Brachyspira hyodysenteriae binding to porcine colonic mucins differs between individuals and is increased to mucins from infected pigs with *de novo* MUC5AC synthesis

Macarena P. Quintana-Hayashi[1], Maxime Mahu[2], Nele De Pauw[2], Filip Boyen[2], Frank Pasmans[2], An Martel[2], Pushpa Premaratne[1], Harvey R. Fernandez[1], Omid Teymournejad[1], Lien Vande Maele[2,3], Freddy Haesebrouck[2] and Sara K. Lindén[1]#

[1]Department of Medical Biochemistry and Cell biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden. [2]Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium. [3]Institute for Agricultural and Fisheries Research (ILVO), Melle, Belgium.

Running title: *B. hyodysenteriae* binding to porcine colonic mucins

Dr. Sara K. Lindén, Department of Medical Chemistry and Cell Biology, University of Gothenburg, Sweden; E-mail: sara.linden@gu.se

M. Mahu and N. De Pauw contributed equally to this work.

Abstract

Brachyspira hyodysenteriae colonizes the pig colon resulting in mucohemorrhagic diarrhea and growth retardation. Fecal mucus is a characteristic feature of swine dysentery; therefore we investigated how the mucin environment changes in the colon during infection with *B. hyodysenteriae*, and how these changes affect this bacterium's interaction with mucins. We isolated and characterized mucins, the main component of mucus, from the colon of experimentally inoculated and control pigs, and investigated *B. hyodysenteriae* binding to these mucins. Fluorescence microscopy revealed a massive mucus induction and disorganized mucus structure in the colon of pigs with swine dysentery. qPCR and antibody detection demonstrated that the mucus composition of pigs with swine dysentery was characterized by *de novo* expression of MUC5AC and increased expression of MUC2 in the colon. Mucins from colon of inoculated and control pigs were isolated by two-steps of isopycnic density-gradient centrifugation. The mucin density was similar between control and inoculated pigs, whereas the mucin quantity was five-fold higher during infection. *B. hyodysenteriae* bound to mucins in a manner that differed between pigs and there was increased binding to soluble mucins isolated from pigs with swine dysentery. The *B. hyodysenteriae* binding ability, in relation to the total mucin content of mucus from sick vs. healthy pigs, increased seven-fold during infection. Together, the results indicate that *B. hyodysenteriae* binds to carbohydrate structures on the mucins, as these differ between individuals. Furthermore, *B. hyodysenteriae* infection induces changes to the mucus niche, which substantially increases the amount of *B. hyodysenteriae* binding sites in the mucus.

Introduction

The gastrointestinal tract is lubricated by a continuously secreted mucus layer which can also act as a barrier against pathogens (1). The main components of the mucus layer are heavily glycosylated gel-forming mucins. Mucin glycans can prevent enzymatic degradation of the mucin protein core, and can also bind water, conferring viscoelasticity (2). Underneath the mucus layer, transmembrane mucins on the mucosal epithelial cells provide barrier and reporting functions (3, 4). Mucins differ in their glycosylation and tissue distribution (5). Murine colonic mucus has been shown to be rich in the MUC2 mucin, which is secreted by goblet cells and is organized in a two-layered mucus system (6). The inner mucus layer is firmly attached to the epithelium, and gives rise to the loosely adherent outer layer (7).

Mucins are a dynamic component of the mucosal barrier, and have been shown to undergo changes in response to intestinal infection and inflammation in mice (8, 9). Mucin glycan structures can bind bacteria, *e.g. Escherichia coli* and *Helicobacter pylori*, limiting colonization and access to the epithelial surface (3, 10-13). Mucin glycosylation can change during bacterial infection, and varies between individuals (14). To date it is unknown whether the large variability in mucin expression and mucin glycosylation arose by chance during evolution, or if the mucin species confer distinct response properties during infection.

Brachyspira hyodysenteriae (*B. hyodysenteriae*) is a recognized swine pathogen, commonly associated with swine dysentery (SD). This anaerobic spirochete colonizes the large intestine of pigs resulting in mucohemorrhagic diarrhea. Ingestion of feces from inoculated pigs, as well as from asymptomatic carriers, is among the main sources of infection (15). SD is responsible for economic losses in the swine industry, posing a threat in countries where antimicrobials are banned for growth promotion, and challenging those where resistant strains have emerged (16-19). The presence of mucus in the feces is a characteristic feature of SD. Recently, colonic specimens from pigs with SD were shown to have increased immunohistochemical staining with an antibody against the human MUC5AC mucin and decreased staining with an antibody against the MUC4 mucin (20).

B. hyodysenteriae pathogenesis is still surrounded by many uncertainties. The mechanisms underlying the bacterial interactions with the colonic mucosal surface, or how the mucin response exerted during infection is regulated, remain to be elucidated. Therefore, the overall aims of the present study were to investigate how the mucin environment changes in the swine colon during infection with *B. hyodysenteriae*; if this bacterium binds to mucins, and if so, how these changes affect binding. We identified that *B. hyodysenteriae* infection causes changes in mucus organization, mucin quantity, identity and expression profile, as well as in the mucin binding ability of this bacterium.

Materials and Methods

Ethics statement

The animal experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2012/01 and EC2013/147) and complied with all ethical and husbandry regulations.

Experimental inoculation and sample collection

Samples from a total of 15 pigs (Danish Large White × Piétrain) from two independent inoculation experiments, 21 months apart, were included in the study (Table 1). The first experiment included 6 control pigs and 6 inoculated pigs, and the second 8 control pigs and 8 inoculated pigs. The pigs from both inoculation experiments were 6-week old, came from two different commercial farrowing to finishing farms in the Flanders region with no previous history of swine dysentery, belonged to different litters, and were fed the same commercial starter feed (crude protein 17%, crude fat 6.09%, crude fiber 3.87%, crude ash 5.09%, phosphorus 0.49%, methionine 0.43%, lysine 1.25%, calcium 0.61%, and sodium 0.23%; Lambers-Seghers, Belgium). At their arrival, the pigs were confirmed negative for *B. hyodysenteriae* in rectal fecal samples by culture and qPCR. The pigs were acclimatized for two weeks in order to recover from transport stress and adapt to diet and housing changes. The pigs were fed twice per day and had ad libitum access to water. A total of 14 pigs were experimentally inoculated with *B. hyodysenteriae* strain 8dII, isolated from a Belgian swine farm with a history of recent dysentery problems. An inoculum of 10^8 CFU/ml in brain heart infusion broth (BHI) (50 ml/pig) was administered orally during three consecutive days, while 14 control pigs received 50 ml of sterile BHI. From the 14 control pigs, 6 samples were randomly selected for use in this study (pigs A-F). From the first infection trial two out of six pigs developed SD, and from the second infection trial three out of eight pigs developed SD (pigs 1-5). Samples from the four inoculated pigs that did not develop SD in the first inoculation experiment were included in the study (pigs 6-9; Table 1).

Infection was confirmed based on clinical signs of mucohemorrhagic diarrhea, and *B. hyodysenteriae* excretion in feces detected by qPCR in fecal samples obtained twice a week. The pigs were sacrificed at day 40 post-inoculation by anesthesia with a combination of xylazine at 4.4 mg/kg (Xyl-M 2%®, VMD, Arendonk, Belgium) and zolazepam/tiletamine at 2.2 mg/kg (Zoletil® 100, Virbac, Carros, France), and final euthanasia by intracardial injection of a formulation comprising embutramide, mebezonium iodide and tetracaine (T61®, Intervet, Brussels, Belgium) at 0.3 ml/kg.

Midsection samples of the spiral colon with a size of 7 × 8 cm were obtained from the inoculated and control pigs for mucin isolation. Fecal material was removed, and the tissues were rinsed with phosphate buffered saline (PBS) and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) before snap freezing and storage at -80°C. Smaller specimens were carefully collected without disturbing the mucus layer (no washing) and immersed in 10-volumes of fresh Carnoy’s methanol fixative (60% dry methanol, 30% chloroform, 10% glacial acetic acid), and embedded in wax for histology/immunohistochemistry. There were also samples collected in RNA*later*® (Life Technologies, Carlsbad, CA, USA) and kept at 4ºC overnight, then stored at -80ºC for RNA extraction.

Detection of B. hyodysenteriae in feces by qPCR

DNA from pig feces was obtained by using the QIAamp DNA stool mini kit (Qiagen, CA, USA) starting from 1 gram of feces. For qPCR, *Brachyspira* spp. specific primers were used in combination with a *B. hyodysenteriae* specific probe as previously described (21).

MUC2 and MUC5AC immunofluorescence

Tissue sections were deparaffinized and antigen retrieval was performed in 10 mM sodium citrate, pH 6.0 at 99°C for 30 min. Slides were cooled to room temperature and washed in PBS. Non-specific background was blocked with serum-free protein block (DAKO, Carpinteria, CA, USA) for 20 min. Primary antibodies anti-MUC2C3 (kindly provided by G. Hansson, University of Gothenburg, Sweden), anti-MUC5AC (45M1, Sigma-Aldrich, St. Louis, MO, USA) and anti-

MUC5CR (kindly provided by G. Hansson, University of Gothenburg, Sweden) were diluted 1/1000 and incubated at 4°C overnight. Sections were washed with PBS and incubated with secondary antibodies conjugated with Alexa Fluor 488 (Life Technologies, Eugene, OR, USA) for MUC2, and Alexa Fluor 594 for MUC5AC, diluted 1/500 for 1 h. After washing in PBS, specimens were mounted with ProLong® antifade containing DAPI (Life Technologies, Eugene, OR, USA). Pig and human gastric specimens were used as positive controls for MUC5AC staining. Similarities in the stomach and colon binding patterns indicated the staining of pig sections was specific, even though the antibodies were raised against human mucins.

qPCR for mucin expression

Pig colon tissue samples were immediately submerged in a 10-fold volume of RNA*later*® (Life Technologies, Carlsbad, CA, USA) at 4°C overnight, and frozen at -80°C until RNA extraction. Isolation of RNA was performed using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA yield and purity was assessed through UV spectroscopy (NanoDrop, Thermo Scientific, MA, USA). Total RNA (5 µg) was DNase treated at 37°C for 45 min, followed by the addition of 5 mM ethylenediaminetetraacetic acid (EDTA) and heat inactivation of DNase at 75°C for 10 min prior to cDNA synthesis. $MgCl_2$ was added to a 5 mM final concentration, and this RNA was used for cDNA synthesis with random hexamers and Superscript III (Life Technologies, Carlsbad, CA, USA) at 50°C for 2 h. The cDNA was used in a real-time PCR reaction using SYBR green (Power SYBR® green mix, Life Technologies, Carlsbad, CA, USA) and primers listed in Table 2. Primers for pig *MUC1*, *MUC2*, and *MUC5AC* mucin genes were designed using the Primer3 program (available at http://frodo.wi.mit.edu/primer3/). qPCR data were normalized using the expression levels of *ACTB* and *RPL4* reference genes (22). Samples were amplified in triplicate, and a negative control without reverse transcriptase was included to verify the absence of contaminating genomic DNA. Data acquisition and analysis was performed using the CFX manager 3.1 software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Mucin isolation and purification

Mucin isolation of colon tissue samples was performed by isopycnic density gradient centrifugation as previously described (23), obtaining guanidinium hydrochloride (GuHCl) soluble and insoluble mucins. Briefly, frozen tissues were drenched with 10 mM sodium phosphate buffer, pH 6.5, containing 0.1 mM phenylmethylsulfonyl fluoride (AppliChem, Darmstadt, Germany). Once thawed, the mucosal surfaces were scraped with a microscope slide, dispersed with a Dounce homogenizer, and stirred slowly overnight at 4°C in ice-cold extraction buffer consisting of 6 M GuHCl (AppliChem, Darmstadt, Germany), 5 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA), 5 mM *N*-ethylmaleimide (Alfa Aesar, Karlsruhe, Germany) and 10 mM sodium di-hydrogen phosphate at pH 6.5. GuHCl soluble mucins were obtained after centrifugation at 23000 × *g* for 50 min at 4°C and the remaining material was re-extracted twice by stirring overnight at 4°C in extraction buffer. The remaining pellets contained the “insoluble” mucins, which were solubilized with 10 mM dithiothreitol (DTT) in reduction buffer (6 M GuHCl, 5 mM EDTA, 0.1 M Tris/HCl, pH 8) for 5 h at 37°C. Finally, residues were alkylated overnight with 25 mM iodoacetamide (IAA, Alfa Aesar, Karlsruhe, Germany).

Both the GuHCl soluble and insoluble material was dialyzed in ten volumes of extraction buffer at 4°C, changing the dialysis solution three times in 24 h. An isopycnic density-gradient centrifugation in cesium chloride (CsCl)/4 M GuHCl with a starting density of 1.39 g/ml was performed at 40000 rpm for 90 h. The mucin containing fractions were pooled and further purified from DNA by a second gradient in CsCl/0.5 M GuHCl. Approximately 25 mucin fractions were recovered per sample using a fraction collector equipped with a drop counter. Fractions were stored at 4°C until further analysis.

Analysis of mucin fractions

First and second CsCl gradient mucin fractions were analyzed as follows. The mucin density was determined by weighing a known volume using a Carlsberg pipette as a pycnometer, results were expressed as g/ml. DNA contamination of mucins were determined using a spectrophotometer. A microtiter-based assay detecting carbohydrates as periodate-oxidizable structures (24) was performed in order to determine the glycan content in the GuHCl soluble and insoluble mucin samples. Briefly, Nunc® 96-well plates (Thermo Scientific, Waltham, MA, USA) were coated overnight at 4°C with mucin fractions diluted in 4 M and 0.5 M GuHCl. Plates were incubated with a 25 mM sodium metaperiodate solution diluted in sodium acetate (NaAc) for 20 min, and blocked with 50 mM Tris-HCl, 0.15 M NaCl, 90 µM $CaCl_2$, 4 µM EDTA, 0.01% NaN_3 and 2 % bovine serum albumin, at pH 8 for 1 h. The wells were then incubated for 1 h with a biotin hydrazid solution diluted 1/50 in NaAc, followed by europium labeled streptavidin diluted 1/1000 in DELFIA® Assay buffer (PerkinElmer, Waltham, MA, USA). Finally, plates were incubated with DELFIA® enhancement solution for 5 min on a shaker. Between each step the plates were washed three times with a solution containing 5 mM Tris-HCl, 0.15 M NaCl, 0.005% Tween 20, and 0.01% NaN3, at pH 7.75, except for the final step where plates were washed six times. Signal was measured in a Wallac 1420 VICTOR^2 microplate reader (PerkinElmer, Waltham, MA, USA) by time-resolved fluorometry.

MUC5AC and MUC2 enzyme-linked immunosorbent assay (ELISA)

Mucin fractions were diluted in 0.5 M GuHCl and coated overnight at 4°C onto 96-well plates (Nunc®, Thermo Scientific). For MUC2 detection, samples were reduced with 80 µl of 2 mM DTT diluted in buffer (6 M GuHCl, 5 mM EDTA, 0.1 M Tris-HCl, pH 8.0), at 37°C for 1 h. On top of the previous solution 20 µl of 5 mM IAA was added and incubated for 1 h in the dark. Plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 1% blocking reagent for ELISA (Roche Diagnostics, Basel, Switzerland) containing 0.05% Tween 20 for 1 h, followed by incubation with the primary antibody anti-MUC5CR and anti-MUC2C3 (both kindly provided by G. Hansson, University of Gothenburg, Sweden) diluted 1/1000. Three more washes with PBS-T were performed before and after wells were incubated with a horse radish peroxidase (HRP) conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1/10000 for 1 h. Subsequently, 100 µl of tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO, USA) was added per well, and the reaction was stopped with an equivalent volume of 0.5 M H_2SO_4. Absorbance at 450 nm was measured in a Wallac 1420 VICTOR^2 plate reader. The 45M1 antibody (Sigma-Aldrich, St. Louis, MO, USA) was used to confirm the specificity of the MUC5AC signal from the isolated mucins obtained with the anti-MUC5CR antibody, verifying the signal obtained with the soluble, but not the insoluble mucins. As a result we performed a range of control analyses with and without reduction and alkylation of the mucin samples, concluding that the absence of MUC5AC signal in the insoluble mucins using the 45M1 antibody was due to the destruction of the epitope recognized by this antibody after reduction and alkylation. Thus, we are convinced the MUC5AC signal is specific, although the antibodies were designed to detect human mucins.

Bacterial strain and culture conditions

B. hyodysenteriae strain 8dII was cultured on tryptone soy agar (TSA, Thermo Fischer Scientific, Waltham, MA, USA) plates supplemented with 5% sheep blood (Thermo Fischer Scientific, Waltham, MA, USA), 0.1% yeast extract (Merck, Darmstadt, Germany), 400 µg/ml spectinomycin, 25 µg/ml colistin and 25 µg/ml vancomycin (AppliChem, Darmstadt, Germany), at 40°C under anaerobic conditions.

Mucin sample preparation and concentration estimation

Gradient fractions containing mucins were pooled together to obtain one sample for each gradient (*i.e.* two from each pig, insoluble and soluble). Mucin concentrations in pooled samples were determined by serial dilutions as well as a standard curve of a fusion protein of the mucin MUC1, 16TR and IgG2a Fc (25), starting at a concentration of 20 mg/ml and using seven 1/2 serial dilutions in a carbohydrate detection assay described above. The mucin concentrations were calculated from the standard curve. Setting the concentration based on the glycan content appears most appropriate as bacterial-mucin interactions largely occur via the mucin glycans (14). Although this is not an absolute measure of concentration it can be used to ensure that the mucins are at the same concentration for comparative assays. Mucin concentration can also be determined by freeze drying, however all mucins do not come into solution after freeze drying, therefore this method of concentration determination can contain large errors as well as remove mucin species selectively.

Binding of B. hyodysenteriae to pig mucins

White 96-well plates (Corning Life Sciences, NY, USA) were coated overnight at 4°C with 6 µg/ml mucins in 0.5 M GuHCl. Wells were washed three times with PBS-T and blocked with 200 µl of 5% fetal bovine serum (FBS) for 1 h. Bacteria were harvested from TSA plates (above), washed in PBS, centrifuged at 2500 × *g* for 5 min, and re-suspended in PBS with 5% FBS. One hundred microliters of a bacterial suspension diluted to10^8 bacterial cells/ml were added per well and the plates were shaken during incubation for 2 h at 40°C in an anaerobic environment. Plates were washed three times with PBS-T and once with PBS. Subsequently, 100 μl of PBS was added to each well, followed by the addition of an equal volume of BacTiter-Glo™ reagent (Promega, Madison, WI, USA). Incubation proceeded for 5 min at room temperature. Relative luminescence (RLU) was measured in an Infinite® M200 microplate reader (Tecan, Männedorf, Switzerland) with an integration time of 1000 ms per well. Controls included wells without the bacteria (PBS only) in mucin coated wells, and non-mucin coated wells incubated with the bacterial suspension followed by addition of PBS and reagent. In order to confirm that differences in *B. hyodysenteriae* binding to pig mucins were not due to variations of the mucin glycan content between samples, a glycan detection assay described above was simultaneously coated and performed with the binding experiments. Only samples with a glycan value of 17000-24000 Eu-counts were included to ensure that the analysis occurred within the linear range of the assay, and the *B. hyodysenteriae* binding signal was normalized against the glycan value for that particular coating and mucin. Results were obtained from three independent experiments with five technical replicates for each mucin, and were plotted as relative luminescence per glycan unit.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 software (La Jolla, CA, USA). Results are expressed as the mean ± SEM for normally distributed data, and median with interquartile range (IQR) for data that did not follow a normal distribution (determined using the D'Agostino-Pearson omnibus test). Data were analyzed using the Mann-Whitney, Kruskall-

Wallis or One-way ANOVA tests wherever applicable, and *p* values ≤ 0.05 were considered as statistically significant.

Results

Clinical signs, bacterial shedding and histology after experimental inoculation

Five inoculated pigs excreted *B. hyodysenteriae* in their feces and developed clinical signs of SD, including mucoid or hemorrhagic diarrhea. A milder case of diarrhea was observed in one of the five pigs with clinical signs of SD. The majority of the pigs had acute dysentery at the time of sacrifice (Table 1). Pig 4 had a longer duration of dysentery, however the clinical signs did not change during the 25 day period, and the macroscopic lesions corresponded with those of acute dysentery. Pig 3 had recovered from clinical signs before the day it was euthanized. Fecal shedding of *B. hyodysenteriae* by the inoculated pigs that developed SD started simultaneously with the onset of clinical signs and continued until the time of sacrifice. The control pigs did not excrete *B. hyodysenteriae* in their feces and did not present any clinical signs of SD at any time point during the experiment. In line with previous reports (20, 26), severe lesions were observed in the colon of pigs with acute dysentery, including necrotic colitis, hyperemic mucosa and fluid content with large amounts of mucus. Microscopically, the mucosa of pigs with SD had a thickened mucus layer (Figure 1), the epithelium contained abundant goblet cells, and the colonic crypts were elongated, dilated and filled with mucus and cell debris. Inflammatory cells were observed in the lamina propria, consisting of lymphocytes, plasma cells, and transmigrating neutrophils. The control group, as well as the inoculated pigs that did not develop SD, had no significant histopathological lesions.

Colonic mucus disorganization during swine dysentery is accompanied by de novo expression of MUC5AC and increased expression of MUC2

Fluorescence microscopy of the pig colon tissue revealed that the mucus layer in the healthy pig colon, including the inoculated pigs that did not develop SD, was organized in striations parallel to the mucosa, and consisted of mainly the MUC2 mucin (Figure 1, A-C), similar to the mucus structure reported for the mouse colon (2, 27). In contrast, during infection with *B. hyodysenteriae*, a massive increase in MUC2 and also *de novo* production of MUC5AC was observed in the four inoculated pigs with severe clinical signs of dysentery (Figure 1, D-F). MUC5AC expression was not observed by immunofluorescence in the inoculated pig with milder clinical signs of dysentery. Both MUC2 and MUC5AC mucins were produced by goblet cells, and when MUC5AC was present in a goblet cell, it was usually present in a cell that also produced MUC2 (Figure 1, D and E). In addition to the massive increase in mucus layer thickness that occurred in dysenteric pigs, the mucus organization was vastly changed by infection as the striated organization was lost and instead the mucus appeared to flow in “rivers” with eukaryotic cells in between, often at a 45° angle from the mucosa.

The antibodies used have previously been shown to detect their specific targets in humans and mice (27, 28), but no MUC2 or MUC5AC antibodies have been verified for use in pigs. To be certain the stain represents MUC5AC and MUC2, we confirmed that the antibodies we used for the immunofluorescence indeed bound to the isolated mucins in a specific manner that differed between the antibodies (Figure 2, A and B). The specificity of the MUC5AC antibody was further supported by the use of a second antibody, both MUC5AC antibodies followed a tissue distribution in the porcine stomach analogous to the distribution observed in the human and murine stomach. In addition, we designed qPCR primers specific for swine *MUC2* and

MUC5AC, and indeed, the mRNA levels of *MUC2* and *MUC5AC* increased four-fold and more than 15-fold, respectively, in the colon tissue of pigs with clinical signs of SD compared to the control pigs (Figure 3). The expression of MUC5AC was not upregulated in the colon tissue of the inoculated pig with milder clinical signs of dysentery. The mRNA levels of *MUC1*, a mucin induced during some bacterial infections in the mouse (3, 11) did not increase in the pigs with SD compared to the controls (Figure 3).

Swine dysentery is associated with a five-fold mucin increase

Mucins from *B. hyodysenteriae* inoculated and control pigs were isolated from the colonic mucus and analyzed in order to determine changes in their composition during infection. Mucins were extracted as previously described (23) and GuHCl soluble and insoluble mucins were obtained. Although insoluble mucins were ultimately solubilized by reduction in DTT they will be referred to from here on as "insoluble". During isopychnic density gradient centrifugation, molecules concentrate as bands where the molecule density matches the density of the surrounding solution. As mucins are highly glycosylated, and sugars have a high density, density gradient centrifugation separates mucins from the less glycosylated non-mucin molecules. The initial CsCl/4M GuHCl isopycnic density gradient procedure rendered the isolated mucins free of non-mucin proteins however they were contaminated with DNA (Figure 4A). Therefore a second CsCl/0.5 M GuHCl gradient was performed in all the samples ensuring removal of DNA contamination (Figure 4B). The median mucin density of the inoculated pigs was 1.527 g/ml (IQR = 0.015). No differences in mucin density were noted between inoculated and control pigs ($p > 0.05$, Figure 4D). Quantification of mucins based on their carbohydrate content revealed that the pig colon mucins of both *B. hyodysenteriae* inoculated and control pigs were mainly insoluble, with less than 20% of the mucins being soluble in GuHCl (Figure 4C). Pigs with clinical signs of SD had a five-fold higher mucin content ($p < 0.05$) compared to the controls (Figure 4C). The amount of mucins isolated from control pigs was similar to the mucin content of the inoculated pigs that did not develop SD ($p > 0.9$, Figure 4C).

In most density gradient samples, MUC5AC and MUC2 antibody reactivity coincided with the glycan peak (Figure 2A), although in one sample there were differences in the MUC2 and MUC5AC curves, demonstrating that the antibodies indeed recognized different mucins (Figure 2B). MUC5AC was present in the GuHCl soluble and insoluble material in similar proportions (45% and 55%, respectively, Figure 2C). Mucins from the pigs with SD contained more MUC5AC compared to the controls and to the inoculated pigs that did not develop SD ($p < 0.05$, Figure 2C). In line with the immunofluorescence and qPCR results, the pig with mild clinical signs of SD had the lowest level of MUC5AC antibody reactivity. Both GuHCl soluble and insoluble mucins contained MUC2, with the majority (80-90%) of MUC2 present as insoluble mucin. In line with the immunofluorescence and qPCR results, the MUC2 protein level was also increased in pigs with SD compared to the controls, as well as to the inoculated pigs that did not develop SD ($p < 0.05$, Figure 2D).

Increased binding ability of B. hyodysenteriae to colonic mucins from pigs with clinical signs of swine dysentery

B. hyodysenteriae bound to colonic mucins isolated from both control and inoculated pigs. The *B. hyodysenteriae* binding pattern to mucins differed between individual pigs (insoluble mucins, overall $p < 0.0001$; soluble mucins, overall $p < 0.0001$; Figure 5A). This suggests that *B. hyodysenteriae* has an adhesin that recognizes specific glycan structure(s), as bacterial adhesins usually recognize these, and the mucin glycans differ between individuals (24). *B.*

hyodysenteriae binding per mucin glycan unit was higher to soluble mucins from pigs with clinical signs of SD compared to controls ($p < 0.0001$), and a similar trend was observed for the insoluble mucins ($p = 0.0595$, Figure 5B). Although *B. hyodysenteriae* bound more to the soluble mucins isolated from pig 3 compared to the other pigs with clinical signs of SD (Figure 5A), the overall binding difference between control and inoculated pig mucins remained statistically significant ($p = 0.0002$) even after excluding pig 3 data. When taking into account the higher total mucin content isolated from pigs with clinical signs of SD than healthy pigs, the total binding ability of *B. hyodysenteriae* to mucins from pigs with clinical signs of SD increased seven-fold ($p < 0.005$, Figure 5C).

Discussion

The present study provides new insights into the composition of pig colonic mucins during health and disease as well as mucin interactions with *B. hyodysenteriae*. This was accomplished through validation, optimization and generation of methods and tools that now can be specifically applied to the swine host. We demonstrated changes in the mucus environment of the swine colon during infection with *B. hyodysenteriae*, evidenced by disorganized mucus, a much thicker mucus layer as well as five-fold higher mucin content, accompanied by *de novo* MUC5AC synthesis. We identified that *B. hyodysenteriae* bound to swine colonic mucins in a manner that differed between individuals and mucin populations, and increased with infection. As a result of these changes, the altered mucin environment provided more bacterial binding sites, increasing the overall binding ability of *B. hyodysenteriae* to colonic mucus seven-fold.

Successful isolation of mucins involves the removal of low density non-mucin proteins as well as DNA contaminants. Pure colonic mucins, soluble and insoluble in GuHCl, were obtained by two isopycnic density gradient centrifugation steps in CsCl with different GuHCl molarities, as previously described (29). As in human colonic mucus (30), we reported in the pig a higher content of insoluble mucins compared to mucins soluble in guanidinium. Mucins are large molecules that form complex networks by connecting the mucin subunits via disulphide bonds. It has been suggested that the higher content of insoluble mucins in the colon denotes the presence of more covalent bonds, needing to be further solubilized by reduction (30). The density we observed for pig colonic mucins was higher than the density (1.38 g/ml) previously reported for human colonic mucins (30). Since human and pig MUC2 are highly homologous and the glycosylation has a similar monosaccharide composition, differences in density are likely to mainly reflect differences in the extent of glycosylation. Thus, pig colonic mucins appeared to be more heavily glycosylated than the human counterparts. The main carbohydrates that compose glycoproteins both in human and pigs are glucosamine, galactosamine, galactose, fucose and sialic acid (30-32).

Besides lubricating the intestinal surface for the transit of the fecal bolus, goblet-cell secreted mucus protects the surface epithelium from bacterial invasion. The mucus layer of healthy pigs was constituted mainly of MUC2 mucin, organized in a striated fashion perpendicular to the mucosal surface, similar to the mucus composition of the mouse colon (6). During *B. hyodysenteriae* infection, we found a loss of the striated organization, and a substantial increase in MUC2 and *de novo* secretion of MUC5AC mucins. We recently reported dynamic changes in the mucus barrier during *Citrobacter rodentium* infection in mice, with structural loss and decrease of the inner mucus layer at the onset and mid time points of infection (27). However, during the clearance phase, the mucus layer thickness increased, but had a similar organization as in uninfected mice, and no Muc5ac was detected (27). The changes observed in *B.*

hyodysenteriae infected pigs are thus completely different to any of the mucus changes identified during *C. rodentium* infection and clearance.

MUC2 and MUC5AC are both gel-forming mucins secreted by goblet cells. The MUC2 mucin is predominantly secreted in the intestine. There is evidence from Muc2 knock out mice that the lack of Muc2 increases the susceptibility to *Salmonella* and *C. rodentium* infections (33, 34). Unlike MUC2, MUC5AC does not form part of the normal mucin repertoire in the colon. Instead, it is commonly found in the normal gastric mucosa (5), airway epithelium (35) and conjunctiva (36). *MUC2* and *MUC5AC* mRNA levels were increased in the colon tissue of pigs with clinical signs of swine dysentery, compared to the control pigs, demonstrating that the mucus change is regulated at the transcriptional level, in contrast to increases in mucus thickness seen in *C. rodentium* infection without changes in mRNA levels (27). Additionally, the fact that *MUC1* expression was not increased in the inoculated pigs compared to the controls further supports the conclusion that *B. hyodysenteriae* infection has a different effect on mucin regulation compared to the *C. rodentium* model where Muc1 is increased (27). Similar to our results, expression of MUC5AC and MUC2 has been described in rabbit ileal loops inoculated with *Shigella flexneri* and *Shigella dysenteriae* (37). In addition, Muc5ac expression is increased in mice infected with the intestinal nematode *Trichuris muris* (38). In pigs, immunohistochemical staining with an antibody against human MUC5AC has indicated that this mucin is increased during infection with *Salmonella* Typhimurium (39), while during infection with *Trichuris suis MUC5AC* mRNA levels were elevated (40). During nematode infection, Muc5ac induction has a protective role in mice, decreasing nematode burden and viability (41). Moreover, Muc5ac deficiency hampers the clearance of the parasite, increasing the susceptibility to chronic infection (41). An altered mucin expression in the colon of pigs with SD was first reported by Wilberts *et al*., after immunohistochemical staining with an antibody against human MUC5AC indicated its presence in pigs with acute dysentery following inoculation with *B. hyodysenteriae* or "*B. hampsonii*" (20), suggesting a common mucin response in the colon during infection with these pathogens. Our results suggest that during infection, *de novo* secretion of MUC5AC in the colon could depend on the stage of the disease, as it was not detected in the pig sacrificed one day after the onset of clinical signs, presenting only mild diarrhea. Furthermore, the similar mucin profile between the inoculated pigs that did not develop SD and the control pigs suggests that *de novo* secretion of MUC5AC in the colon depends on the ability of the bacterium to colonize the host. Further experiments are required to determine whether the *de novo* MUC5AC secretion plays a protective role during *B. hyodysenteriae* infection in the pig.

Successful colonization of the host by enteric pathogens involves penetration of the mucus layer overlying the epithelium. Genomic evidence shows that *B. hyodysenteriae* carries genes associated with potential virulence factors involved in motility, chemotaxis, and tissue injury by proteases and hemolysins, that if expressed could facilitate colonization of the colon (42). Thus far, the importance of motility and chemotaxis in *B. hyodysenteriae* colonization has not been thoroughly demonstrated. A strong chemotactic response to pig mucins and components like fucose and serine has been described (43, 44), although a decreased attraction to mucins at concentrations greater than 6 % has also been reported (45). Colonization of the gastrointestinal tract can also be mediated by bacterial adhesion to carbohydrate structures such as blood group antigens that act as receptors. For example, *H. pylori* strains that express the BabA adhesin bind to the Lewis b blood group antigen expressed in the human gastric mucosa, resulting in a blood group and strain dependent binding (46), and the FedF adhesin expressed in F18 fimbriated *E. coli* binds to glycosphingolipids isolated from intestinal epithelium of blood group A and O pigs

(47). In the pig intestine AO blood group antigens are expressed, with a predominance of blood group A (48), and thus individual pigs carry different glycan structures in their intestines. Here we showed that *B. hyodysenteriae* bound to mucins from all pigs in the study, but that the level of binding per mucin glycan unit differed between the mucin populations and with disease status. In light of that other infections have previously shown to induce changes in mucin glycosylation (49), it is likely that the differences in *B. hyodysenteriae* binding reflect differences in the pig/mucin glycan repertoire rather than differences in the mucin density or extent of glycosylation.

Mucins from pigs with clinical signs of SD bound more *B. hyodysenteriae* compared to mucins from the control pigs. Potentially, during infection the mucin secretion provides distinct carbohydrate structures for *B. hyodysenteriae* binding. The mucous niche is very unstable, and pathogen binding to mucins may prevent the more intimate adherence that can occur between the pathogen and, for example, glycolipids of the cell membrane. Indeed, mucin binding to the human gastric pathogen *H. pylori* acts as a decoy and prevents prolonged adherence (13). Furthermore, in the rhesus monkey model of *H. pylori* infection, animals with mucins that bind *H. pylori* more effectively have a lower *H. pylori* density in their stomachs, indicating that mucin binding to *H. pylori* aids in removing the bacteria from the gastric niche (50). However, it is not certain if these principles apply to *B. hyodysenteriae*; the massively thick disorganized mucus layer may not be as unstable as a normal mucus layer, and there is a possibility that the protective function of the mucus changes under these conditions. *B. hyodysenteriae* may indeed induce these mucus changes to create a more favorable niche instead.

In conclusion, *B. hyodysenteriae* bound to mucins from all pigs, in a manner that differed between the mucin populations and increased with SD. Together with the massive mucus induction and disorganization that occurred during infection, this demonstrates major changes in the colon mucus niche during *B. hyodysenteriae* infection.

Acknowledgments

This work was supported by the Swedish research council Formas (221-2011-1036 and 221-2013-590), the Swedish Research Council (no 521-2011-2370 and 522-2007-5624**)** The Swedish Cancer Foundation, Ragnar Söderbergs Stiftelser, the Jeansson Foundation and the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT Vlaanderen), Brussels, Belgium (grant IWT LO 100850). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. **McGuckin MA, Linden SK, Sutton P, Florin TH.** 2011. Mucin dynamics and enteric pathogens. Nature reviews. Microbiology **9:**265-278.
2. **Johansson ME, Sjovall H, Hansson GC.** 2013. The gastrointestinal mucus system in health and disease. Nature reviews. Gastroenterology & hepatology **10:**352-361.
3. **McAuley JL, Linden SK, Png CW, King RM, Pennington HL, Gendler SJ, Florin TH, Hill GR, Korolik V, McGuckin MA.** 2007. MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. The Journal of clinical investigation **117:**2313-2324.
4. **Singh PK, Hollingsworth MA.** 2006. Cell surface-associated mucins in signal transduction. Trends in cell biology **16:**467-476.

5. **Nordman H, Davies JR, Lindell G, de Bolos C, Real F, Carlstedt I.** 2002. Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution. The Biochemical journal **364:**191-200.

6. **Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC.** 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A **105:**15064-15069.

7. **Atuma C, Strugala V, Allen A, Holm L.** 2001. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. American journal of physiology. Gastrointestinal and liver physiology **280:**G922-929.

8. **Linden SK, Florin TH, McGuckin MA.** 2008. Mucin dynamics in intestinal bacterial infection. PloS one **3:**e3952.

9. **Boltin D, Perets TT, Vilkin A, Niv Y.** 2013. Mucin function in inflammatory bowel disease: an update. Journal of clinical gastroenterology **47:**106-111.

10. **Sando L, Pearson R, Gray C, Parker P, Hawken R, Thomson PC, Meadows JR, Kongsuwan K, Smith S, Tellam RL.** 2009. Bovine Muc1 is a highly polymorphic gene encoding an extensively glycosylated mucin that binds bacteria. Journal of dairy science **92:**5276-5291.

11. **McGuckin MA, Every AL, Skene CD, Linden SK, Chionh YT, Swierczak A, McAuley J, Harbour S, Kaparakis M, Ferrero R, Sutton P.** 2007. Muc1 mucin limits both *Helicobacter pylori* colonization of the murine gastric mucosa and associated gastritis. Gastroenterology **133:**1210-1218.

12. **Parker P, Sando L, Pearson R, Kongsuwan K, Tellam RL, Smith S.** 2010. Bovine Muc1 inhibits binding of enteric bacteria to Caco-2 cells. Glycoconjugate journal **27:**89-97.

13. **Linden SK, Sheng YH, Every AL, Miles KM, Skoog EC, Florin TH, Sutton P, McGuckin MA.** 2009. MUC1 limits *Helicobacter pylori* infection both by steric hindrance and by acting as a releasable decoy. PLoS pathogens **5:**e1000617.

14. **Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA.** 2008. Mucins in the mucosal barrier to infection. Mucosal immunology **1:**183-197.

15. **Songer JG, Harris DL.** 1978. Transmission of swine dysentery by carrier pigs. American journal of veterinary research **39:**913-916.

16. **Hidalgo A, Carvajal A, Vester B, Pringle M, Naharro G, Rubio P.** 2011. Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of *Brachyspira hyodysenteriae* in Spain. Antimicrobial agents and chemotherapy **55:**3330-3337.

17. **Sperling D, Smola J, Cizek A.** 2011. Characterisation of multiresistant *Brachyspira hyodysenteriae* isolates from Czech pig farms. The Veterinary record **168:**215.

18. **Rohde J, Kessler M, Baums CG, Amtsberg G.** 2004. Comparison of methods for antimicrobial susceptibility testing and MIC values for pleuromutilin drugs for *Brachyspira hyodysenteriae* isolated in Germany. Veterinary microbiology **102:**25-32.

19. **Alvarez-Ordonez A, Martinez-Lobo FJ, Arguello H, Carvajal A, Rubio P.** 2013. Swine dysentery: aetiology, pathogenicity, determinants of transmission and the fight against the disease. International journal of environmental research and public health **10:**1927-1947.

20. **Wilberts BL, Arruda PH, Kinyon JM, Madson DM, Frana TS, Burrough ER.** 2014. Comparison of lesion severity, distribution, and colonic mucin expression in pigs with acute swine dysentery following oral inoculation with "*Brachyspira hampsonii*" or *Brachyspira hyodysenteriae*. Veterinary pathology.

21. **Song Y, Hampson DJ.** 2009. Development of a multiplex qPCR for detection and quantitation of pathogenic intestinal spirochaetes in the faeces of pigs and chickens. Veterinary microbiology **137:**129-136.

22. **Nygard AB, Jorgensen CB, Cirera S, Fredholm M.** 2007. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC molecular biology **8:**67.

23. **Nordman H, Davies JR, Herrmann A, Karlsson NG, Hansson GC, Carlstedt I.** 1997. Mucus glycoproteins from pig gastric mucosa: identification ofdifferent mucin populations from the surface epithelium. The Biochemical journal **326 (Pt 3):**903-910.

24. **Skoog EC, Sjoling A, Navabi N, Holgersson J, Lundin SB, Linden SK.** 2012. Human gastric mucins differently regulate *Helicobacter pylori* proliferation, gene expression and interactions with host cells. PloS one **7:**e36378.

25. **Backstrom M, Link T, Olson FJ, Karlsson H, Graham R, Picco G, Burchell J, Taylor-Papadimitriou J, Noll T, Hansson GC.** 2003. Recombinant MUC1 mucin with a breast cancer-like O-glycosylation produced in large amounts in Chinese-hamster ovary cells. The Biochemical journal **376:**677-686.

26. **Jacobson M, Fellstrom C, Lindberg R, Wallgren P, Jensen-Waern M.** 2004. Experimental swine dysentery: comparison between infection models. Journal of medical microbiology **53:**273-280.

27. **Gustafsson JK, Navabi N, Rodriguez-Pineiro AM, Alomran AH, Premaratne P, Fernandez HR, Banerjee D, Sjovall H, Hansson GC, Linden SK.** 2013. Dynamic Changes in Mucus Thickness and Ion Secretion during *Citrobacter rodentium* Infection and Clearance. PloS one **8:**e84430.

28. **Navabi N, McGuckin MA, Linden SK.** 2013. Gastrointestinal cell lines form polarized epithelia with an adherent mucus layer when cultured in semi-wet interfaces with mechanical stimulation. PloS one **8:**e68761.

29. **Fogg FJ, Hutton DA, Jumel K, Pearson JP, Harding SE, Allen A.** 1996. Characterization of pig colonic mucins. The Biochemical journal **316 (Pt 3):**937-942.

30. **Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, Carlstedt I.** 1999. Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. The Journal of biological chemistry **274:**15828-15836.

31. **Marshall T, Allen A.** 1978. The isolation and characterization of the high-molecular-weight glycoprotein from pig colonic mucus. The Biochemical journal **173:**569-578.

32. **Podolsky DK, Isselbacher KJ.** 1983. Composition of human colonic mucin. Selective alteration in inflammatory bowel disease. The Journal of clinical investigation **72:**142-153.

33. **Zarepour M, Bhullar K, Montero M, Ma C, Huang T, Velcich A, Xia L, Vallance BA.** 2013. The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during *Salmonella enterica* serovar Typhimurium colitis. Infection and immunity **81:**3672-3683.

34. **Bergstrom KS, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, Ryz N, Huang T, Velcich A, Finlay BB, Chadee K, Vallance BA.** 2010. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS pathogens **6:**e1000902.

35. **Hovenberg HW, Davies JR, Herrmann A, Linden CJ, Carlstedt I.** 1996. MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. Glycoconjugate journal **13:**839-847.

36. **Inatomi T, Spurr-Michaud S, Tisdale AS, Zhan Q, Feldman ST, Gipson IK.** 1996. Expression of secretory mucin genes by human conjunctival epithelia. Investigative ophthalmology & visual science **37:**1684-1692.

37. **Radhakrishnan P, Halagowder D, Devaraj SN.** 2007. Altered expression of MUC2 and MUC5AC in response to Shigella infection, an in vivo study. Biochimica et biophysica acta **1770:**884-889.

38. **Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI.** 2010. Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. Gastroenterology **138:**1763-1771.

39. **Kim CH, Kim D, Ha Y, Cho KD, Lee BH, Seo IW, Kim SH, Chae C.** 2009. Expression of mucins and trefoil factor family protein-1 in the colon of pigs naturally infected with *Salmonella* typhimurium. Journal of comparative pathology **140:**38-42.

40. **Kringel H, Iburg T, Dawson H, Aasted B, Roepstorff A.** 2006. A time course study of immunological responses in Trichuris suis infected pigs demonstrates induction of a local type 2 response associated with worm burden. International journal for parasitology **36:**915-924.

41. **Hasnain SZ, Evans CM, Roy M, Gallagher AL, Kindrachuk KN, Barron L, Dickey BF, Wilson MS, Wynn TA, Grencis RK, Thornton DJ.** 2011. Muc5ac: a critical component mediating the rejection of enteric nematodes. The Journal of experimental medicine **208:**893-900.

42. **Bellgard MI, Wanchanthuek P, La T, Ryan K, Moolhuijzen P, Albertyn Z, Shaban B, Motro Y, Dunn DS, Schibeci D, Hunter A, Barrero R, Phillips ND, Hampson DJ.** 2009. Genome sequence of the pathogenic intestinal spirochete *Brachyspira hyodysenteriae* reveals adaptations to its lifestyle in the porcine large intestine. PloS one **4:**e4641.

43. **Kennedy MJ, Yancey RJ, Jr.** 1996. Motility and chemotaxis in *Serpulina hyodysenteriae*. Veterinary microbiology **49:**21-30.

44. **Milner JA, Sellwood R.** 1994. Chemotactic response to mucin by *Serpulina hyodysenteriae* and other porcine spirochetes: potential role in intestinal colonization. Infection and immunity **62:**4095-4099.

45. **Naresh R, Hampson DJ.** 2010. Attraction of *Brachyspira pilosicoli* to mucin. Microbiology **156:**191-197.

46. **Linden S, Nordman H, Hedenbro J, Hurtig M, Boren T, Carlstedt I.** 2002. Strain- and blood group-dependent binding of *Helicobacter pylori* to human gastric MUC5AC glycoforms. Gastroenterology **123:**1923-1930.

47. **Coddens A, Diswall M, Angstrom J, Breimer ME, Goddeeris B, Cox E, Teneberg S.** 2009. Recognition of blood group ABH type 1 determinants by the FedF adhesin of F18-fimbriated *Escherichia coli*. The Journal of biological chemistry **284:**9713-9726.

48. **King TP, Kelly D.** 1991. Ontogenic expression of histo-blood group antigens in the intestines of suckling pigs: lectin histochemical and immunohistochemical analysis. The Histochemical journal **23:**43-54.

49. **Linden S, Mahdavi J, Semino-Mora C, Olsen C, Carlstedt I, Boren T, Dubois A.** 2008. Role of ABO secretor status in mucosal innate immunity and *H. pylori* infection. PLoS pathogens **4:**e2.

50. **Linden S, Mahdavi J, Semino-Mora C, Olsen C, Carlstedt I, Boren T, Dubois A.** 2008. Role of ABO secretor status in mucosal innate immunity and *H. pylori* infection. Plos Pathog **4:**e2.

Table 1. Experimental design and data of *B. hyodysenteriae* inoculated and control pigs

Pig ID (Exp. N°)[#]	**Treatment group**	**Samples analyzed[†]**	**Start of clinical signs (dpi)**	**Days from start of clinical signs until necropsy**	**Clinical signs at day of necropsy**	***Bh* in feces the day of necropsy**	**Macroscopic signs of SD at necropsy**	**Histological signs of SD at necropsy**
1 (2)	Inoculated with *Bh*	Yes	39	1	Yes	Yes	Yes	Yes
2 (2)			29	11	Yes			
3 (1)			12	28	No*			
4 (1)			15	25	Yes			
5 (2)			29	11	Yes			
6 (1)			No SD	N/A	No	No	No	No
7 (1)								
8 (1)								
9 (1)								
10-14 (1-2)		No						
A-F (1-2)	Controls	Yes	N/A					
G-N (1-2)		No						

Exp. N° states whether the pigs belonged to the 1st or 2nd infection trial; *Bh* = *Brachyspira hyodysenteriae*; dpi = days post inoculation; SD = swine dysentery, N/A = not applicable; † Inoculated pigs without clinical signs of SD and control pigs were randomly selected to match the number of pigs with clinical signs of SD; * Pig 3 presented clinical signs of mucoid hemorrhagic diarrhea before sacrifice, and severe necrotic lesions in the colon at necropsy.

Table 2. List of primers used in qPCR

Target	Direction	Sequence (5’- 3’)	Reference
MUC1	Forward	TCCGACCCGGGATGCCTACCA	This study
	Reverse	GGCTGCCCCCACCGTTGCCT	This study
MUC2	Forward	CCTTGCTCTCGTGTGGAACA	This study
	Reverse	ACTTCTCCTCGGGCTTGTTG	This study
MUC5AC	Forward	TGCGCCGTGCCACGCGGAGAT	This study
	Reverse	GCGGGGCAGGGGAAGGGGCA	This study
ACTB	Forward	CACGCCATCCTGCGTCTGGA	(22)
	Reverse	AGCACCGTGTTGGCGTAGAG	(22)
RPL4	Forward	CAAGAGTAACTACAACCTTC	(22)
	Reverse	GAACTCTACGATGAATCTTC	(22)

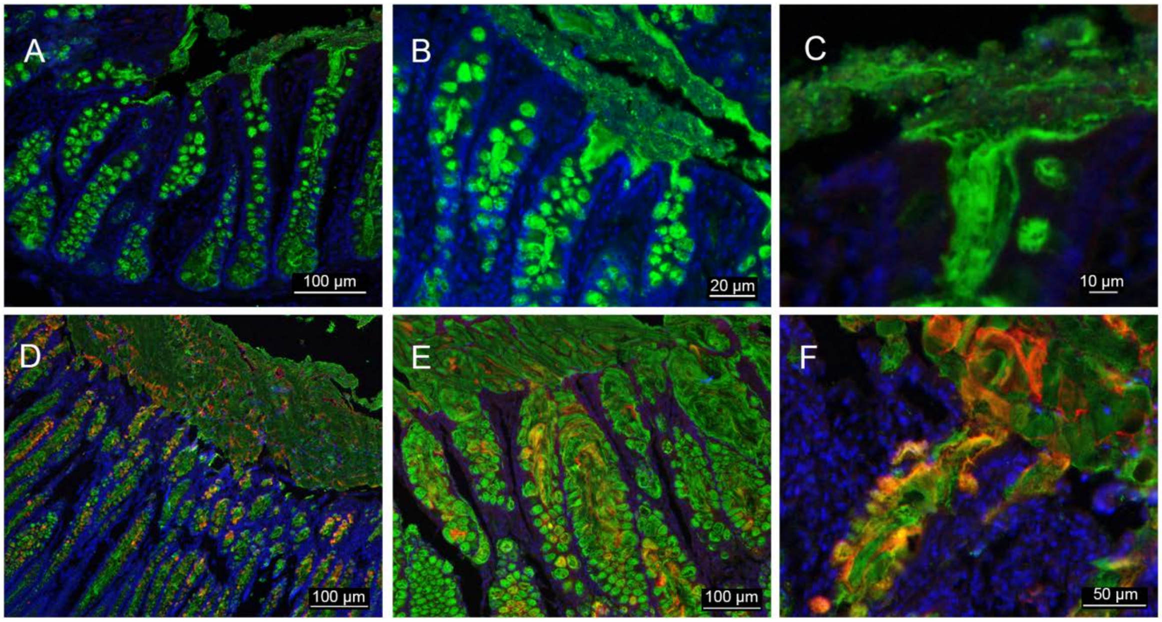

Figure 1. Colon tissue sections from control and *B. hyodysenteriae* inoculated pigs stained for MUC2 and MUC5AC. Immunofluorescence of MUC2 (green) and MUC5AC (red) in colon tissue counterstained with DAPI (blue). Panels A-C show the striated organization of the mucus in the colon of control pigs along with expression of MUC2. In contrast, panels D-F show a

disorganized mucus barrier with *de novo* expression of MUC5AC and increased expression of MUC2 in the colon of pigs with clinical signs of SD.

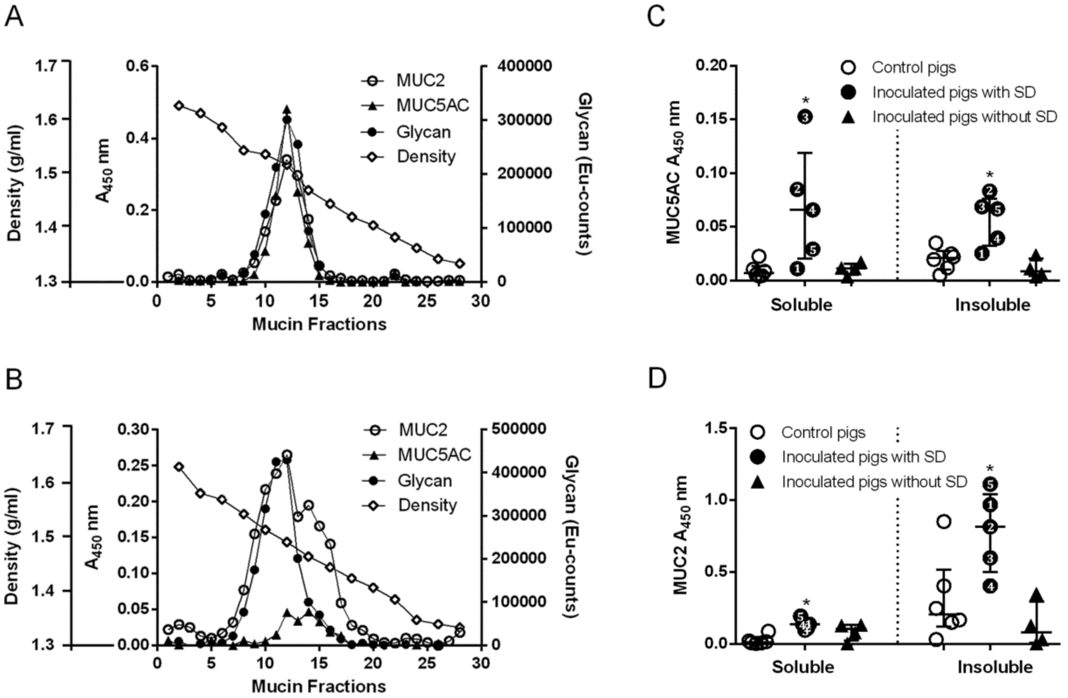

Figure 2. MUC5AC and MUC2 content in the colon of *B. hyodysenteriae* inoculated pigs. (A) The peak of antibody reactivity against MUC5AC and MUC2 coincided with the glycan detection peak in GuHCl soluble mucin fractions isolated from a *B. hyodysenteriae* inoculated pig with clinical signs of SD. (B) The mucin population of one pig was more heterogeneous with distinctly different mucin peaks, demonstrating that the antibodies against MUC2 and MUC5AC recognize different mucins. (C) MUC5AC and (D) MUC2 antibody reactivity against GuHCl soluble and insoluble mucins isolated from *B. hyodysenteriae* inoculated pigs that developed SD (❶ pig 1, ❷ pig 2, ❸ pig 3, ❹ pig 4, and ❺ pig 5), inoculated pigs that did not develop SD, and control pigs. Results are expressed as the median with interquartile range. Kruskall-Wallis test with Dunn's correction for multiple comparisons, * $p < 0.05$.

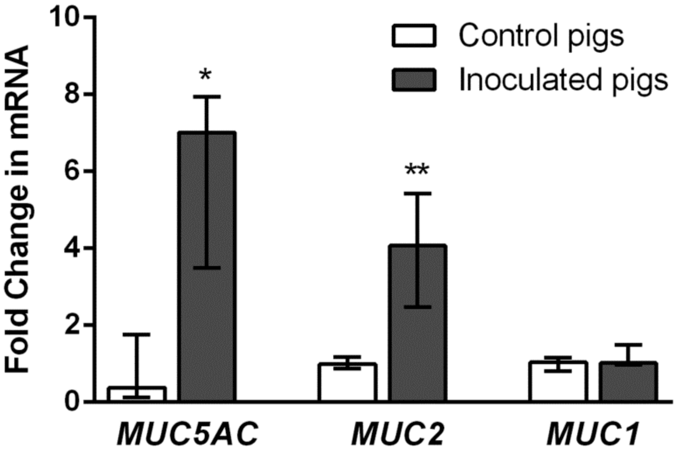

Figure 3. *MUC5AC, MUC2* and *MUC1* mRNA expression of *B. hyodysenteriae* inoculated and control pigs. Normalized fold expression of *MUC5AC*, *MUC2,* and *MUC1* mRNA levels in the colon tissue of *B. hyodysenteriae* inoculated pigs with clinical signs of SD and controls by qPCR analysis. Expression data were normalized against *ACTB* and *RPL4* reference genes. Fold changes were calculated using ΔΔCT. Results are expressed as the median with interquartile range. Mann-Whitney test, * $p < 0.05$ and ** $p < 0.005$.

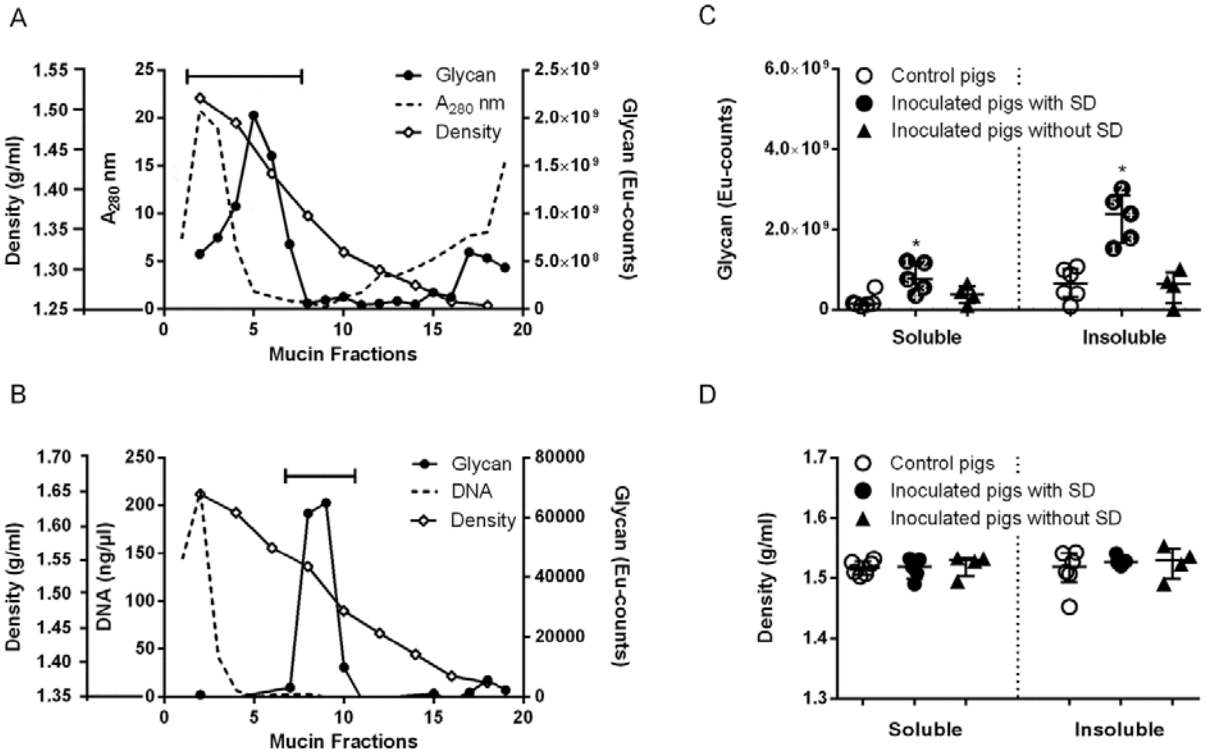

Figure 4. Isolation, density and glycan content of colonic mucins from *B. hyodysenteriae* inoculated and control pigs. (A) Mucin fractions were recovered from the density gradients and analyzed for their glycan content. Here, a representative sample of soluble mucins isolated from a *B. hyodysenteriae* inoculated pig with clinical signs of SD after the first gradient in CsCl/4 M GuHCl (starting density of 1.39 g/ml), shows that low density non-mucin proteins (A_{280} nm) are excluded from the pooled mucin fractions. Bar: pooled mucin fractions 2-7. (B) Representative sample of soluble mucins isolated from a control pig, shows baseline separation between the glycan peak and DNA after a second gradient in CsCl/0.5 M GuHCl (starting density 1.5 g/ ml). Bar: pooled mucin fractions 1-2. (C) Glycan content of GuHCl soluble and insoluble mucins isolated from inoculated pigs with clinical signs of SD (❶ pig 1, ❷ pig 2, ❸ pig 3, ❹ pig 4, and ❺ pig 5), inoculated pigs that did not develop SD, and control pigs. The mucin content in the colon was five-fold higher in inoculated pigs with clinical signs of SD compared to the controls.

(D) Density (g/ml) of GuHCl soluble and insoluble mucins isolated from control and *B. hyodysenteriae* inoculated pigs (with and without clinical signs of SD) showing no differences between the groups. Results are expressed as the median with interquartile range. Kruskall-Wallis test with Dunn's correction for multiple comparisons, * $p < 0.05$.

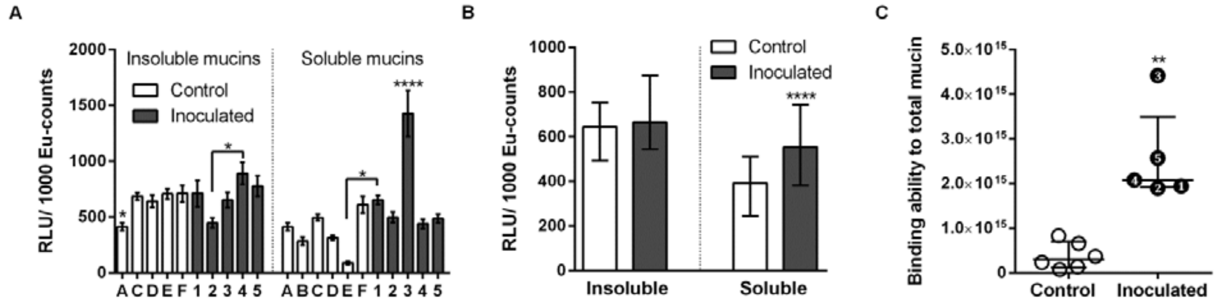

Figure 5. Binding of *B. hyodysenteriae* to colonic mucins. (A) Binding pattern of *B. hyodysenteriae* to soluble and insoluble mucins isolated from control (pigs A-F) and inoculated pigs with clinical signs of SD (1-5). Results are expressed as the mean ± SEM of technical replicates. One-way ANOVA, with Tukey's correction for multiple comparisons, * $p < 0.05$ and **** $p < 0.0001$. (B) *B. hyodysenteriae* binding to soluble and insoluble mucins isolated from control and pigs with SD showing higher binding to the soluble mucins isolated from pigs with clinical signs of SD compared to the control group. Results are expressed as the median with interquartile range. Mann-Whitney test, **** $p < 0.0001$. (C) Binding ability of *B. hyodysenteriae* to the total mucin content observed in pigs with SD (❶ pig 1, ❷ pig 2, ❸ pig 3, ❹ pig 4, and ❺ pig 5) and control pigs (*i.e* binding to mucin at a set concentration × the total amount of mucin recovered from that sample). Results are expressed as the median with interquartile range. Mann-Whitney test, ** $p < 0.005$. Data shown are representative of three independent experiments.