# *Helicobacter* spp.-host interaction in the mucus niche

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# ABSTRACT

*Helicobacter pylori* is the most common human gastric pathogen, colonizing half of the world's population. *Helicobacter suis* colonizes the stomach of 60-95% of pigs at slaughter age and it is the most prevalent non-*Helicobacter pylori Helicobacter* species found in the human stomach causing severe gastric disorders. The first barrier that gastric pathogens encounter is the mucus layer, of which the main components are highly glycosylated mucin glycoproteins. Mucins carry a high diversity of mucosal glycan chains terminating with glycan structures that vary between species, individuals and tissue locations and provides an extensive repertoire of interaction surfaces for bacteria.

In this thesis, we describe a constant dynamic interplay between *Helicobacter* spp. and host gastric mucins. *Helicobacter* infection induces changes in host gastric mucin composition and glycosylation, and these alterations affect the binding avidity, growth and gene expression of the bacteria. The mucin interaction with pathogens is mediated by its glycan composition and shows high inter-individual difference. We show that *H. pylori* and *H. suis* bind to human and pig gastric mucin glycans and glycolipids via different binding modes and with different specificity. *H. suis* binding to gastric mucins and glycolipids occurs via two modes of adhesion: to structures with terminal galactose at both neutral and acidic pH, and to negatively charged structures at acidic pH. These binding modes enable *H. suis* adhesion to mucins at lower pH close to the gastric lumen and in parietal cells and a more intimate adhesion to mucin glycans and glycolipids closer to the host epithelial cells.

We demonstrated that mucins play important role in host defense mechanism against gastric pathogens. Mucins are able to limit bacterial growth by adhesion and aggregation of *H. pylori* and they affect the adhesin gene expression of the bacteria. *Helicobacter* infection changes host mucin glycosylation in a way that decreases the amount of mucin glycan structures targeted in binding and impairs the growth regulating effects of the mucins maintaining a more inhabitable niche in the stomach.

Understanding the dynamic interplay between Helicobacters and host gastric mucins and alleviating the impairments of the host defense by these pathogens can contribute to the development of preventive strategies against *Helicobacter* infection.



**Keywords**: *Helicobacter*, adhesion, mucin, glycosylation

# SAMMANFATTNING PÅ SVENSKA

*Helicobacter pylori* (*H. pylori*) är den vanligaste sjukdomsalstrande organismen i magen och halva jordens befolkning bär på denna bakterie. *Helicobacter suis* (*H. suis*) är nära besläktad med *H. pylori*. *H. suis* koloniserar grisens mage och är även den vanligaste icke-*H. pylori* Helicobacter-arten i magen hos människa. Infektion med både *H. pylori* och *H. suis* orsakar inflammation magen och kan leda till magsår och magcancer. Behandling av dessa infektioner med antibiotika blir alltmer problematisk på grund av den globala ökningen av förvärvad antimikrobiell resistans. Sjukdomsalstrande organismer i lantbruksdjur utgör ett hot mot människors hälsa både på grund av att zoonotiska bakterier såsom *H. suis* kan infektera både människor och lantbruksdjur och på grund av att antibiotikabruk i djur ökar belastningen av antibiotikaresistenta bakterier i omlopp. Den vanligaste vägen bakterier kommer in i kroppen på är via slemhinnan. Denna yta är täckt av ett kontinuerligt utsöndrat slem som tvättar bort bundna partiklar. Huvudkomponenterna i detta slem är utsöndrade muciner. Mucinerna bär ett stort antal kolhydratstrukturer, vilket ger många potentiella ställen för bakterier att binda till.

I denna avhandling har vi visat att *H. suis* i likhet med *H. pylori* lever i slemlagret som täcker magslemhinnan. Båda dessa bakterier binder till muciner, fast till olika kolhydratstrukturer på mucinerna och därigenom binder de till olika muciner. Infektion och inflammation kan orsaka kvalitativa och kvantitativa förändringar i kolhydratstrukturerna som sitter på muciner. Eftersom bakteriens bindning, tillväxt och genuttryck påverkas av mucinernas kolhydratstrukturer förändrar detta relationen mellan bakterien och värden. Vi visade att mucinerna, förutom att bära antimikrobiella glykanstrukturer, kan begränsa bakteriell tillväxt genom att binda bakterierna. *Helicobacter* spp. infektion kan minska mängden kolhydratstrukturer som binder till *H. suis* och kan försämra de tillväxtreglerande effekterna av mucinerna, vilket kan leda till en mer gästvänlig nisch för *H. suis* i magen. Vi observerade ett dynamiskt samspel mellan *Helicobacter* spp. och värdmuciner. Vi tror att störning av dessa värd-mikrob interaktioner och utveckling av strategier för att förändra slemhinnans beteende vid infektion skulle kunna användas både förebyggande och som 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.** **BabA dependent binding of *Helicobacter pylori* to human gastric mucins cause aggregation that inhibits proliferation and is regulated via ArsS.**
Skoog EC*, Padra M*, Åberg A, Gideonsson P, Obi I, Quintana-Hayashi MP, Arnqvist A, Lindén SK
*Sci Rep. 2017. 20;7:40656.** Equal contribution

**II.** ***Helicobacter suis* binding to carbohydrates on human and porcine gastric mucins and glycolipids occurs via two modes**.
Padra M, Adamczyk B, Benktander J, Flahou B, Skoog EC, Padra JT, Smet A, Jin C, Ducatelle R, Samuelsson T, Haesebrouck F, Karlsson NG, Teneberg S, Lindén SK
*Virulence. 2018. 31;9(1):898-918.*

**III.** ***Helicobacter suis* infection alters glycosylation and decreases the pathogen growth inhibiting effect and binding avidity of gastric mucins.**
Padra M, Adamczyk B, Flahou B, Erhardsson M, Chahal G, Smet A, Jin C, Thorell A, Ducatelle R, Haesebrouck F, Karlsson NG, Lindén SK
*Manuscript*

**IV.** **Carbohydrate-dependent and antimicrobial peptide defense mechanisms against *Helicobacter pylori* infections**.
Médea Padra, John Benktander, Karen Robinson and Sara K. Lindén
Book chapter in *"Current Topics in Microbiology and Immunology (CTMI)" by Springer,* volume *"Molecular mechanisms of inflammation: induction, resolution and escape by Helicobacter pylori"*
*Accepted*

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| Alp | Adherence-associated lipoprotein |
| BabA | Blood group antigen binding adhesin |
| CEACAM | Carcinoembryonic antigen-related cell adhesion molecule |
| CFU | Colony forming unit |
| ELISA | Enzyme-linked immunosorbent assay |
| FBS | Fetal bovine serum |
| GalNAc | N-acetylgalacosamine |
| GlcNAc | N-acetylglucosamin |
| GuHCl | Guanidinium chloride |
| HpaA | *Helicobacter pylori* adhesin A |
| *H. pylori* | *Helicobacter pylori* |
| HSA | Human serum albumin |
| *H. suis* | *Helicobacter suis* |
| HRP | Horseradish peroxidase |
| IL | Interleukin |
| IFN | Interferon |
| LabA | LacdiNAc specific adhesin |
| Le | Lewis |
| LNT | Lacto-N-tetraose |
| LPS | Lipopolysaccharide |
| MALT | Mucosa-associated lymphoid tissue |
| NapA | Neutrophil activating protein A |
| OD | Optical density |
| OipA | Outer inflammatory protein A |
| OMPs | Outer membrane proteins |
| SabA | Sialic acid binding adhesin |
| $SLe^a$ | Sialyl-$Le^a$ |
| $SLe^x$ | Sialyl-$Le^x$ |
| SP-D | Surfactant binding protein D |
| TNF | Tumor necrosis factor |

# 1 INTRODUCTION

## 1.1 Mucus

An adherent mucus layer covers the epithelial surfaces of the eye and gastrointestinal, respiratory and reproductive tracts in order to protect the mucosa from mechanical damage or entrance of harmful chemicals, such as drugs, toxins and heavy metals (1-3). In the stomach and duodenum, the mucus contributes to surface neutralization of luminal acid by mucosal bicarbonate secretion (4) and prevents access of luminal pepsin to the mucosal surface (5). The viscous mucus layer is the first barrier in the gastrointestinal tract nutrients and enteric drugs must interact with and diffuse through, in order to gain access to their targeted organs through the circulatory system (6).

The thickness of the mucus layer varies greatly among organs, ranging between 70-100 μm in the oral cavity (7), approximately 250 μm in the stomach, 150-400 μm in the small intestine and reaching 800-900 μm in the colon (8). In the gastrointestinal tract, the mucus is continuously cleared by the peristaltic movement of luminal food and faecal material and can be rapidly replaced from goblet cells by continuous secretion or by compound exocytosis in response to chemical or physical irritation (8-10). Mucus consists approximately 95% water but it also contains salts, lipids (11) and proteins with protective function, such as lysozymes, immunoglobulins, defensins, growth factors and trefoil factors (12-14). The main component of the mucus is the gel-forming mucin glycoprotein, which is responsible for its viscous properties (6, 15).

## 1.2 Mucins

Mucins are highly glycosylated glycoproteins with a molecular weight ranging between 0.5 and 20 MDa (6). Mucins consist of a protein core with tandemly repeating amino acids rich in serine and threonine, where *O*-linked carbohydrate chains are added in the Golgi apparatus during biosynthesis (16). The two major types of mucins are transmembrane and secretory mucins. Transmembrane (cell-surface) mucins are located on the apical surface of the mucosal epithelial cells. In the human gastrointestinal tract, cell surface mucins include MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC16 and MUC17 (17). They participate in mucosal defense translating external stimuli to cellular responses (18). They can also play an important role under host-pathogen interactions (19). The expression of MUC1 is upregulated in

response to infection (20) and acts as a decoy to limit adhesion of the bacteria to the cell surface (21). Secretory mucins are one of the major components of the extracellular mucus barrier and they are characterized by high molecular weight. They can be gel-forming (MUC2, MUC5AC, MUC5B, MUC6 and MUC19) or non-gel-forming secreted mucins (MUC7). The gel-forming mucins have cysteine-rich motifs that are important in the formation of oligomers via inter-molecular disulphide bonds (22). In a healthy human stomach, MUC5AC and MUC6 are the major gel-forming mucins located in the surface and glandular region, respectively (23), whereas in the intestine the main secreted mucin is MUC2. In the gastrointestinal tract, secretory mucins are produced and secreted by mucous cells of glandular tissues and by goblet cells which are specialized epithelial cells (24). The intestinal goblet cells migrate from the bottom of the crypts to the villus tip where the mucus shed to the lumen and they constantly get replaced (25). The migration along the crypt-surface axis causes changes in morphology and mucin composition of the goblet cells (26, 27). Mucin secretion can happen via vesicle secretion or compound exocytosis and can be regulated by numerous environmental stimuli, including cholinergic agonist, hormones, neurotransmitters and intracellular messengers, such as $Ca^{2+}$ and cAMP (28, 29). In response to infection, both innate and adaptive immunity can regulate the expression of mucins. Inflammatory cytokines, such as IL-1β, IL-4, IL-6, IL-9, IL-13, IFNγ, tumor necrosis factor (TNF), nitric oxide and granulocyte proteases can directly upregulate the expression of mucins (30).

## 1.3 Mucin glycosylation

Posttranslational modifications of proteins by glycosylation can occur in *N*-linked and *O*-linked form. Mucin-type *O*-glycosylation takes place in the Golgi complex and is initiated by the addition of α-N-acetylgalactosamine (GalNAc) to the hydroxyl group of Ser/Thr side chains of the folded protein (31, 32). The complex oligosaccharides on proteins have three regions: core region (core 1 - core 8), backbone region (type 1 and type 2) and peripheral region. This latter region can be terminated by fucose, galactose, GalNAc or sialic acid residues, forming histo-blood group antigens such as A, B, H, Lewis a ($Le^a$), Lewis b ($Le^b$), Lewis x ($Le^x$), Lewis y ($Le^y$), as well as sialyl-$Le^a$ and sialyl-$Le^x$ structures (33) (Table 1). The structure of carbohydrates depends on glycosyltransferases expressed in the cells (34). The mucin oligosaccharide terminal structure varies between species (35), individuals (36) and between tissue locations within one individual (37, 38). For instance, type 1 blood group-related antigens are expressed in the cells of the surface epithelium, whereas type 2 antigens are found mainly in the glandular region of the human

gastric mucosa (39). The majority of normal gastric mucin *O*-glycans are neutral and fucosylated (36), increased sialylation and /or sulphation as well as expression of Tn and T antigens can indicate aberrant or incomplete glycosylation (40).

Mucin *O*-glycans contribute up to 80% of the molecular weight of mucins (6). The high level of glycosylation enables mucins to function as a protective barrier by lubricating the epithelium (41) and preventing the degradation of the protein backbone by proteases (42). Mucin glycans can modulate cell adhesion (43), serve as ligands for cell surface receptors (44) and take part in host-pathogen interaction (20, 45, 46). Glycan structure alterations are associated with many pathological conditions. Cancer associated mucin glycosylation changes have been reported in tumor tissues from different organs (47, 48) and these modifications can be potentially used as biomarkers of the development or progression of tumors (49-51). Bacterial infection can also alter mucin production and glycosylation quantitatively as well as qualitatively at both cellular and subcellular level (52-54).

| **Histo bloodgroup antigens** | **Glycan structure** |
|---|---|
| Blood group H | Fucα1-2Galβ1- |
| Blood group A | Fucα1-2(GalNAcα1-3)Galβ1- |
| Blood group B | Fucα1-2(Galα1-3)Galβ1- |
| Lewis a ($Le^a$) | Galβ1-3(Fucα1-4)GlcNAcβ1- |
| Lewis b ($Le^b$) | Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1- |
| Sialyl-$Le^a$ | NeuAc(α2-3)Galβ1-3(Fucα1-4)GlcNAcβ1- |
| Sialyl-$Le^x$ | NeuAcα2-3 Galβ1-4(Fucα1-3)GlcNAcβ1- |
| Lewis x ($Le^x$) | Galβ1-4(Fucα1-3)GlcNAcβ1- |
| Lewis y ($Le^y$) | Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1- |

*Table 1. Histo-blood group antigens carried by mucin O-glycans.*

## 1.4 Pig gastric mucins

The stomach mucosa of pigs can be divided into two main parts: a glandular part (containing cardiac gland zone, fundic gland zone and antrum with pyloric glands) and a non-glandular part called *pars esophagea* that is covered by a stratified squamous epithelium (55). The non-glandular region and the cardiac gland zone have a pH around 5-7 due to the presence of saliva and cardiac gland bicarbonate secretions (56), while the fundic and pyloric glands provide lower pH in the distal part of the stomach with high inter-individual variability

(57). The mucus secreted in the different regions of the pig stomach shows big variations regarding density, size, viscosity and amino acid and glycan content (58-60). Mucins produced by the surface epithelium also differs from the gland mucins in apoprotein content and length of the glycosylated domains indicating that the surface epithelium and the glands produce different mucins, which mucins might represent the porcine equivalents of the human MUC5AC and MUC6 mucins, respectively (61).

Alterations in pig mucins can be induced by certain environmental factors and conditions, e.g., weaning associated mucin glycosylation changes play an important role in the adaptation to new dietary constituents, physical environment as well as commensal and pathogenic bacteria (62, 63). In weaned pigs, increased mucin secretion occurs (64) and an elevated level of fucosylated mucin glycans has also been reported (62). These glycosylation changes can be further modified with dietary changes (63-66) and by microbial activities (67).

## 1.5 Host-pathogen interactions in the mucus niche

The mucus layer serves as the first barrier between pathogens and host cells on several organs providing a surface for host-pathogen interaction. Microbes commonly interact with the glycan structures of the host glycocalyx to colonize mucosal surfaces (68). The high variety of mucin oligosaccharides forms an extensive repertoire of attachment sites for bacteria (69). Microorganisms attach to mucosal glycans via adhesins with different carbohydrate specificities (46, 70, 71) and the high diversity of mucin glycan chains can lead to region-specific colonization by the bacteria (72). Binding of pathogens to the cell surface mucins supports the barrier function of mucus by releasing the extracellular domain together with the bound bacteria from the cell surface, acting as a releasable decoy (21).

Mucus can serve as a reservoir for numerous pathogens (73-75), and be used as a matrix for replication and colonization (76). Mucus can provide an important source of nutrients for bacterial growth (77, 78). A number of bacterial strains are able to degrade mucins by producing specific enzymes, including glycosidases, sulphatases, sialidases and use the released glycans as energy source (79-84). The microbiota inhabiting the mucus layer is able to modulate the mucus niche in a way that it becomes beneficial for the bacteria. Studies on germ-free mice have revealed that *Bacteroides thetaiotaomicron*, by secreting signaling molecules, induces the expression of fucose on cell surface glycoconjugates and these fucosylated glycans can be utilized by these

bacteria as carbon source (67). Some pathogens can also alter the pH of the mucus in their microenvironment decreasing its viscoelasticity that can facilitate bacterial motility (85). In response to infection, host mucins have the ability to affect the behavior of the bacteria by regulating the growth and gene expression of pathogens (86-88) which leads to a constant dynamic host-pathogen interaction.

## 1.6 *Helicobacter pylori*

*Helicobacter pylori* (*H. pylori)* is a Gram-negative spiral-shaped microaerophilic bacterium that colonizes the stomach of half the human population. In 1983, Dr. J. Robin Warren and Dr. Barry Marshall reported the hypothesis that peptic ulcers are caused by spiral shaped bacteria in the stomach and they were the first who successfully isolated *H. pylori* from gastric biopsies (89). *H. pylori* infection usually occurs at a very young age and becomes persistent (90). Although the majority of *H. pylori* infections are asymptomatic, infected patients may develop non-ulcer dyspepsia, peptic ulcer disease, adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (91-93). The International Agency for Research on Cancer (IARC) classified *H. pylori* as a class I carcinogenic agent based on epidemiologic evidence (94). The clinical outcome of *H. pylori* infection can be determined by the interplay of several bacterial, host or environmental factors, reviewed in (95).

## 1.7 *H. pylori* interaction with mucins

*H. pylori* colonizes the human gastric mucosa and is predominantly located in the mucus layer that covers the surface epithelial cells and only a small percentage are found in close association to the epithelial cells (96). Because of the rapid gastric mucus turnover, the bacteria need to develop strategies to avoid being shed into the gastric lumen and to maintain a stable niche in the stomach. The pH gradient across the gastric mucus layer ranges between 2 and 7, being very acidic close to the lumen and approximately neutral at the epithelium. *H. pylori* can use the pH gradient in the mucus for chemotactic orientation, which plays an important role in the persistence in the stomach (97). *H. pylori* secretes urease enzyme, which is responsible for the tolerance of the acidic environment and facilitates the motility of *H. pylori* in the gastric mucus (98). Experiments using urease-negative *H. pylori* mutants demonstrated that urease activity is crucial for gastric colonization and survival of the bacteria (99). Urease level detection is a commonly used rapid diagnostic tool for *H. pylori* infection in the stomach (100).

## 1.8 *H. pylori* adhesion to mucins

*H. pylori* adhesion to gastric mucins allows the bacteria to gain access to nutrients from host tissues (71, 101), and triggers host inflammatory responses (102-104). Binding to membrane bound mucins protects the bacteria from being shed by the passage of luminal content (21), which is crucial for maintaining a stable niche in the mucus layer. The glycan environment that *H. pylori* is exposed to constantly changes in response to different environmental effects, such as bacterial infection and development of diseases (53, 105-107). *H. pylori* requires a wide range of adhesive molecules to adapt to the dynamic microenvironment in the stomach. The genome of *H. pylori* codes for numerous outer membrane proteins (108) which allows adhesion to several different carbohydrate structures on mucins. *H. pylori* binds to glycan structures present on both glycolipids and mucins, the former providing a more intimate adhesion to the host cells and the latter can serve as a decoy and be part of the host defense system (21, 71, 109).

The blood group antigen binding adhesin (BabA) recognizes fucosylated structures, such as $Le^b$ and H-type 1 antigen (46, 110) and mediates a high affinity bacterial binding to these structures (71). *H. pylori* strains expressing BabA have been considered more virulent, since they are more commonly associated with the development of severe gastric diseases (110-113). BabA-mediated binding can be influenced by certain environmental factors. It has been shown that BabA-$Le^b$ adhesion is acid sensitive but fully reversible by pH neutralization (114). This type of binding can also be reduced by treatment with the redox-active pharmaceutical *N*-acetylcysteine that has been suggested to be used in *H. pylori* eradication therapy development (115).

The sialic acid binding adhesin (SabA) mediates adhesion to α2,3-sialylated structures, such as sialyl-$Le^a$ and sialyl-$Le^x$ (116). In a healthy human stomach, sialyl-$Le^x$ antigen containing glycoconjugates are rarely expressed, whereas it has been shown to be upregulated after *H. pylori* infection and inflammation (116, 117). The acid responsiveness of *sabA* expression can be controlled by the ArsRS two-component signal transduction system (118, 119). *In vitro* studies demonstrated that *sabA* transcription is repressed by the acid-responsive ArsS and the *H. pylori* J99 isogenic mutant lacking ArsS histidine kinase locus (J99Δ*arsS*) had a 10 fold SabA-dependent binding to human gastric cells compared to the wild-type strain (118).

Several other adhesion molecules have been described to mediate *H. pylori* binding (Table 2). The lacdiNAc specific adhesin (LabA) has been suggested

to bind to the lacdiNAc structure (GalNAcβ1-4GlcNAc) on gastric mucins (120). The neutrophil activating protein A (NapA) has been shown to mediate binding to sulphated carbohydrate structures on high-molecular-mass salivary mucins (121, 122) as well as to $Le^x$ blood group antigen (121) and to sialylated glycans (122). Studies using isogenic mutants of *H. pylori* discovered a role of adherence-associated lipoprotein A and B (AlpA and AlpB) in binding to the host extracellular molecule laminin (123, 124) and a role of outer inflammatory protein A (OipA) in binding to gastric epithelial cells (125), although, the target receptor of the latter has not yet been identified. The *Helicobacter pylori* adhesin A (HpaA) lipoprotein has been characterized as an *N*-acetylneuraminyllactose-binding hemagglutinin (126). The HopZ membrane protein of *H. pylori* has also been associated with adhesion to gastric cancer cells (127). The type IV Cag secretion apparatus can also contribute to *H. pylori* adhesion carrying the CagL adhesive protein that has been shown to mediate $\alpha_5\beta_1$ integrin binding on gastric epithelial cells (128).

Lipopolysaccharide (LPS) on the surface of most *H. pylori* strains express Lewis blood group antigens with structural identity to the ones on host cells (129, 130). This molecular mimicry helps the survival of *H. pylori* in the stomach making the bacteria less recognizable by host immune cells (131). The most likely mechanism of adhesion of *H. pylori*-expressed *O*-glycans to host cells happens via the galactoside-binding lectine, galectin-3 (132). *H. pylori* LPS is able to bind to surfactant binding protein D (SP-D) which is a C-type lectin involved in antibody-independent pathogen recognition and clearance (133).

| **Adhesion molecule** | **Adhesion target** | **Reference** |
|---|---|---|
| BabA | $Le^b$, H-type-1 | (46, 110) |
| SabA | Sialyl-$Le^a$, sialyl-$Le^x$ | (116) |
| LabA | LacdiNAc | (120) |
| NapA | Sulphated, sialylated glycans, $Le^x$ | (121, 122) |
| AlpA, AlpB | Laminin | (123, 124) |
| OipA | Unknown | (125) |
| HpaA | N-acetylneuraminyllactose | (126) |
| HopZ | Unknown | (127) |
| HopQ | CEACAM | (134, 135) |
| CagL | Integrin | (128) |
| LPS | Galectin-3, SP-D, E/L-selectin | (132) |

*Table 2. Adhesion molecules and adhesion targets of H. pylori.*

Bacteria that penetrate the mucus layer get in contact with the large membrane-bound mucins before they reach the host tissue (136, 137). In a healthy human stomach, MUC1 is the most highly expressed cell surface mucin. MUC1 serves as an adhesion target for *H. pylori* since it can carry ligands for the BabA and SabA adhesins (21). After bacterial adherence, MUC1 can act as a releasable decoy shedding together with the bound bacteria from the epithelial surface to the gastric juice (19, 21).

## 1.9 Effects of mucins on *H. pylori* growth

Besides serving as attachment sites, mucins can be important for bacterial colonization by providing energy source for the bacteria. Depending on the origin and type of the mucin, it can have a stimulatory or inhibitory effect on bacterial growth (86). *In vitro* proliferation assays revealed that culturing *H. pylori* in the presence of purified human gastric mucins from tumor tissue and from surface mucosa had a growth promoting effect, whereas mucins from the glandular region tended to inhibit the growth of *H. pylori* (86). Glandular mucins with α1,4-linked N-acetylglucosamine (α1,4-GlcNAc) terminating *O*-glycans have been suggested to have an antimicrobial effect by inhibiting the synthesis of a vital cell wall component, cholesteryl-α-D-glucopyranoside (138, 139). The α1,4-GlcNAc glycan structure can be found on MUC6 produced by gland mucus cells which can explain *H. pylori* colonizing the surface mucous layer and only rarely appear in deeper mucus (139).

## 1.10 *H. pylori* infection induced mucin glycosylation changes

*H. pylori* infection can alter the expression of normal gastric mucins (53) and impair mucin production and turnover rate (54). Prolonged infection with this pathogen showed a decrease in fucosylation and an increase in sialylation of mucin glycans in human stomach (140). Increased sialyl-Lewis antigen expression have also been observed in *H. pylori* infected rhesus monkeys one week after infection where the glycosylation returned to baseline level by 10 months post-infection (107). In addition, increased sialyl-Lewis antigen expression upon *H. pylori* infection was observed in mice and mongolian gerbils (141-143). These alterations in mucin glycosylation enable further *H. pylori* infection by increasing receptors for the SabA adhesion molecule (144). These mucin glycosylation changes can be explained by the ability of *H. pylori* to alter the expression of several genes involved in glycan biosynthesis. Infection experiments on human gastric cell lines demonstrated that *H. pylori*

infection alters the expression of GlcNAc-transferase (β3GnT5) gene that drives the biosynthesis of sialyl-Le$^x$ (145).

## 1.11 *Helicobacter suis*

Tightly coiled spiral shaped bacteria in the pig stomach were first described by Mendes *et al.* and Queiroz *et al.* (146, 147). The temporary name of this bacterium was '*Gastrospirillum suis*' because of its morphological similarity to the bacterium '*Gastrospirillum hominis'* (148). However, sequencing of the 16S rRNA gene of this bacterium revealed that it belongs to the *Helicobacter* genus and the bacterium was renamed as '*Candidatus Helicobacter suis'* (149).

*Helicobacter suis* is a Gram negative tightly coiled spiral-shaped bacterium that requires highly enriched biphasic medium at pH 5 and a microaerobic atmosphere for *in vitro* growth (150). In its main host, the pig, *H. suis* colonizes mainly the antrum and the fundic gland zone of the stomach (151) and the presence of *H. suis* DNA was also shown in the *pars oesophagea* (152). The prevalence of *H. suis* in pigs gradually increases with age reaching up to 90% at slaughter age (150, 153). In pigs, *H. suis* infection is associated with chronic gastritis (55), decreased daily weight gain (154) and the presence of ulcers in the *pars oesophagea* (155). *H. suis* infection may result in increased gastric acid secretion, contributing to the contact of the non-glandular part of the stomach with hydrochloric acid (55). *H. suis* can often be found in close contact with the acid producing parietal cells (156, 157) and they are able to affect the viability and function of these cells (158). The outcome of *H. suis* infection in pigs was shown to be dependent on the phase of infection and the age of the pigs (159).

*H. suis* is the most prevalent non-*Helicobacter pylori Helicobacter* (NHPH) species in the human stomach, with a prevalence ranging between 0.2 and 6%, which refers to its zoonotic importance (55). Pigs may serve as source of infection for humans by direct contact or by consuming raw or undercooked meat (160). Direct human-to-human transmission of *H. suis* has not yet been reported. In the human host, *H. suis* can contribute to the development of peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and chronic gastritis (161). The risk of developing MALT lymphoma after infection with NHPH species is higher than after *H. pylori* infection (162).

Apart from pigs and humans, *H. suis* infection has also been described in rhesus monkeys and cynomolgus monkeys (163) where it caused relatively mild gastric disorders (164, 165).

# 2 AIM

***General aim:***

The overall aim of the thesis was to investigate the dynamic interplay between *Helicobacter* spp. and host gastric mucins. A better understanding of this host-pathogen interaction can contribute to the development of therapeutic strategies that can be used to manage *Helicobacter* infection.

***Specific aims:***

- To investigate the relationship between *H. pylori* adhesin mediated binding, aggregation, growth and adhesin gene expression using purified human gastric mucins and synthetic glycoconjugates.

- To identify and characterize pig gastric mucins, investigate *H. suis* binding to pig and human gastric mucins and glycolipids and define bacterial binding-active structures on mucins.

- To study the effect of experimental *H. suis* infection on pig gastric mucin glycosylation.

- To study how *Helicobacter* spp. infection related mucin glycosylation changes affect *H. suis* binding and growth.

# 3 METHODOLOGY

The methods used in the thesis are described in details in the attached papers. Here I discuss reasons behind the choice and required optimization and adjustments of certain methods.

## 3.1 Bacterial growth detection

Mucins can promote or inhibit bacterial growth depending on the source and the type of the mucin. To study the effect of purified gastric mucins on the growth of different *Helicobacter* strains, we cultured the bacteria in the presence or absence of mucins. Optical density (OD) is a widely used measure of bacterial growth with the interpretation that the OD value of bacteria is directly related to the bacterial cell count**.** Although this method appears accurate in many cases, we have discovered that several *H. pylori* cultures with strong binding to the added mucins resulted in high OD values despite low colony forming unit (CFU) counts which can be explained by bacterial aggregate formation **(**Paper I**).** If the aggregates are not fully dispersed prior to the CFU plating, several bacteria can appear as one colony leading to an enhancing error in OD measurements, and a diminishing error using the CFU counting method. Another disadvantage of the bacterial growth detection with OD measurement is that this method does not give information about the viability of the bacteria (i.e. the ratio of live and dead cells in the bacterial culture) that makes this method less informative about the growth response of bacteria to different agents. Therefore, there was a need to develop a method for the accurate assessment of *H. pylori* growth and viability**.** The metabolic activity of bacteria can be measured by adding alamarBlue cell viability reagent to the bacterial cultures (166). Our growth experiments demonstrated that, the relationship between the alamarBlue signal and CFU counts is similar to the relationship between OD measurement and CFU counts in the absence of bacteria binding elements in the culture, thus metabolic activity measurement with alamarBlue seems to be accurate in *H. pylori* growth detection.

The same method, however, could not be used for *H. suis* growth detection, since this pathogen requires a culture media with pH5 for *in vitro* growth which is below the optimum pH for alamarBlue bioassays. Therefore, we needed to develop a method that measures the metabolic activity of the bacteria in the environment that is crucial for the bacterial growth. For this purpose, we used the RealTime-Glo™ viability assay that is a luciferase reaction based assay, where the detected light production is proportional to the number of live

bacterial cells in the culture media (Paper III). With this method, we can continually monitor the bacterial growth while maintaining an optimal environment for the bacteria (Figure 1).

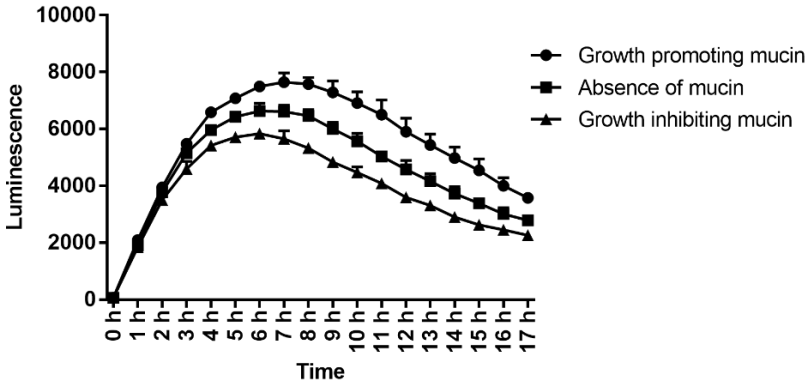


***Figure 1. H. suis growth detection by RealTime-Glo™ viability assay.*** *H. suis growth curve when cultured in the absence (■) or presence of a growth promoting (●) or a growth inhibiting (▲) mucin.*

## 3.2 Bacterial binding detection

We used four different assays to study bacterial binding to purified mucins, glycoconjugates and glycolipids.

### 3.2.1 Binding assay using antibody detection (Paper I, II)

*Helicobacter* spp. binding to purified mucins and glycocojugates 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 conjugated secondary antibody are added to the wells. The bound bacteria are visualized by adding 3,3',5,5'-Tetramethylbenzidine (TMB) to the wells that is a substrate for horse radish peroxidase 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. During the binding evaluation process, 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.

### 3.2.2 Binding inhibition assay (Paper II)

To investigate the binding of bacteria to monosaccharides that are not conjugated to carrier protein, hence cannot be coated on polysorp plates, binding inhibition assay was performed. In this method, a mucin with strong bacterial binding ability was coated on the 96-well plate and the bacteria were pre-incubated with the sugars of interest prior to the incubation with mucins. If the glycans that are used during pre-incubation are targets of the bacterial adhesins, they are expected to inhibit binding of the bacteria to the mucin sample by occupying the binding site of the adhesion molecules.

### 3.2.3 Binding to purified mucins and glycoconjugates using biotinylated bacteria (Paper II, III)

The *H. suis* binding signal detected with the antibody detection method was relatively weak compared to the level of adhesion found with *H. pylori*. The low binding signal and high background signal due to the cross-reaction between the antibody and mucins was technically challenging, thus we performed an adhesion assay using biotinylated *bacteria*. To avoid damaging the adhesins by biotinylation, mild biotinylation of bacteria was performed, as previously described (167). Similarly to the binding detection with antibody, the mucin and glycoconjugate samples were coated on 96-well polysorp plates, and incubated with biotinylated bacteria. Bound bacteria were detected by the reaction between biotin carried by the bacteria and horseradish peroxidase conjugated streptavidin and the binding was visualized with TMB substrate. The results obtained with this method were similar to the results obtained with the antibody detection method, but with higher signal to noise ratio.

### 3.2.4 Binding of *Helicobacter* spp. to glycosphingolipids on thin-layer chromatograms (Paper II)

Glycolipids can carry similar glycoepitopes as mucins that can serve as binding sites for bacteria providing a more intimate bacterial adhesion to the host. The use of glycolipids in binding studies simplifies the investigation of the binding specificity, since these molecules carry only one glycan, contrary to the multiple glycans carried by mucins. Binding detection to glycolipids was performed by using $^{35}$S-methionine labeled bacteria. The labeling of *Helicobacter* spp. was performed as described (168). The glycosphingolipids were separated on aluminum-backed silica gel plates and the bound $^{35}$S-labeled bacteria were detected by autoradiography. Due to that the low incorporation of radioactive label into *H. suis* made the glycolipid binding results difficult to reproduce, we also demonstrated binding of this pathogen to glycoconjugates

terminating with the same epitopes using binding methods independent of metabolic labeling of the bacteria (i.e. the above methods).

# 4 RESULTS AND DISCUSSION

## 4.1 BabA mediated binding of *H. pylori* affects the growth and gene expression of the bacteria (Paper I)

The presence of certain gastric mucins in bacterial culture has been shown to promote or inhibit *H. pylori* growth, depending on the type and origin of the mucin (86). Mucin molecules carry a vast array of different oligosaccharide structures that can be utilized as nutrition source by mucin degrading bacteria, hence can stimulate bacterial growth (83, 84, 169). Glandular mucins that carry α1,4-GlcNAc terminating *O*-glycans have been suggested to have an antimicrobial effect inhibiting the biosynthesis of a major *H. pylori* cell wall component (138, 139), although, this cannot be the only explanation for the growth inhibitory effect of mucins, since not all the mucin samples that inhibit *H. pylori* growth carry this structure (86). To investigate the growth of *H. pylori* in response to mucins and mucin glycans, we cultured the bacteria in the presence of $Le^b$ and $SLe^x$ glycoconjugates that are the adhesion targets of *H. pylori* adhesin BabA and SabA, respectively, and in the presence of mucins that carry these structures. In these experiments, we used strains with different adhesin gene expression: strain J99 that carries both the *babA* and *sabA* gene and strain P12 that carries the *babA* gene only. Both strains had stronger binding avidity to $Le^b$ than to $SLe^x$ and both strains bound better to the mucin derived from healthy stomach that carries $Le^b$ and is lacking $SLe^x$, than to the tumor-derived mucin that carries both $Le^b$ and $SLe^x$ glycan structures. *H. pylori* growth measurement by detecting alamarBlue reduction revealed that $Le^b$-conjugates decreased the metabolic activity of both *H. pylori* strains, suggesting that adhesion to $Le^b$ and to mucins carrying these glycan structures inhibit the growth of the bacteria. This hypothesis was supported by further binding and growth experiments using adhesin deletion mutants. As expected, *babA* deletion mutants of both strain J99 and P12 had lower binding avidity to $Le^b$ glycoconjugate and to mucins that carry this structure than the isogenic wt strains and they showed low or no binding to $SLe^x$. Deletion of *babA* reversed the growth inhibiting effect on strain J99 and enhanced the growth of strain P12 in the presence of the mucin and $Le^b$, indicating that strain P12 has a positive growth response to mucin glycans, which is suppressed by the BabA mediated binding. These results together indicate that the interaction of both *H. pylori* strains with mucin glycans is mediated by BabA and that the effect of mucins on bacterial growth is highly dependent on the glycosylation of mucins.

To visualize the morphology and viability of the bacteria in the presence of mucin glycans, the bacterial culture of strain J99 wt and its isogenic mutants after co-culturing with $Le^b$ and $SLe^x$ glycoconjugates were stained with Live/Dead double staining kit that stains viable and dead bacterial cells in the culture media with different colors. This staining revealed aggregate formation with J99Δ*sabA* and J99 wt in the presence of $Le^b$-glycoconjugate, which was accompanied by decreased metabolic activity as measured by alamarBlue reduction. There was no aggregate formation when J99Δ*babA* was cultured in the presence of $Le^b$, and it did not affect the metabolic activity of the bacteria. Adhesive interaction between bacteria has been shown to be induced by unfavorable growth conditions (170, 171), and the role of aggregate formation in bacterial resistance towards antimicrobial agents has been also described (172). These observations suggest that aggregate formation might be beneficial for the bacteria as part of the bacterial survival strategy in response to environmental factors. In this thesis, we describe the benefits of bacterial aggregate formation for the host and we hypothesize that in addition to facilitating washing away the bacteria from the stomach, mucin binding controls the pathogen number in the stomach by the growth limiting effect of aggregate formation. As it was revealed by Live/Dead staining, the majority of the aggregate forming bacteria were alive, suggesting that there is no direct antimicrobial effect of aggregate formation, the growth limiting effect can be instead explained by the slow replication due to physical hindrance or inter-bacterial communication.

Apart from affecting the growth of the bacteria, attachment to mucins can alter the expression of *H. pylori* genes relevant to colonization. We have previously shown that culturing *H. pylori* in the presence of mucins from different individuals can affect the adhesin expression of the bacteria (86). Here we demonstrated that *H. pylori* BabA and SabA adhesin expression in response to mucins negatively correlates with the binding avidity of the bacteria to these mucins. To verify the effect of adhesion on bacterial gene expression, we analyzed adhesion gene expression level after co-culturing the bacteria with $SLe^x$- and $Le^b$-glycoconjugates and we observed that the presence of $Le^b$ decreased *babA* expression, whereas the level of this gene was not affected by the presence of $SLe^x$. The repression of *babA* gene expression in response to $Le^b$ binding might be part of the bacterial defense mechanism avoiding excessive binding to mucins that would lead to the removal of the bacteria along with shedding mucus. Decreasing the amount of adhesins in response to binding might serve as a negative feedback loop that can enable long-term colonization by *H. pylori*.

## 4.2 ArsS affects *H. pylori* growth and BabA-dependent binding (Paper I)

*H. pylori* expresses the ArsS pH sensor histidine kinase protein that plays a role in urease gene transcription and in urease protein delivery in order to enhance acid acclimation (173). Increased of SabA expression level has been previously shown in an *H. pylori* strain lacking the ArsS (118). In line with these results, we detected increased binding of J99Δ*arsS* to $SLe^x$ and to $SLe^x$ containing mucins compared to that of J99 wt and it was accompanied by decreased binding to $Le^b$ and $Le^b$ containing mucins with the *arsS* deletion mutants of both J99 and P12 strains. Deleting the *arsS* slightly increased BabA protein expression in J99Δ*arsS*, whereas decreased in P12Δ*arsS* to a similar degree. These results suggest that level of BabA-dependent binding is more dependent on the topographical localization rather than the number of adhesins present on the bacteria. Contrary to the growth inhibitory effect of mucin glycans on the *H. pylori* wt strains, J99Δ*arsS and* P12Δ*arsS* growth did not decrease in the presence of the mucin samples or $Le^b$ glycoconjugate, which can be due to the lack of aggregate formation. The adhesion and growth experiments using the *H. pylori arsS* deletion mutants further confirmed the role of bacterial aggregate formation in the growth inhibitory effect of mucins.

## 4.3 *H. suis* resides in the mucus layer and can also be found associated with parietal cells (Paper II, III).

In *H. pylori* infected human stomachs, the majority of *H. pylori* have been detected in the surface mucus layer (96) that protects the bacteria from the low acidity in the stomach and provides surface for host-pathogen interactions (70). Here we analyzed the mucus layer of the pig stomach and the spatial distribution of the pig gastric pathogen, *H. suis*. On pig gastric tissue sections, we detected a thick mucus layer covering the epithelial cells. Antibody detection on these sections as well as proteomic analysis on purified gastric mucin samples revealed that MUC5AC is the predominant secreted mucin in the pig stomach, similarly to the human stomach. We performed fluorescent *in situ* hybridization on pig gastric tissue sections obtained from pigs experimentally or naturally infected with *H. suis* and detected *H. suis* in the mucus layer lining the surface epithelium and throughout the gastric pits. We and others also detected this bacterium in close association with the acid producing parietal cells (156). These findings suggest that *H. suis* is exposed to neutral pH in the mucus layer closer to the epithelial cells and in the lamina

propria and exposed to acidic pH closer to the gastric lumen as well as when inside the parietal cells.

## 4.4 Gastric mucin glycosylation differs between pigs and humans as well as between *H. suis* infected and non-infected pigs (Paper II, III)

*H. suis* is associated with the development of severe gastric disorders in its main host, the pig and also in humans. Since *H. suis* resides in the gastric mucus layer, interaction with mucin glycans can be crucial for colonization and survival of the bacteria and can also support the host defense system maintaining a dynamic host-pathogen interplay in the stomach. To get a better understanding of this host-pathogen interaction, we analyzed the glycosylation of purified pig gastric mucin samples by mass spectrometry analysis, and since *H. suis* is of zoonotic importance, we studied the differences between pig and human gastric mucin glycosylation. The glycan profile of both pig and human mucins showed high inter-individual differences. We detected higher number of different glycan structures in the human mucins than in the pig mucins, which does not necessarily provide evidence for higher inter-individual variability in humans than in pigs, since the pig samples analyzed here only include non-infected pig mucins, whereas the human samples also include pathological specimens. The length of mucin *O*-glycans varied between 2 and 14 residues in pig samples and from 2 to 12 residues in human mucins. Both pig and human gastric mucin oligosaccharides were mainly extended core 1 and core 2 *O*-glycans, although, structures with core 3 and 4 were also detected. The relative abundance of extended core 1 *O*-glycans was higher in pig than in human mucins. The terminal residues on the mucin glycans are usually vital parts in mucin-pathogen interaction, therefore we quantified the relative abundance of these glycan epitopes on pig and human gastric mucins (Figure 2A). The most abundant terminal residue was galactose on pig mucin glycans and fucose on human mucin glycans. The level of sialylation of gastric mucins was low both in pigs and in humans. The relative abundance of mucin glycans with terminal galactose were higher in pig mucins, whereas the relative abundance of fucose terminating glycans were higher in human mucins. The main difference between human and pig glycan terminal epitopes was the level of sulphation which was around 50% in pig mucins and very low, around 0.6% in the human samples.

Since *H. pylori* infection has been shown to trigger qualitative and quantitative changes in host gastric mucins (53, 54, 141-143), we studied the alterations of

pig gastric mucins in *H. suis* infection. With mass spectrometry analysis on purified pig gastric mucins, we identified that MUC5AC is the major secreted mucin in the pig stomach. A decreased level of MUC5AC was detected among mucins from *H. suis* infected pigs compared to the samples from non-infected pigs, similarly to the effect of *H. pylori* infection in the human stomach (53). We detected in total 118 different oligosaccharides on the pig gastric mucin samples, out of which 18 structures with low fucose and sialic acid content were detected only in the non-infected samples and 7 structures containing higher fucose and sialic acid level were only detected in the infected group. The number of different oligosaccharide structures detected in the mucin samples was lower in the infected group, implying a decrease in the number of glycan structures on pig gastric mucins upon infection limiting the variety of glycan epitopes for bacterial interactions. In mucins from the infected group, we detected an increase in the relative abundance of acidic (mostly sulphated) and fucosylated glycan structures and a decrease in glycan structures terminating with galactose (Figure 2B). These terminal glycan structures play important role in the binding and growth of *H. suis* in the presence of mucins, as it is described later in the thesis.

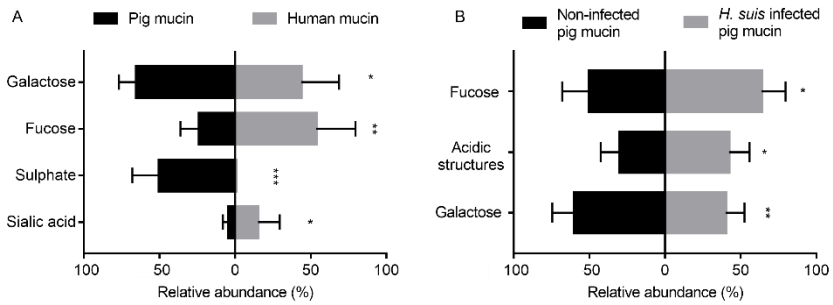


***Figure 2. Differences in the relative abundance of terminal glycan epitopes between pigs and humans as well as between H. suis infected and non-infected pigs.*** *A. Differences in the relative abundance of terminal glycan structures between pig and human gastric mucins B. Changes in pig gastric mucin glycosylation upon H. suis infection. Stars indicate statistically significant difference between mucin glycans, *, ** and *** indicate $p \leq 0.05$, 0.01 and 0.001, respectively, Two-way ANOVA.*

## 4.5 *H. suis* binding to gastric mucins in health and disease (Paper II, III).

We analyzed *H. suis* binding to purified pig and human gastric mucins at the pH range present in the stomach and compared it with the binding of *H. pylori*

to the same human mucins. *H. suis* and *H. pylori* binding to purified mucins and glycoconjugates showed different pattern both regarding their pH preference and glycan specificity (Figure 3). *H. suis* binding level was highest at pH 2 with a gradual decrease towards neutral pH, whereas *H. pylori* bound better at neutral pH. *H. suis* and *H. pylori* bound to the same mucin samples as well as glycoconjugates with different avidity, suggesting that the two *Helicobacter* species use different modes of adhesion with different glycan specificities. *H. pylori* binding to mucins has been described to happen via four modes of adhesion (70), the most well characterized of which are the BabA and SabA mediated binding to $Le^b$ and sialylated glycans, respectively. *H. suis* genome analyses revealed that *H. suis* lacks homologs of BabA and SabA, although, contains some OMPs similar to the major OMP families described in *H. pylori*, which might be involved in binding to gastric mucins (161). Our bacterial binding experiments showed that *H. suis* binding avidity differed between the mucin samples investigated. Some samples bound the bacteria only at acidic pH, whereas other samples bound also at neutral pH. Furthermore, *H. suis* binding to GuHCl soluble mucins was more pronounced than to insoluble ones. The differences in *H. suis* binding avidity to the different mucin samples can be explained by the glycosylation differences between the samples. Here we suggest that *H. suis* uses two ways of adhesion to mucins: one binding mode that is dependent on acidic pH and one that is functional also at neutral pH, and both binding modes depend on the glycan structures carried by the mucins.

To find the glycan structures on the mucins that might serve as adhesion targets for *H. suis*, we analyzed the relation between the bacterial binding amplitude to the mucins and the abundance of the different glycan structures carried by these mucins. We focused primarily on the terminal residues, since these glycans are more exposed for bacterial binding. At pH 2, the relative abundance of acidic (i.e. sulphated and/or sialylated) glycans correlated with the level of *H. suis* binding, indicating that *H. suis* can bind to acidic structures via charge dependent mode. This hypothesis was also confirmed by *H. suis* binding to the highly charged DNA at pH 2, but not at pH 7. The stronger binding avidity to mucins at lower pH cannot be the consequence of protein denaturing because not all the mucin samples that were tested bound *H. suis* at low pH. In addition, binding at low pH to DNA also suggests that binding at acidic pH occurs to charged structures, not to denatured proteins (Figure 3A).

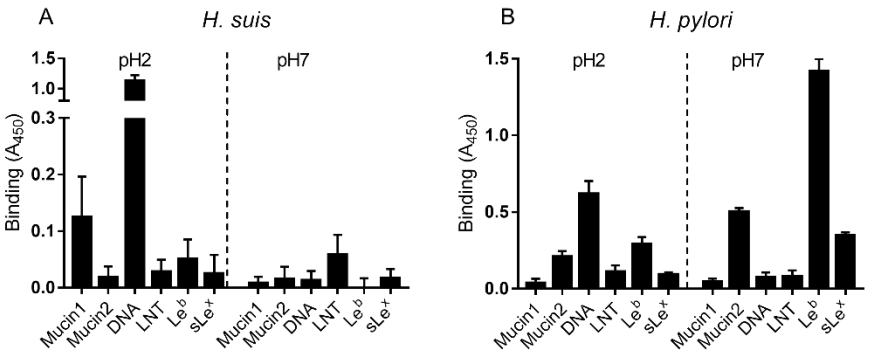


***Figure 3. H. suis and H. pylori binding differences regarding pH and glycan specificity.*** *A. H. suis binding to purified mucins, glycoconjugates and DNA at pH 2 and pH 7. B. H. pylori binding to purified mucins, glycoconjugates and DNA at pH 2 and pH 7. The binding values are shown after subtracting background signal (bacteria binding to plastic well).*

Glycolipids can carry similar glycan structures as mucins and they represent a more intimate adherence to the host by *Helicobacter* species. To further investigate *H. suis* binding specificity, we examined *H. suis* binding to glycosphingolipids isolated from porcine stomach, where binding to lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) was detected. To confirm the binding specificity to this structure, *H. suis* binding to Lacto-N-tetraose (LNT, Galβ3GlcNAcβ3Galβ4Glc) conjugated to human serum albumin (HSA) was also tested. In line with the glycolipid binding results, *H. suis* bound to LNT conjugated to HSA at pH 7 and the binding remained functional at pH 2. We also demonstrated that binding to pig mucins can be inhibited by pre-treating the bacteria with LNT or sialylated LNT. Together these data indicate that *H. suis* can bind to mucins with terminal galactose and that acidic modification may have beneficial effects on binding.

*H. pylori* induced gastric mucin glycosylation changes have been demonstrated to influence the mucin binding avidity of the pathogen (107). The decreased $Le^b$ level and increased sialylation in the gastric mucosa decrease BabA mediated binding and increased the adhesion via SabA and via the charge-dependent binding modes (70, 107) leading to an overall decreased adhesion, since BabA mediated binding is generally higher than binding via SabA. To study the effect of *Helicobacter* infection on the binding avidity of *H. suis*, we used purified pig gastric mucins with or without *H. suis* infection as well as human gastric mucins with and without *Helicobacter* spp. infection and tested *H. suis* binding to these mucins at acidic and neutral pH. In line with previous

observations, *H. suis* binding to pig gastric mucins was higher at pH 2 than at pH 7, regardless of the infection status of the pigs (Figure 4A, B), whereas pH did not affect the binding avidity to human gastric mucins. Infection caused a decreased *H. suis* binding avidity to pig gastric mucins at pH 7 (Figure 4B) but not at pH 2 (Figure 4A). At neutral pH, the decreased binding after infection can be explained by the infection-induced loss of adhesion targets by the decreased terminal galactose on mucins and at acidic pH, *H. suis* also binds via charge dependent mode to acidic structures, the abundance of which structures increased during infection.

Bacterial adherence to gastric mucins can serve as important part of the host defense mechanism protecting the epithelial cells from the invasion by Helicobacters. This hypothesis has been supported by observations where *H. pylori*-infected children and rhesus monkeys secreting mucins with less *H. pylori* binding capacity, develop higher *H. pylori* density and more severe gastritis (107, 174). Our results demonstrate that *Helicobacter* spp. infection decreases the ability of mucins to bind *H. suis*, thereby avoiding the removal of the pathogen from the gastric niche.

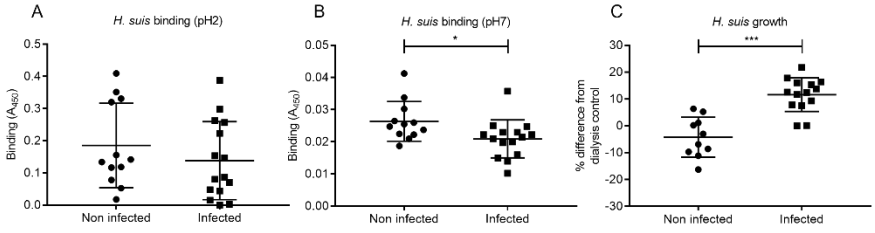


***Figure 4. H. suis binding and growth in the presence of gastric mucins derived from H. suis infected or non-infected pigs.*** *A. H. suis binding to H. suis infected or non-infected pig gastric mucin samples at pH 2. B. H. suis binding to H. suis infected or non-infected pig gastric mucin samples at pH 7. C. H. suis growth in the presence of gastric mucin samples derived from H. suis infected or non-infected pigs. (*p<0.05, ***p<0.001, Two-way ANOVA).*

## 4.6 *Helicobacter* spp. infection induced mucin glycosylation changes increase *H. suis* growth (Paper III).

The growth of *H. pylori* has been shown to have different response to mucins, depending on the origin and the type of the mucin (86). For instance, when *H.*

*pylori* is cultured in the presence of mucins that carry glycan structures that the bacteria adheres to, the binding-induced aggregate formation slows down the growth of the bacteria (Paper I). *H. pylori* growth can be also inhibited by mucins containing α1,4GlcNAc-capped *O*-glycans (139), a structure that primarily is associated with the glandular mucins in the stomach. In this thesis, we demonstrated that the growth of *H. suis* is affected by both porcine and human gastric mucins and the response of the bacteria to mucins is dependent on the infection status of the individual the mucin was isolated from. Mucins from non-infected individuals inhibited the growth of *H. suis*, whereas mucins from infected individuals had a growth promoting effect (Figure 3C). To find the possible glycan structures on the mucins that affect the bacterial growth, we studied the relationship between the effect of mucin samples on *H. suis* growth and the abundance of glycan structures carried by these mucins. The growth inhibitory effect of mucins correlated with the abundance of galactose terminating structures and was independent of α1,4GlcNAc-capped *O*-glycan abundance. Positive correlations between growth and the abundance of acidic and fucosylated structures on mucin glycans were observed. These results suggest that *Helicobacter* spp. infection induced host mucin glycosylation changes create a more stable and growth-promoting environment for *H. suis*, and possibly for other *Helicobacter* species in the stomach that facilitates the long term colonization by these pathogens.

# 5 CONCLUSIONS

In this thesis, we investigated host-pathogen interactions in the mucus niche focusing on *Helicobacter* spp. and gastric mucins. We analyzed the mucus alterations in infection and how these changes affect bacterial behavior. Based on our results of mucin characterization as well as bacterial adhesion and growth assays we can conclude that:

- *Helicobacter* spp. infection induces a constant host-pathogen adaptation and response process in the stomach. The mucin interaction with pathogens is mediated by the mucin glycan composition, which is able to inhibit *H. pylori* growth by adhesion and aggregation of bacteria. Mucins also have the ability to influence *H. pylori* pathogenicity by affecting adhesin gene expression.
- *H. pylori* and *H. suis* binding to human and pig gastric mucins differ in specificity and pH preference and show high inter-individual variation, which can be explained by mucin glycosylation differences.
- *H. suis* binding to gastric mucins and glycolipids occurs via two modes of adhesion: to structures with terminal galactose at both neutral and acidic pH, and to negatively charged structures at acidic pH. These binding modes may enable bacterial adhesion at low pH close to the gastric lumen and in parietal cells and a more intimate adhesion to mucin glycans and glycolipids close to the epithelial cells.
- *Helicobacter* spp. infection alters host mucin composition and glycosylation in a way that decreases the amount of *H. suis* binding glycan structures on gastric mucins and the *H. suis* growth regulating effects of the mucins. By these alterations, Helicobacters create a more stable and inhabitable niche in the stomach which may be crucial for long-term colonization.

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