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BACTERIAL INFECTIONS

Role of Sialic Acid in *Brachyspira hyodysenteriae* Adhesion to Pig Colonic Mucins

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ABSTRACT Infection with *Brachyspira hyodysenteriae* results in mucoid hemorrhagic diarrhea. This pathogen is associated with the colonic mucus layer, mainly composed of mucins. Infection regulates mucin *O*-glycosylation in the colon and increases mucin secretion as well as *B. hyodysenteriae* binding sites on mucins. Here, we analyzed potential mucin epitopes for *B. hyodysenteriae* adhesion in the colon, as well as the effect of colonic mucins on bacterial growth. Associations between *B. hyodysenteriae* binding to pig colonic mucins and mucin glycan data showed that *B. hyodysenteriae* binding was associated with the presence of *N*-glycolylneuraminic acid (NeuGc) on mucins. The role of sialic acid in *B. hyodysenteriae* adhesion was analyzed after the removal of sialic acid residues on the mucins by enzymatic treatment with sialidase A, which decreased bacterial binding to the mucins. The effect of pig colonic mucins on *B. hyodysenteriae* growth was determined in carbohydrate-free medium. *B. hyodysenteriae* growth increased in the presence of mucins from two out of five infected pigs, suggesting utilization of mucins as a carbon source for growth. Additionally, bacterial growth was enhanced by free sialic acid and *N*-acetylglucosamine. The results highlight a role of sialic acid as an adhesion epitope for *B. hyodysenteriae* interaction with colonic mucins. Furthermore, the mucin response and glycosylation changes exerted in the colon during *B. hyodysenteriae* infection result in a potentially favorable environment for pathogen growth in the intestinal mucus layer.

KEYWORDS *Brachyspira hyodysenteriae*, adhesion, colon, mucins, pig, sialic acid

The mucosal surface lining the gastrointestinal tract is covered by a mucus layer that protects the epithelial surface and acts as a barrier against pathogens (1). This mucus layer is mainly composed of heavily glycosylated gel-forming mucins secreted by goblet cells (1). Similar to the colonic mucus layer in mice and humans, the colonic mucus layer in healthy pigs is organized in striations parallel to the mucosal surface and is mainly composed of MUC2 mucin (2–5). Mucins carry an array of carbohydrate structures that vary between species and individuals, in tissue distribution, and in response to bacterial infection (1, 6–13). The mucin glycan structures are also available for host-pathogen interactions, such as bacterial attachment via adhesins, limiting colonization and access to the epithelial surface (14–18). Mucin glycosylation changes due to bacterial infections can affect pathogen attachment (19–23). Furthermore, pig intestinal bacteria can degrade mucins and utilize mucin carbohydrates as an energy source for growth (13, 24–26).

Brachyspira hyodysenteriae is a causative agent associated with swine dysentery (SD), a disease characterized by mucohemorrhagic colitis. SD results in decreased performance parameters, such as reduced feed conversion and weight gain, accompanied by 30% mortality and 90% morbidity in weaned pigs (27, 28). The emergence of antimicrobial-resistant *B. hyodysenteriae* strains (29–32) suggests the need for alternative strategies to treat infections. Colonization of the colon with

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the pathogen *B. hyodysenteriae* profoundly alters the mucus layer organization and mucin composition in the colon with the loss of the striated mucus organization and aberrant mucin production, characterized by the increased expression of MUC2 and the *de novo* expression of MUC5AC (2, 33). This mucin increase is accompanied by an increase in the ability of *B. hyodysenteriae* to bind to mucins (2). The host's colonic mucosal immune response to *B. hyodysenteriae* infection is involved in the regulatory networks determining mucin expression. Neutrophil elastase and interleukin-17 (IL-17), part of the colonic mucosal immune response to *B. hyodysenteriae* infection, induce mucin production synergistically with *B. hyodysenteriae* via mitogen-activated protein kinase 3 (34). *B. hyodysenteriae* infection regulates mucin glycosylation synthesis in the colon, resulting in the loss of interindividual variation, shorter glycan chains, and a higher abundance of neutral, core 2, and *N*-glycolylneuraminic acid (NeuGc)- and sulfate-containing structures compared to healthy pigs (8).

B. hyodysenteriae infection increases the bacterial binding sites on mucins, and since binding differs between pigs, it is highly likely that binding occurs via the mucin glycans (2). However, the glycan residues that *B. hyodysenteriae* interacts with in the colon remain unknown. We recently characterized the mucin *O*-glycome in the colon of *B. hyodysenteriae*-infected and healthy pigs, providing a database of glycan structures, some of which could be relevant for host-pathogen interactions (8). Therefore, the overall aims of the present study were to identify potential mucin glycan epitopes involved in *B. hyodysenteriae* binding to colonic mucins, as well as the effect of mucins and their composition on bacterial growth. The results highlight a role of sialic acid as an adhesion epitope for *B. hyodysenteriae* interaction with colonic mucins. Furthermore, *B. hyodysenteriae* can utilize mucins from infected pigs, sialic acid, and *N*-acetylglucosamine (GlcNAc) as energy sources.

RESULTS AND DISCUSSION

***B. hyodysenteriae* binding to pig colonic mucins is associated with the presence of NeuGc on mucins.** We have previously shown that *B. hyodysenteriae* adheres to colonic mucins in a manner that differs between pigs (2) and that bacterial infection results in changes of the mucin glycan profile in the colon (8). Although these data have been presented before, they have not been previously intercorrelated. Associations between the previously reported *B. hyodysenteriae* binding to colonic mucins from infected and healthy pig data (2) and pig mucin *O*-glycome data (8) were determined to identify potential epitopes involved in *B. hyodysenteriae* adhesion to colonic mucins. Mass spectrometry data for the 94 mucin *O*-glycan structures, as well as groups of structures with similar features (i.e., acidic, neutral, sulfated, sialylated, core type 1, 2, 3, or 4, blood group H or A, Lewis type a/x or b/y, terminal galactose, and NeuGc or NeuAc) were considered in the correlation analysis. More than 80% of the pig colonic mucins from both *B. hyodysenteriae*-infected and healthy pigs are insoluble in guanidinium hydrochloride (GuHCl), and bacterial binding to these was higher than to mucins soluble in GuHCl (2). Overall, there was a statistically significant correlation between *B. hyodysenteriae* adhesion to GuHCl-insoluble mucins and the abundance of sialic acid-containing structures on pig colonic mucins (Table 1; $r = 0.80$, $P = 0.02$). The association between bacterial binding and the presence of sialylated structures on mucins was mainly given by the NeuGc residue (Table 1; $r = 0.72$, $P = 0.04$), as there was no statistically significant association between the amount of NeuAc-based structures alone and *B. hyodysenteriae* binding to mucins ($r = 0.39$, $P = 0.36$). Thus, an increased *B. hyodysenteriae* binding ability was associated with a higher abundance of NeuGc on mucins. These results are in line with the higher abundance of NeuGc detected on *B. hyodysenteriae*-infected pig mucins and the equal proportion of NeuGc and NeuAc detected in healthy pigs (8), paralleling the higher binding to *B. hyodysenteriae*-infected pig mucins than to noninfected pig mucins (2). Overall, this indicates a potential interaction between *B. hyodysenteriae* and NeuGc residues on mucins. Additionally, associations were observed between *B. hyodysenteriae* binding and mucin glycan chains mainly containing NeuGc or terminal galactose with a β(1-3)

TABLE 1 Associations between *B. hyodysenteriae* binding to pig mucins and mucin *O*-glycan data from *B. hyodysenteriae*-infected and healthy pigs[a]

Mucin	*r*	*P* value	CI
Insoluble mucins[b]			
Sialic acids (NeuAc + NeuGc)	0.80	0.02	0.22–0.96
NeuGc	0.72	0.04	0.03–0.94
Galβ1,3(NeuGcα2,6)GalNAcol	0.73	0.04	0.04–0.94
Galβ1,3(NeuAc-GlcNAcβ1,6)GalNAcol	0.76	0.03	0.11–0.95
NeuGc-Galβ1,3(Galβ1,3/4(Fuc)GlcNAcβ1,6)GalNAcol	0.82	0.01	0.28–0.97
NeuGc-Galβ1,3(GlcNAc-Galβ1,4(Sul)GlcNAcβ1,6)GalNAcol	0.71	0.05	0.005–0.94
Gal-GlcNAc-Galβ1,3(GalNAcβ1,4(Sul)GlcNAcβ1,6)GalNAcol	0.75	0.03	0.09–0.95
Soluble mucins			
NeuGc-Galβ1,3(Fuc-Galβ1,3/4(Fuc)GlcNAcβ1,6)GalNAcol	0.7	0.02	0.17–0.93
GlcNAcβ1,3/4Galβ1,3(Fuc-Galβ1,3/4(Fuc)GlcNAcβ1,3/4Galβ1,3/4GlcNAcβ1,6)GalNAcol	0.8	0.00	0.36–0.95
NeuGcα2,3Galβ1,3(GalNAcβ1,4(Sul)GlcNAcβ1,4)Galβ1,3(Fucα1,2Gal(Fuc)GlcNAcβ1,6)GalNAcol	0.6	0.05	0.004–0.90

[a]Data for associations between *B. hyodysenteriae* binding to pig mucins are from reference 2, and mucin *O*-glycan data from *B. hyodysenteriae*-infected and healthy pigs are from reference 8. *r*, Pearson product-moment correlation coefficient determining the relationship between binding to mucins and mucin glycan data from *B. hyodysenteriae*-infected ($n = 5$) and healthy ($n = 5$) pigs; CI, confidence interval; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; Fuc, fucose; Sul, sulfate; GlucNAc, *N*-acetylglucosamine.

[b]Mucins insoluble in GuHCl were solubilized by reduction and alkylation, breaking the disulfide bonds that link the mucin monomers into large complexes.

linkage, out of which Gal-GlcNAc-Galβ1,3(GalNAcβ1,4(Sul)GlcNAcβ1,6)GalNAcol and GlcNAcβ1,3/4Galβ1,3(Fuc-Galβ1,3/4(Fuc)GlcNAcβ1,3/4Galβ1,3/4GlcNAcβ1,6)GalNAcol were unique to mucins from *B. hyodysenteriae*-infected pigs (Table 1).

Removal of sialic acid residues decreases *B. hyodysenteriae* adhesion to pig colonic mucins. The findings of an association between *B. hyodysenteriae* adhesion and mucin glycan chains carrying NeuGc or terminal galactose led us to study the potential role of sialic acid and galactose residues in *B. hyodysenteriae* adhesion. Bacterial binding was analyzed after sialidase A and β-galactosidase enzymatic treatment of the insoluble mucins. Sialidase A cleaves both NeuGc and NeuAc structures from complex carbohydrates, while β-galactosidase hydrolyzes nonreducing terminal galactose β(1-3) and β(1-4) linkages. In line with the statistical correlation between *B. hyodysenteriae* binding and the relative abundance of sialic acids, we observed that *B. hyodysenteriae*'s ability to bind to insoluble mucins decreased after sialidase A treatment compared to that for the non-enzyme-treated mucin control ($P < 0.0001$; Fig. 1). In contrast, adhesion to

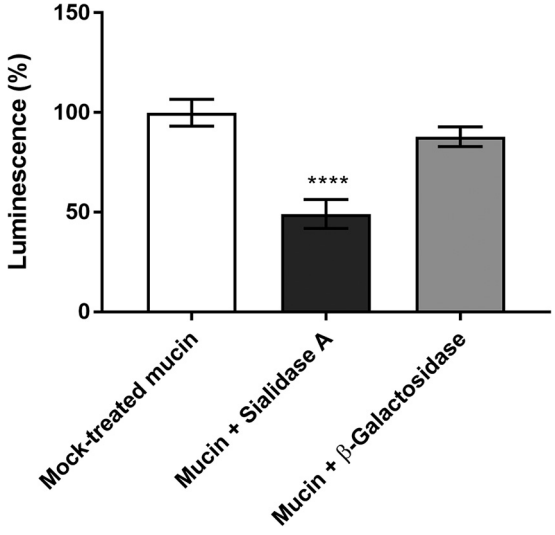


FIG 1 *B. hyodysenteriae* adhesion to enzyme-treated mucins. The binding of *B. hyodysenteriae* to sialidase A- and β(1-4)-galactosidase-treated pig colonic mucins compared to mock (non-enzyme)-treated mucins is shown. The results are expressed as the mean ± SEM for technical replicates from three independent experiments. *P* values were determined by one-way ANOVA with Dunnett's correction for multiple comparisons. ****, $P < 0.0001$.

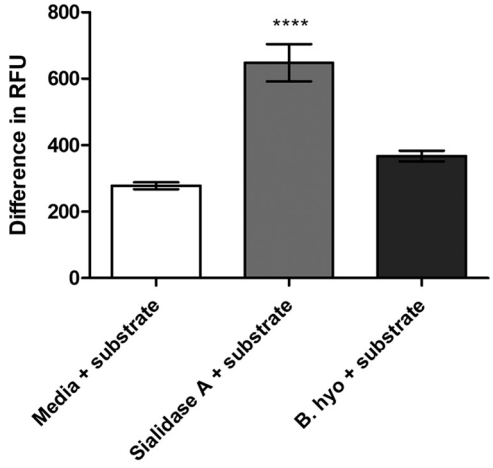


FIG 2 Functional analysis of *B. hyodysenteriae* (B. hyo) sialidase activity. The neuraminidase activity of *B. hyodysenteriae* was measured as the number of relative fluorescence units (RFU) after 10 h of incubation with a 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid sodium salt hydrate substrate. Medium and substrate were used as a negative control, while sialidase A and substrate were used as a positive control. Results are expressed as the mean ± SEM for technical replicates from two independent experiments. *P* values were determined by one-way ANOVA with Tukey's correction for multiple comparisons. ****, $P < 0.0001$.

β-galactosidase-treated mucins remained unchanged ($P > 0.05$; Fig. 1). These findings infer that *B. hyodysenteriae* binds to sialic acid-containing structures on mucins, as the removal of sialic acid hampered adhesion to mucins. An affinity for sialic acid epitopes on intestinal pig mucins has also been demonstrated in *Escherichia coli* K99 (35). It is possible that the sialic acids present on mucins play a role in *B. hyodysenteriae* colonization of the intestinal mucosa, as adhesion of a gastric pathogen to mucins can limit access to the epithelial surface: mice lacking the Muc1 mucin are more susceptible to infection by *Helicobacter pylori* and MUC1 acts as a releasable decoy for *H. pylori* binding to the gastric epithelial surface (15, 18). Furthermore, *H. pylori*-infected children and rhesus monkeys secreting mucins with less *H. pylori* binding capacity develop higher-density *H. pylori* infections and gastritis (9, 19). Together, these results support the suggestion that the ability of mucins to bind *H. pylori* protects the gastric epithelium. It is likely that these types of mechanisms are also relevant as protection against *B. hyodysenteriae* colonization; however, during infection the mucus layer is so disorganized (2) that these protection mechanisms may be impaired.

Functional analysis of *B. hyodysenteriae* sialidase activity. Several mucosal pathogens and commensal bacteria carry sialidases, also known as neuraminidases, that can hydrolyze the glycosidic linkages of the terminal sialic acid residue in oligosaccharides and glycoproteins (36, 37). The sequenced *B. hyodysenteriae* BHWA1 strain carries a predicted/hypothetical sialidase gene coding for the sialidase protein (38); however, there are no functional studies confirming its sialidase activity or sialidase protein expression. For this purpose, *B. hyodysenteriae* was incubated with the 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate substrate, unexpectedly showing no difference in the luminescent signal from that with the substrate alone ($P \geq 0.05$; Fig. 2). Thus, *B. hyodysenteriae* was unable to cleave the fluorescent NeuAc substrate, as demonstrated by a luminescent signal significantly lower than that for the substrate and sialidase A-positive control ($P < 0.0001$; Fig. 2), overall suggesting that the ability of *B. hyodysenteriae* to cleave sialic acid on mucins is limited.

Colonic mucins from infected pigs enhance *B. hyodysenteriae* growth. As we previously reported, *B. hyodysenteriae* infection regulates mucin glycosylation in the colon (8); however, it is unknown whether the resulting changes have a stimulatory, inhibitory, or no effect on pathogen growth. The effect of pig colonic mucins on *B. hyodysenteriae* growth was determined in carbohydrate-free medium in coculture with

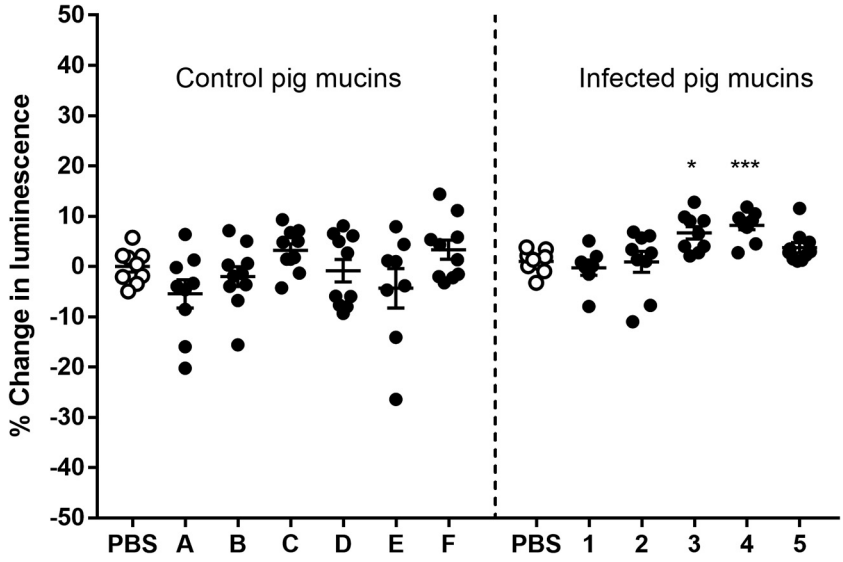


FIG 3 Effect of pig colonic mucins on *B. hyodysenteriae* growth. *B. hyodysenteriae* growth was determined in carbohydrate-free medium in coculture with dialyzed colon-insoluble mucins from healthy pigs (pigs A to F, $n = 6$) and *B. hyodysenteriae*-infected pigs (pigs 1 to 5, $n = 5$) and compared to the growth of the non-mucin-treated control (PBS). Results are expressed as the mean ± SEM for technical replicates from two independent experiments. *P* values were determined by one-way ANOVA with Dunnett's correction for multiple comparisons. *, $P < 0.05$; ***, $P < 0.001$.

dialyzed mucins from healthy ($n = 6$) and *B. hyodysenteriae*-infected ($n = 5$) pig colon. Growth was measured as a luminescent signal that is dependent on the reduction of the cell viability assay substrate by viable cells and rapid turnover by NanoLuc luciferase. The resulting luminescent signal is proportional to the number of *B. hyodysenteriae* live cells in culture. *B. hyodysenteriae* growth increased in the presence of mucins from two out of five infected pigs compared to the nonmucin control (pig 3, $P = 0.01$; pig 4, $P = 0.001$; Fig. 3). Mucins from pigs 3 and 4 moderately increased *B. hyodysenteriae* growth by 6.7% and 8.2% respectively (Fig. 3). Pigs 3 and 4 had an earlier onset of SD (clinical signs started at 12 to 15 days postinfection) and a longer duration of clinical signs (25 to 28 days) than pigs 1, 2, and 5 (clinical signs started at 29 to 39 days postinfection and lasted for 1 to 11 days) (2). In contrast, colonic mucins from healthy pigs did not have a growth-enhancing effect compared to phosphate-buffered saline (PBS) (Fig. 3). No statistically significant differences in overall proliferation means were observed between the PBS, control pig mucin, and infected pig mucin groups ($P > 0.05$).

B. hyodysenteriae growth correlated with the presence of overall sialic acid ($r = 0.7$, $P = 0.03$), NeuAc/Gc with an α2,3-galactose linkage ($r = 0.7$, $P = 0.01$), and terminal galactose ($r = 0.8$, $P = 0.002$). The results suggest that *B. hyodysenteriae* utilizes mucins as a nutrient source, consistent with data from other pig intestinal bacterial species that can degrade mucins and use their components for growth (25, 26). It is possible that the host's altered mucin environment in response to *B. hyodysenteriae* infection is advantageous for the pathogen, modulating its growth.

***B. hyodysenteriae* growth increases in the presence of free sialic acid and N-acetylglucosamine.** To investigate the mucin epitopes that could contribute to the growth-enhancing effect of mucins on *B. hyodysenteriae*, bacteria were cultured in the presence of galactose, fucose, NeuAc, NeuGc, GalNAc, and GlcNAc, monosaccharides that are present as terminal residues on mucins. Among the studied carbohydrates, the NeuGc and NeuAc sialic acids had a surprisingly stimulatory effect on *B. hyodysenteriae*, increasing its growth by 105.3% and 107.9%, respectively, in comparison to that of the no-carbohydrate control ($P < 0.0001$ for all; Fig. 4). In line with our results, intestinal sialic acid catabolism has been demonstrated to be advantageous for pathogens like

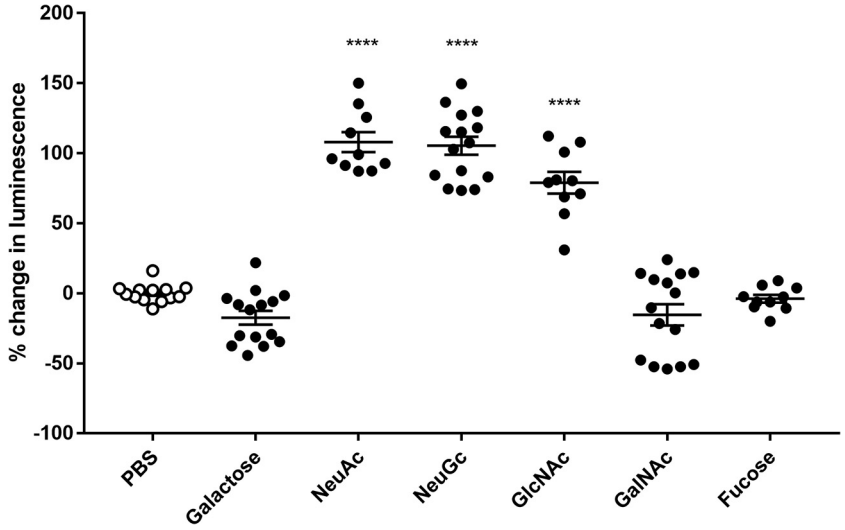


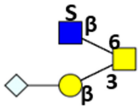
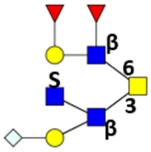
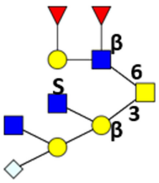
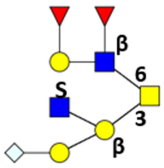
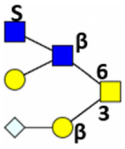
FIG 4 *B. hyodysenteriae* growth in the presence of monosaccharides. Bacterial growth was determined in defined medium (without glucose) in coculture with carbohydrates and compared to that in the non-carbohydrate-treated control (PBS). Results are expressed as the mean ± SEM from three independent experiments. *P* values were determined by one-way ANOVA with Dunnett's correction for multiple comparisons. ****, $P < 0.0001$.

Vibrio cholerae, which can degrade and grow on sialic acid (39). In line with the growth-enhancing effects of GlcNAc on *Aeromonas salmonicida* (40), GlcNAc increased *B. hyodysenteriae* growth by 78.9% compared to that of the no-carbohydrate control ($P < 0.0001$; Fig. 4). The role of GlcNAc in the synthesis of the bacterial cell wall peptidoglycan may possibly contribute to the growth-promoting effects of GlcNAc. Similarly, the intestinal mucosa-associated commensal bacterium *Cloacibacillus porcorum* has mucolytic activity and can grow on mucin and its components, including NeuAc and GlcNAc (26).

Although *B. hyodysenteriae* was not capable of cleaving NeuAc *in vitro*, we cannot rule out the potential cleavage of NeuGc on mucins. There is evidence of interplay between *B. hyodysenteriae* and other members of the microbiota during SD, as the presence of other anaerobes, along with *B. hyodysenteriae*, is required for the development of SD (41). Furthermore, a higher relative abundance of *Campylobacter*, *Fusobacterium*, and *Mogibacterium* was found in mucosal scrapings and luminal samples from pigs with SD compared to pigs without the disease (42). It is possible that *B. hyodysenteriae* can scavenge free sialic acid cleaved by commensal bacteria, such as *Clostridium* and *Bacteroides* species (43, 44), from the surrounding environment or the host pig's intestinal mucosa (42). Thus, the ability to use the free sialic acids and GlcNAc present on the mucosal surface of the pig colon as energy and carbon sources could be beneficial for *B. hyodysenteriae*'s survival in the colon.

Several factors, including infections, diet, weaning, and age, can affect pig intestinal glycosylation (8, 45, 46). Differences in the NeuAcα2,6 versus NeuAcα2,3 content of the pig small intestine have been reported at birth and at weaning (47), and experimental infection of conventional versus specific-pathogen-free pigs with *Trichinella spiralis* results in differential sulfation and sialylation patterns (48), suggesting that numerous circumstances can lead to glycosylation changes that in turn have the potential to affect pathogen interactions with its host. *B. hyodysenteriae* infection results in a higher abundance of NeuGc-containing structures, compared to the equal distribution of NeuGc- and NeuAc-containing structures in healthy pigs (8). Furthermore, expression of sulfated mucins is decreased in the colon of *B. hyodysenteriae*-infected pigs (8, 33). This opens up the question as to whether the loss of sulfated residues exposes sialic acids for *B. hyodysenteriae* binding. However, sulfate moieties in the vicinity of sialic acids were rare: we identified only five sulfated glycan structures that carried sialic

TABLE 2 Sulfated sialoglycans in pigs with and without SD

Structure[a]	Soluble mucins[b]			Insoluble mucins		
	Mean % ± SEM			Mean % ± SEM		
	Control	Infected	*P* value	Control	Infected	*P* value
	—	2.1 ± 1.2	—	0.6 ± 0.2	1.9 ± 0.6	0.06
	0.9 ± 0.4	0.3 ± 0.3	0.25	0.5 ± 0.3	1.4 ± 0.5	0.28
	0.3 ± 0.3	1.8 ± 0.8	0.14	1.0 ± 0.4	0.5 ± 0.2	0.28
	0.7 ± 0.3	—	—	1.5 ± 0.4	2.4 ± 0.7	0.56
	—	—	—	0.5 ± 0.2	0.7 ± 0.1	0.70

[a]Yellow squares, GalNAc; purple diamonds, *N*-acetylneuraminic acid; gray diamonds, *N*-glycolylneuraminic acid; red triangles, fucose; blue squares, GlcNAc.
[b]—, structure absent.

acids, including NeuAc and NeuGc (Table 2). In contrast to the decrease in overall sulfation among mucins from infected pigs, the abundance of sulfated glycan structures that carry sialic acids tended to be higher among mucins from infected pigs (4.1% and 6.8% for soluble and insoluble mucins, respectively) than among mucins from uninfected pigs (1.9% and 4.1% for soluble and insoluble mucins, respectively) ($P = 0.11$). As a common theme among these glycans, the sulfation was present on the branch opposite that of the sialylation. Therefore, exposure of sialic acids due to decreased sulfation is unlikely to be the cause of the increased sialic acid-dependent *B. hyodysenteriae* binding to colonic mucins from infected pigs. However, sulfated glycans could potentially inhibit bacterial neuraminidases via intermolecular ionic interactions (49), suggesting that the decrease in overall sulfation among infected pigs may contribute to making mucin glycans more available as a microbial nutrient.

Conclusion. Here, we provided insights into the interactions of *B. hyodysenteriae* with pig intestinal mucin glycans, highlighting the relevance of NeuGc as an adhesion epitope for bacterial binding to mucins. We identified that sialic acids are key players in the interactions between *B. hyodysenteriae* and host mucins: *B. hyodysenteriae* binding to pig colonic mucins occurs in a sialic acid-dependent manner, and both GlcNAc and sialic acids enhance *B. hyodysenteriae* growth. The fact that *B. hyodysente-*

riae grew only in the presence of mucins from pigs with prolonged clinical signs of SD suggests a potential time-dependent component change in the mucus composition. Additionally, infected pig mucins carried a larger array of glycan structures, indicating that *B. hyodysenteriae* can adapt to the glycan-rich environment in the mucus layer during infection. Earlier studies have shown that infection cannot be established in gnotobiotic pigs after inoculation with pure cultures of *B. hyodysenteriae* (41, 50). Thus, in order to develop clinical signs of swine dysentery, *B. hyodysenteriae* requires the presence of other anaerobic enteric bacteria in the mucus layer, such as *Bacteroides vulgatus* and *Fusobacterium necrophorum* (41). It is possible that *B. hyodysenteriae* benefits from the free sialic acids cleaved by sialidase-producing commensal bacteria in the colonic mucus layer. Overall, the mucin response and glycosylation changes exerted in the colon during SD potentially result in a favorable environment for pathogen growth in the mucus layer.

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.

Isolation and purification of pig colonic mucins. For a detailed description of the experimental inoculation of pigs and sample collection procedures, refer to the work of Quintana-Hayashi et al. (2). Briefly, a total of five 6-week-old pigs (Danish Large White × Piétrain) developed swine dysentery after oral inoculation with brain heart infusion broth (BHI) containing 10^8 CFU of *B. hyodysenteriae* strain 8dII, while control pigs received sterile BHI. Pigs were fed a commercial starter feed twice per day and had *ad libitum* access to water. The pigs were sacrificed at 40 days after inoculation. Midsection samples of the spiral colon of the five infected pigs and healthy controls were collected for mucin isolation. Fecal material was removed, and the tissues were rinsed with phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), followed by snap-freezing and storage at −80°C. The isolation of mucin from colon tissue samples was performed by isopycnic density gradient centrifugation as previously described, obtaining guanidinium hydrochloride (GuHCl)-soluble and -insoluble mucins (2). 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 dihydrogen phosphate at pH 6.5. GuHCl-soluble mucins were obtained after centrifugation at 23,000 × *g* for 50 min at 4°C, and the remaining material was reextracted 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 10 volumes of extraction buffer at 4°C, with the dialysis solution being changed 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 40,000 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. The fractions were stored at 4°C until further analysis.

Analysis of mucin fractions. The first and second CsCl gradient mucin fractions were analyzed as previously described (2). A microtiter-based assay detecting carbohydrates as periodate-oxidizable structures (20) 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. The 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 hydrazide solution diluted 1/50 in NaAc, followed by europium-labeled streptavidin diluted 1/1,000 in DELFIA assay buffer (PerkinElmer, Waltham, MA, USA). Finally, the 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% NaN_3 at pH 7.75, except for the final step, where the plates were washed six times. The signal was measured in a Wallac 1420 Victor^2 microplate reader (PerkinElmer, Waltham, MA, USA) by time-resolved fluorometry.

Mucin sample preparation and concentration estimation. Mucin sample preparation and concentration estimation were performed as previously described (2). Briefly, gradient fractions containing mucins were pooled to obtain one sample for each gradient. Liquid chromatography-tandem mass spectrometry spectra (8) were analyzed for the presence of carbohydrates of bacterial origin, which were not observed in any mucin samples. Mucin concentrations in pooled samples were determined by the use of serial dilutions as well as a standard curve of a fusion protein of the mucin MUC1, 16TR (tandem

repeat), and IgG2a Fc (51), starting at a concentration of 20 mg/ml and using seven 1/2 serial dilutions in the carbohydrate detection assay described above. The mucin concentrations were calculated from the standard curve. Setting the concentration based on the glycan content appears to be the most appropriate, as bacterium-mucin interactions largely occur via the mucin glycans (1). Although this is not an absolute measure of the concentration, it can be used to ensure that the mucins are at the same concentration for comparative assays.

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

***B. hyodysenteriae* adhesion to enzyme-treated mucins.** The roles of sialic acid and galactose in *B. hyodysenteriae* adhesion were analyzed after sialidase A and β-galactosidase treatment of pig colon insoluble mucins isolated as previously described (2). White 96-well plates (Corning Life Sciences, Corning, NY, USA) were coated at 4°C for 12 h with 100 μl pig colonic mucins at 6 μg/ml in 0.5 M GuHCl. The wells were washed three times with PBS with Tween 20 (PBS-T) and blocked with 200 μl of 5% fetal bovine serum (FBS) for 1 h. For enzyme treatment of mucins, 75 μl of sialidase A (1 U/200 μl; Prozyme, CA, USA) diluted 1:200 and β(1-4)-galactosidase (0.5 U/100 μl; Prozyme, CA, USA) diluted 1:50 in 50 mM Na_3PO_4, pH 6.0, were added per well, and the plates were incubated for 11 h at 37°C.

Subsequently, the wells were washed with PBS-T and blocked with 200 μl of 5% FBS for 1 h. Bacteria were harvested from tryptic soy agar plates, washed in PBS, centrifuged at 2,500 $\times$ *g* for 5 min, and resuspended in PBS containing 5% FBS. One hundred microliters of a bacterial suspension diluted to 10^8 bacterial cells/ml was added per well, and the plates were shaken during incubation for 2 h at 40°C in an anaerobic environment. The 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. The BacTiter-Glo microbial cell viability method results in a luminescent signal proportional to the amount of ATP present, which in turn is proportional to the number of viable bacterial cells in the culture. Relative luminescence (RLU) was measured in an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) with an integration time of 1,000 ms per well. Controls included mucin-coated wells with enzyme, mucin-coated wells without enzyme (50 mM Na_3PO_4, pH 6.0), non-mucin-coated wells (0.5 M GuHCl) with enzyme, and non-mucin-coated wells without enzyme (50 mM Na_3PO_4, pH 6.0) incubated with bacteria, followed by addition of PBS and reagent. Results were obtained from three independent experiments with six technical replicates and were plotted as relative luminescence (RLU).

***B. hyodysenteriae* growth in the presence of mucins and monosaccharides.** Bacterial growth in the presence of mucins was performed as previously described (52). Mucins were dialyzed against four changes of 2 M NaCl, followed by two changes against sterile PBS. Twenty-five microliters of colonic mucins from five *B. hyodysenteriae*-infected pigs and six healthy pigs (2) and the carbohydrates galactose, fucose, NeuAc, NeuGc, GlcNAc, and GalNAc (Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS (100 μg/ml) were added to a sterile Falcon 96-well plate (Corning Life Sciences, Corning, NY, USA). *B. hyodysenteriae* was harvested from the agar plates, washed in PBS, and resuspended in carbohydrate-free medium (40) to an optical density at 600 nm (OD_{600}) of 0.1. RealTime-Glo cell viability assay reagent (Promega, Madison, WI, USA) was diluted 1/1,000 in the bacterial suspension, and 225 μl of the bacterial suspension with the reagent was added per well. The microtiter plate was covered with a sterile gas-permeable sealing membrane (Diversified Biotech, Dedham, MA, USA) and incubated at 40°C inside a plate reader (CLARIOstar; BGM Labtech GmbH, Ortenberg, Germany) connected to an atmospheric unit set to 0.1% O_2. Luminescence readings were measured every 2 h for 24 h. Proliferation control wells contained PBS instead of mucins or monosaccharides, in addition to the bacterial suspension and reagent. To ensure that the residual CsCl and GuHCl in the pig colonic mucins had no adverse effects on *B. hyodysenteriae* growth, a control consisting of CsCl dissolved in 0.5 M GuHCl was dialyzed in parallel to the mucins, with the results on *B. hyodysenteriae* growth being similar to those for the PBS control.

Fluorometric assay of *B. hyodysenteriae* neuraminidase. Eighty microliters of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate substrate (500 μM; catalog number 69587; Sigma) and 20 μl of a *B. hyodysenteriae* suspension in defined medium (OD_{600}, 0.1) were added to a Falcon 96-well plate. Sialidase A (5 U/ml; ProZyme; Hayward, Canada) diluted 1,000-fold in a 100-μl final volume was added along with substrate as a positive control, while defined medium and substrate were used as a negative control. Sialidase activity was measured by analyzing the fluorescence of liberated 4-methylumbelliferyl from the 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate substrate as relative fluorescence units (RFU) at an excitation wavelength of 340 nm and an emission wavelength of 420 nm in a plate reader (BMG Labtech, Ortenberg, Germany) every 10 min for 11 h.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 6) software (GraphPad Software, La Jolla, CA, USA). Results are expressed as the mean $\pm$ standard error of the mean (SEM) for normally distributed data (determined by the D'Agostino-Pearson omnibus test). Equality of variances between groups was assessed by Barlett's test. Data were analyzed using one-way analysis of variance (ANOVA), and *P* values of $\leq$0.05 were considered statistically significant. The Pearson product-moment correlation coefficient (*r*) was computed to assess the relationship between two variables.

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