliva mucins.

2 AIMS

General aim:

The overall aim of this thesis was to identify glycan epitopes on the mucins in the oral and gastric niche that are of importance for interactions with *S. oralis* and *Helicobacter* spp. A comprehensive understanding of this host-pathogen interaction can help to determine the precise glycan epitopes and their functions so that more targeted strategies can be developed against these pathogens.

Specific aims:

1. To study the gastric mucin glycome and how *H. pylori* infection-related changes in mucin glycosylation affect *H. pylori* binding using purified host gastric mucins.

2. To investigate the effects of *Helicobacter* spp. infection on the pig and human gastric mucin *O*-glycome and mucin-*H. pylori* interactions.

3. To study the effects of *H. suis* infection-induced glycosylation changes on *H. suis* growth and mucin binding ability.

4. To examine the role of glycans in *S. oralis* subspecies binding to purified human salivary mucins.

3 PATIENTS AND METHODS

In this thesis, the methods are described in the attached papers and manuscripts. Here, the choice and adjustments made to certain methods are discussed.

Materials and ethics

All experiments involving human gastric tissue were approved by the regional ethics board (Regionala etikprövningsnämnden i Göteborg, Dnr 753-14) and written informed consent was obtained from all study subjects.

In the studies on which the present thesis is based, a large number of subjects was included. In Paper I and II, the material came from patients referred for vertical sleeve gastrectomy for obesity in whom the gastrectomy and the macroscopic appearance of the mucosa were normal. The material used for the current studies would have been discarded if not used for research, so the use of the material did not compromise material that could have been used for diagnosis or similar. The patient`s indentity was kept confidential duing data collection. In paper IV, healthy subjects with different blood groups and without known inflammation in the mouth were included and the donors' identities were kept confidential.

3.1 Detection of bacterial binding

We used three different assays to study bacterial binding to purified mucins and other glycoconjugates.

3.1.1 Binding assay using antibody detection (Paper I, II and III)

Helicobacter spp. binding to purified mucins and glycoconjugates can be analyzed in a microtiter-based binding assay using *Helicobacter*-specific antibody. In this assay, the samples are coated on 96-well polysorp plates and incubated with the bacteria. *Helicobacter*-specific primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibody are added to the wells. The bound bacteria are visualized by adding 3,3', 5,5'-Tetramethylbenzidine (TMB), a substrate for the HRP on the secondary antibody. After color development, the reaction is stopped with 0.5 M H_2SO_4 and absorbance is measured in a microplate reader at 450 nm. To evaluate the binding, the background signal given by the binding of bacteria to the plastic wells and the binding of antibody to the mucin are subtracted from the detected binding values.

3.1.2 Binding and binding inhibition assay using relative luminescence detection (Paper IV)

S. oralis binding to purified mucins and glycoconjugates can be analyzed in a microtiter-based assay. In this assay, the samples are coated on a 96-well white polystyrene microplates and incubated with bacteria. Due to that it is not known how well existing antibodies recognize the different subspecies, we used the BacTiter-Glo reagent (Promega, Madison, WI, USA), which is added to the wells. This method (BacTiter-Glo™ Microbial Cell Viability Assay) measures the viable number of bacterial cells in the plate. Luciferase enzyme in the reagent generates luminescent signal proportional to the ATP levels and intracellular concentration of ATP directly relates to the metabolic activity of bacterial cells. Unbound bacteria are washed. The bound bacteria are detected by measuring relative luminescence unit (RLU) in an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) with an integration time of 1,000 ms per well. The background signal given by the binding of bacteria to the plastic wells and binding of reagent to the mucin-coated wells are subtracted from the detected binding values.

Microtiter plates provide stable attachment of mucins to their surface ensuring that observed differences for both bacterial binding and inhibition of binding are due to quality/quantity of attached mucins. This method is simple to use, rapid, highly reproducible and fits our experimental needs to analyze large numbers of mucin samples and to detect various sugars of interest. Several other affinity-based methods could be used instead to study the binding of *S. oralis* to purified human salivary mucins. For example, surface plasmon resonance (SPR) binding analysis methodology studies molecular interactions of two different unlabeled molecules in real time, in which one is mobile and one is fixed on a thin gold film [190] and the interaction is characterized/quantified by change in refractive index at the gold surface. However, SPR method measures binding of all bacteria, whereas the BacTiter-Glo™ Microbial Cell Viability Assay (ATP-based) measures only live bacteria. Binding results from SPR are also affected by the size of the pathogen since SPR has high sensitivity for size and detects binding based on the mass. Another challenge of SPR method is that it is hard to control for the accurate flow rate during adhesion as fluid flow affects host-microbial interactions [191]. When analyzing few samples, SPR assay allows for faster analysis and offers significant advantages over both Bactitre glow method as well as antibody detection in that it minimizes experimental time (a few minutes) of the experiment. However, the disadvantage of SPR is that it is difficult to discriminate between specific and non-specific interactions with the sensor

surface. Additionally, non-specifically bound material is not completely removed with elaborate washing, thus, requiring reference material or control samples to correct for the non-specific binding. Moreover, it is not feasible to run analyses with 212 mucins on SPR, considering the cost of gold chips and the amount of time needed for coupling and verification of bound mucins.

Microarrays technology could also be used but is not as sensitive due to various sources of error, systematic or otherwise, and is harder to control for false positives as well as non-specific noise and errors introduced during the course of the experiment [192].

3.2 Binding inhibition assay

In this assay, mucins were coated on the 96-well polysorp plate. Bacteria were pre-incubated with the sugars of interest (glycan-adhesin interaction) prior to the incubation with mucins. If the glycans used during pre-incubation are the targets of bacterial adhesins, they are expected to inhibit binding of the bacteria to the mucin sample by occupying the target binding sites for the adhesion molecules.

4 RESULTS AND DISCUSSION

4.1 Enormous diversity of gastric mucin *O*-glycans exists in the human stomach (Paper I)

In the stomach, gastric mucins and their *O*-glycans provide the first point of interaction for *H. pylori. H. pylori* carries several carbohydrate-binding adhesins (BabA, SabA, and LabA) characterized to promote binding to the gastric mucosa. Information on the human stomach glycome remains relatively limited due to technical limitations associated with analyzing glycans.

Recent studies have focused on the identification of potential host receptors for microbes, the majority of which appears to be glycoproteins together with glycolipids [193-195]. The expression of glycans varies between individuals [37] and within one individual differs between cell- and tissue locations [32, 33]. Thus, recognition of specific carbohydrate structures on the host determines the specificity of microbial binding. A recent study by Jin et al. [37] showed a large diversity in glycosylation in the stomach from 10 individuals (3 healthy and 7 tumor-affected stomachs), and identified in total 258 glycan structures. Another study identified 90 glycan structures among mucins purified from gastric juice or gastric tissue of 10 healthy uninfected individuals, 25 *H. pylori* infected asymptomatic individuals and 5 infected individuals with intestinal metaplasia in the gastric mucosa [196]. Jin et al. found that the glycosylation varied between individuals and from each individual 34-103 *O*-glycan structures were identified extending from 2-13 residues [37]. Core 2 capped *O*-glycans dominated and the majority of glycans were neutral and fucosylated [37, 196]. In line with these results, here we further show that an enormous diversity of gastric mucin *O*-glycans exists from 28 human stomachs. We identified 650 distinct oligosaccharides out of which only six were common to all individuals. From each individual, we characterized 60-189 structures, demonstrating high diversity, both within and between individuals.

Using mass spectrometry, 156 structures could be fully characterized and core 2 structures were the dominating type, although core 1 and 3 were also detected. Neutral glycans had higher prevalence than acidic oligosaccharides and sulfation was very low. Among the fucosylated structures, blood group H type 2 and H type 3 and blood group A were the dominating structures and H type 1 was very low. Additionally, fucosylation was found in Lea/x and Leb/y including extension into A Leb/y and B Leb/y. Terminal sialylation was very

low and was predominantly 3-linked although 6-linked was also identified. The total number of structures identified was similar between surface epithelia and glandular tissue and 79% structures were common to both tissues. Among surface mucins, unique fucosylated structures were abundant, whereas in glandular tissue, the unique sialylated structures were dominant.

Gastric glycosylation differs between *H. pylori* infected and non-infected stomachs

H. pylori causes severe gastric diseases in humans and since it lives in gastric mucus layer, interaction with mucin glycans can be vital for its survival to colonize and to maintain a dynamic host-pathogen interaction within in the stomach. To gain a better understanding of the changes induced by the *H. pylori* infection, we studied the difference in glycosylation of *H. pylori* infected and non-infected purified gastric mucins. Mucins from *Helicobacter* infected individuals showed a larger inter-individual variation than mucins from non-infected individuals. We identified higher number of glycan structures (535) among *Helicobacter* spp. infected individuals compared to non-infected individuals (481), whereof 393 (60 %) were detected in both groups. In the *H. pylori* infected group, both the number and the relative abundance of unique structures were larger than in the non-infected group. These results suggest an increased glycan diversity among *H. pylori* infected individuals.

Furthermore, the glycan profiles of each sample within groups were correlated using Spearman's rank correlation coefficient (ρ). The distribution of ρ measures diversity within groups, with a low ρ indicating high diversity. Among glycans from the non-infected groups, the distribution of ρ was significantly higher than the distribution of ρ from the *Helicobacter* spp. infected group. The average number of monosaccharides in the glycans was larger on mucins from *Helicobacter* spp. infected individuals, implying an increased diversity of possible glycans that can be biosynthesized. Furthermore, in mucins from infected individuals, the variation in the intra-individual glycan size was greater compared to non-infected individuals.

We found that fucosylation contributes to overall increased diversity in *Helicobacter* spp. infected individuals. The relative abundance as well as the number of unique fucosylated structures were higher from infected group. The lewis type (a/x, b/y) structures were overrepresented among infected individuals and the lewis type structures clustered the samples into infected and non-infected within Leb positive and Leb negative groups. In Leb positive infected group, the heterogeneity of fucosylation within a sample was larger

and the inter-individual correlation for the fucosylated structures was higher suggesting that fucosylation contributes to the overall increased diversity in *Helicobacter* spp. infected individuals.

Taken together, these results demonstrate that infection is associated with increased glycan diversity, larger inter-individual as well as intra-individual diversity.

4.2 *H. pylori* binding to gastric mucins (Paper I and II)

It has previously been shown that *H. pylori* infection induces qualitative/quantitative changes in host gastric mucosa [197, 198], and here we studied *H. pylori* binding to purified gastric mucins divided into four groups: Leb positive and negative, infected and non-infected. *H. pylori* avidity was highest to mucins from *Helicobacter spp.* infected individuals among Leb positive mucins and to non-infected individuals among Leb negative mucins. To identify the glycan structures on the mucins that might serve as adhesion targets for *H. pylori* binding and differences in binding between groups, we analyzed the glycan structure on mucins for *H. pylori* binding from infected and non-infected Leb positive and Leb negative individuals.

We focused primarily on the terminal carbohydrate structures previously characterized for *H. pylori* binding. *H. pylori* uses BabA to bind to Leb and other fucosylated structures [121, 122]. Overall fucosylation was found to not differ between groups but among Leb negative mucins, two distinct populations were found. Among infected individuals (Leb positive as well as Leb negative), the Lea/x (mono-fucose glycan) was higher and Leb (di-fucose glycan) was found increased only among Leb positive infected individuals compared to non-infected, implying that increase in *H. pylori* binding is driven by increased Leb levels. However, infected mucins negative for Leb showed decreased binding. The cause of this decreased binding is less clear to explain in terms of which glycan structures are involved, but fucosylation may play a role as we found a trend towards decreased fucosylation in infected Leb negative individuals. Another possible explanation could be that majority of the individuals in this group were either low- or non-secretors as they express little or no H type 1 structures [52]. As for other structures such as SLea/x (SabA binding), LacdiNAc (LabA binding) and Galβ3GlcNAc structures, no differences were detected between the groups.

Furthermore, J99 binding to mucins and glycoconjugates is known to be dominated by BabA dependent adhesion (24). In line with this study, we also found that BabA dependent binding to fucosylated structures dominates both the overall binding and differences in binding between groups. Of the structures identified to correlate well with *H. pylori* binding, most were fucosylated and included blood group like epitopes indicating their role in host-pathogen interactions. The BabA-dependent binding to terminal fucoyslated structures play a crucial role in *H. pylori* binding to mucins, whereas no clear LabA- or SabA-dependent binding to mucins was detected.

4.3 Effect of *Helicobacter* spp. infection on human and pig gastric glycome (Paper II, III)

We showed that *Helicobacter* spp. infection alters the binding avidity of *H. suis* to gastric mucins isolated from naturally infected humans and experimentally infected pigs. Additionally, *Helicobacter* infection was shown to increase the growth of *H. suis* in response to mucins from both infected pigs and humans.

We also investigated the effect of infection and inflammation on the different modes of binding to gastric mucins isolated from pigs and humans with and without *Helicobacter* spp. infection using a range of *H. pylori* strains with different adhesins and their isogenic deletion adhesin mutants. We assessed the binding specificities of four *H. pylori* strains J99 (carries BabA and SabA adhesin), P12 (carries BabA adhesin), 26695 and G27 (carries SabA adhesion) to the purified gastric mucins from human and pig with and without *Helicobacter* spp. infection (212 mucin samples from 53 individuals). We found that the four *H. pylori* strains show notable differences in their binding preferences. Binding of *H. pylori* J99 to mucins and glycolipids is known to be mediated dominantly by BabA adhesin. We detected large differences in binding preferences between BabA carrying strains J99 and P12 strains implying that these strains use additional sites for binding. *H. pylori* J99 strain showed increased binding to gastric mucins from infected humans and pigs. P12 strain binding was decreased to infected pig gastric mucins but no effect was observed with infected human gastric mucins. *H. pylori* G27 binding was increased to infected human and pig gastric mucins compared to non-infected. Infection decreased the charge-dependent binding of 26695 to human gastric mucins at pH 2, but infection had no effect on binding of 26695 to pig gastric mucins at either pH 2 or pH 7.

Analyzing the relation between bacterial binding amplitude and the relative abundance of different glycan structures carried by these mucins revealed that these strains prefer different structures for binding. *H. pylori* strain J99 binding correlates with fucose, blood group O, H and the glycan chain length. P12 and G27 strains binding correlated with blood group A and terminal GlcNAc, whereas 26695 binding to pig and human gastric mucins correlates with charged glycans at pH 2 and with terminal GlcNAc at pH 7. Using multivariate orthogonal partial least square (OPLS) analysis, we identified that both *Helicobacter* spp. infected individuals as well as experimentally infected pigs display a different gastric mucin glycosylation profile compared to non-infected individuals and pigs, respectively. Overall, this points to that *Helicobacter* infection increases the adhesion of different strains differently and that different strains prefer different glycan structures for their binding.

4.4 *S. oralis* binding to host salivary mucins (Paper IV)

Here we investigated binding of *S. oralis* subsp. *oralis* and *S. oralis* subsp. *dentisani* strains (isolated from infective endocarditis vegetations in humans) and isogenic mutants to oral mucins isolated from blood group A and B positive individuals.

S. oralis binding occurred to density gradient fractions of purified human salivary mucins MUC5B, MUC7 and the salivary agglutinin (SAG) and differed between strains. The main *S. oralis* avidity colocalized with MUC7/SAG. The *S. oralis* subsp. *oralis* strain IE12 wt avidity to soluble MUC7/SAG containing fractions from the density gradients was 3-fold higher than the avidity of *S. oralis* subsp. *oralis* ATCC 10557 wt and 10-fold higher than the avidity of *S. oralis dentisani* strain F0392 wt. These differences in the amplitude of binding suggest that *S. oralis* uses different modes of adhesion to bind salivary mucins and binds with different glycan specificities. Several *Streptococcus* species have been shown to have different specificities for MUC7 and MUC5B. For example, MUC7 aggregates *S. gordonii* but MUC5B has no effect on binding (63) and *S. sanguinis*, *S. sobrinus* and *S. oralis* bind to MUC7 but not MUC5B (62). In line with these results, we show that the *S. oralis* subsp. *oralis* and *S. oralis* subsp. *dentisani* main avidity is to MUC7, whereas binding to MUC5B is weaker. The differences in *S. oralis* avidity to the different mucin samples can be explained by the glycosylation differences between the blood groups and mucin samples.

To find the glycan structures on the salivary mucins that are responsible for *S. oralis* binding and difference in binding between mucins, we compared glycan

epitopes on the MUC7/SAG rich samples with the epitopes on MUC5B samples. The MUC7/SAG rich samples contained higher relative abundances of Lewis type structures including SLea/x as well as both total sialylated structures and α2,3-linked sialic acid. In contrast, the MUC5B rich samples contained notably higher relative abundance of fucosylated structures, H type, blood group A/B as well as α2,6-linked sialic acid and sulfate structures compared to MUC7/SAG rich samples. MUC5B rich samples contained an overall higher relative abundance of terminal galactose than MUC7/SAG rich samples, but with less pronounced differences in relation to other epitopes.

Since the terminal glycan residues are exposed for binding, we found that among the fucosylated characterized structures, blood group H was the most dominating epitope followed by lewis type structures. Furthermore, to find the glycan structures responsible for the binding to salivary mucins, we pre-incubated the parent strain with Lewis b, SLex and LNT prior to addition to MUC5B and MUC7/SAG mucins. We found that Leb and LNT inhibited binding to both MUCB as well as MUC7/SAG, whereas SLex inhibited binding only to MUC7/SAG, implying that these structures, or similar structures, present on salivary mucins were mediating this binding to *S. oralis*.

Surface proteins in *S. oralis* contribute to binding to salivary mucins

The sortase gene (srtA) codes for the transpeptidase sortase that covalently links LPXTGX-containing surface proteins to the cell wall of gram-positive bacteria [199]. To evaluate the contribution of srtA to the binding of *S. oralis* subsp. *oralis* strains, we compared the binding avidities of the wild type and *srtA* mutant of *S. oralis* subsp. *oralis* IE12 strain. We found the avidity of IE12Δ*srtA* was significantly reduced compared to that of the parental strain to both the MUC5B and MUC7/SAG rich samples from both the A positive and the B positive donors, demonstrating that srtA is required for *S. oralis* subsp. IE12 binding to these salivary mucins.

Taken together, these results indicate that Leb, SLex and LNT related structures on salivary mucins mediate the binding to SrtA anchored *S. oralis* protein(s). The fact that *S. oralis* binding to MUC7 was higher than MUC5B and together with results from binding inhibition studies also indicate that *S. oralis* uses different mechanisms to bind to MUC7 and MUC5B.

5 CONCLUSIONS

In this thesis, we studied host-pathogen interactions in the mucus niche focusing on *S. oralis* and salivary mucins and *Helicobacter* spp. and gastric mucins isolated from human stomachs. We studied the mucus changes in infections and inflammation and how these changes influence the bacterial binding. Based on our results of mucin characterization as well as bacterial binding we can conclude that:

- The human gastric glycome contains an enormous diversity of mucin *O*-glycans. *Helicobacter* spp. infection is associated with an increased number of unique glycan structures and increased intra-individual as well as inter-individual diversity. *H. pylori* J99 binds to both Leb/H type 1 positive and negative mucins from infected and non-infected individuals. BabA dependent binding to fucosylated structures dominates the overall binding to gastric mucins regardless of Leb status.

- *H. pylori* strains display clear differences in binding preferences for different gastric mucins and these differences in binding preferences to human gastric mucins can be attributed to mucin glycosylation differences.

- Infection by *Helicobacter* spp. changes the mucin composition and glycosylation of the host in a manner that decreases the amount of *H. suis* binding glycan structures on gastric mucins, decreases the *H. suis* binding ability to mucins and enhances the growth of the *H. suis*. By such changes, *Helicobacter* spp. infections impair the mucus barrier to create a more stable and inhabitable niche in the stomach.

- *S. oralis* strains (isolated from infective endocarditis vegetations) bind to salivary mucins MUC5B and MUC7 and salivary agglutinin and the binding differs between different *S. oralis* strains. The main binding colocalizes with MUC7 which contains higher levels of lewis type structures and 2,3 linked sialic acid. *S. oralis* binding to salivary mucins is mediated by cell wall anchored surface LPXTG protein and Leb, Slex and LNT glycans present on MUC7 and MUC5B mucins.

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