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Structural analysis of broiler chicken small intestinal mucin *O*-glycan modification by *Clostridium perfringens*

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ABSTRACT *Clostridium perfringens* is a Gram-positive opportunistic pathogen that is the principal etiological agent of necrotic enteritis (**NE**) in poultry. The ability of *C. perfringens* to incite NE depends upon its ability to penetrate the protective mucus barrier within the small intestine, which is largely composed of heavily glycosylated proteins called mucins. Mucins are decorated by *N*- and *O*-linked glycans that serve both as a formidable gel-like barrier against invading pathogens and as a rich carbon source for mucolytic bacteria. The composition of avian *O*-linked glycans is markedly different from mucins in other vertebrates, being enriched in sulfated monosaccharides and *N*-acetyl-D-neuraminic acid (**Neu5Ac**, sialic acid). These modifications increase the overall negative charge of mucins and are believed to impede colonization by enteric pathogens. The mechanism by which *C. perfringens* penetrates the poultry intestinal mucus layer during NE is still unknown. However, the CAZome (i.e., the total collection of proteins encoded within a genome active on carbohydrates) of *C. perfringens* strain CP1 encodes several putative and known enzymes with activities consistent with the modification of mucin. To further investigate this relationship, *O*-glycans from *Gallus gallus domesticus* mucus were extracted from the small intestine and characterized using gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. Chicken mucin monosaccharides included L-fucose (**Fuc**), D-mannose (**Man**), D-galactose (**Gal**), *N*-acetyl-D-galactosamine (**GalNAc**), *N*-acetyl-D-glucosamine (**GlcNAc**), and Neu5Ac (sialic acid). Using these monosaccharides as sole carbon sources, we showed that *C. perfringens* CP1 grew on Neu5Ac, Man, Gal, and GlcNAc but not on Fuc and GalNAc. We also demonstrated *C. perfringens* grew on different native-state preparations of intestinal mucins and mucus including porcine mucins, chicken mucus, and chicken mucins. Finally, anaerobic incubation of chicken mucin *O*-glycans with *C. perfringens* and subsequent analysis of the glycans revealed that there was preferential removal of Neu5Ac. These observations are discussed in the context of the predicted metabolic potential of *C. perfringens* CP1 and the mucolytic enzymes encoded within its CAZome.

Key words: *Clostridium*, mucin *O*-glycan, intestinal mucus, carbohydrate chromatography, mass spectrometry

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INTRODUCTION

The intestines of poultry harbor trillions of bacteria and other microorganisms that collectively are termed the intestinal microbiota. Chicken intestines are predominantly colonized by 3 prokaryotic phyla: Firmicutes, Bacteroidetes, and Proteobacteria (>90%) (Pan and Yu, 2014; Xiao et al., 2017). A major function of the intestinal microbiota is the metabolism of dietary fiber into short chain fatty acids that are absorbed by the host as an energy source (Pan and Yu, 2014; Koh et al., 2016; Makki et al., 2018). In addition to dietary glycans, select populations of intestinal bacteria can

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also metabolize glycans present on host glycoconjugates, such as mucin glycoproteins, which comprise the intestinal mucus layer. The mucus layer is the first line of innate host defense and is the single largest physical barrier that protects the host epithelia from pathogenic bacteria (McGuckin et al., 2011). Mucin glycoproteins are produced by goblet cells, which package the glycoproteins into granules and transport them to the cell surface of the epithelium where they can be docked at the cell-surface or secreted into the lumen to form a gelatinous matrix (Kim and Khan, 2013). Mucins are *O*-glycosylated at serine or threonine residues or *N*-glycosylated at asparagine residues (Bansil and Turner, 2006). *O*-linked glycans are more abundant than *N*-linked glycans in intestinal mucins and are complex in nature, consisting of variable sequence and branching of L-fucose (**Fuc**), D-mannose (**Man**), D-galactose (**Gal**), *N*-acetyl-D-galactosamine (**GalNAc**), *N*-acetyl-D-glucosamine (**GlcNAc**), and *N*-acetyl-D-neuraminic acid (**Neu5Ac**, sialic acid). In contrast, *N*-linked glycans contains a chitobiose core (β-GlcNAc-1,4-GlcNAc) with high mannose or hybrid decorations (Brockhausen and Stanley, 2015; Stanley et al., 2017). Mucin glycans account for over 80% of the total mucin molecule by mass and provide an abundant energy source for mucolytic intestinal bacteria (Bansil and Turner, 2006; Pan and Yu, 2014).

Mucins are sloughed into the intestinal lumen and degraded by mucolytic bacteria, and therefore are continually replenished to maintain barrier integrity (McGuckin et al., 2011). This relationship is dynamic. For example, increased mucin secretion has been linked to the onset of enteric infection (Linden et al., 2008a) and in response to immune modulation (Linden et al., 2008b) in murine models. Newly shed mucins infuse into the intestinal mucus matrix and are stabilized by the formation of disulfide bonds and further modifications, such as increasing the density of negatively charged carbohydrates (i.e., "acidification"), which can also greatly alter mucus viscosity (McGuckin et al., 2011). Thus, secreted mucin structure and chemistry affects the functional components of mucus, and defects in mucin *O*-glycosylation compromise the fidelity of mucus formation (Bergstrom and Xia, 2013), resulting in an increased susceptibility to infection (Taylor et al., 2018).

Mucin glycans are compositionally and structurally complex. Liquid chromatography-mass spectrometry (**LC-MS**) of a single mucin type expressed in the human colon, Muc2, revealed the presence of >100 different glycan structures linked to the mucin protein core (Smirnov et al., 2004; McGuckin et al., 2011). Furthermore, comparisons between mucin *O*-glycans across species reveal novel structures; chicken intestinal mucin *O*-glycans show considerable differences to human intestinal mucins (Struwe et al., 2015). Sialomucins and sulfomucins, through addition of sialic acid and sulfate, respectively, can further alter the chemical and biological properties of mucins. These unique glyco-landscapes provide a diverse range of substrates for mucolytic bacteria. To adapt, mucolytic bacterial species have evolved a variety of carbohydrate binding proteins (e.g., carbohydrate binding modules, adhesins) and carbohydrate-active enzymes (CAZymes) to selectively adhere to and modify mucin glycans, respectively (Ficko-Blean et al., 2009; Ficko-Blean and Boraston, 2012a; Etzold and Juge, 2014). Given the structural diversity of mucin glycans, utilization of mucus carbohydrates by intestinal microorganisms is a complex process (Cantarel et al., 2009; Etzold and Juge, 2014).

Clostridium perfringens is a Gram-positive, endospore-forming, obligate anaerobe commonly found in the intestines of animals (Flores-Díaz et al., 2016). *Clostridium perfringens* has the ability to produce more than 15 different toxins as well as various other enzymes that can contribute to disease in many animal hosts (Petit et al., 1999; Flores-Díaz et al., 2016). One such disease in poultry is necrotic enteritis (**NE**), which has emerged as a leading cause of economic losses in global poultry production (est. USD $6 billion annually) from morbidities (e.g., lowered harvest weights) and mortalities (Flores-Díaz et al., 2016). Necrotic enteritis in broiler chickens begins with the rapid overgrowth and colonization of *C. perfringens* at the mucus layer of the small intestine. Following penetration of the mucus layer, the release of cytological toxins by *C. perfringens*, such as α toxin and NetB, results in epithelial cell death and tissue necrosis (Prescott et al., 2016).

The mechanism by which *C. perfringens* dismantles broiler chicken intestinal mucus during NE and the role of *O*-glycan modification in subclinical and clinical disease have not been defined. Previous work with *Campylobacter jejuni* has shown that differences in the fine-chemistry of mucus glycans between humans and chickens may explain differences in host outcomes for enteric infections (Alemka et al., 2010; Struwe et al., 2015). Further investigation of chicken intestinal mucin glycan structure and the dynamics by which mucolytic bacteria deconstruct chicken mucin glycans may help illuminate differences in species-specific infection and disease. Studying these relationships, however, is limited by the lack of analytical methods available for determining changes in the structure of mucin glycans and the effect of physiological stimuli, such as stress or biological interactions, including interactions with mucolytic bacteria. In this study, we evaluated the fidelity of 3 different chemical *O*-glycan extraction methods for chicken mucus and present a reproducible assay for studying *C. perfringens* mucin utilization and modification in vitro and informing the role(s) of *C. perfringens* in the modification and utilization of chicken mucin *O*-glycans in vivo. These techniques have allowed the observation of glycan compositional and structural changes in broiler chicken intestinal mucin caused by *C. perfringens* and may help to inform the design of more effective intervention methods for NE in poultry production.

MATERIALS AND METHODS

Clostridium perfringens

Wild-type *C. perfringens* CP1, a strain known to cause NE in chickens, was obtained from Prof. John Prescott (Ontario Veterinary College, University of Guelph). To ensure the retention of the *netB* toxin-plasmid (88 kb), quality checks were conducted by PCR using plasmid-specific primers, CP4_3449 (*netB*) forward (GCTGGTGCTGGAATAAATGC) and CP4_3449 (*netB*) reverse (GCTGGTGCTG-GAATAAATGC), as previously described (Lepp et al., 2013). *Clostridium perfringens* cultures were grown on 1.5% Columbia sheep blood agar plates, and glycerol stocks were prepared for all n*etB*-positive isolates and stored at −80°C. Prior to all growth studies, the purity of each culture was determined by plating on Columbia sheep blood agar and tryptose-sulfite-cycloserine agar plates, the latter of which is a selective medium for *C. perfringens* (Harmon et al., 1971).

Defined C. perfringens *Growth Medium*

To determine the ability of *C. perfringens* to degrade chicken intestinal mucins and metabolize released mucin carbohydrates, *C. perfringens* CP1 was grown in a medium adapted from Deplancke et al. (2002). For a 2 × concentrated solution, this defined medium (**MM**) contained the following: 0.46 g L^{-1} K_2HPO_4 (VWR), 0.46 g L^{-1} KH_2PO_4 (VWR), 0.46 g L^{-1} $(NH_4)_2SO_4$ (BDH Chemicals), 0.92 g L^{-1} NaCl (VWR), 0.18 g L^{-1} $MgSO_4{\cdot}7H_2O$ (Sigma), 80 mg L^{-1} $CaCl_2{\cdot}2H_2O$ (Sigma), 4 g L^{-1} tryptone (BD Biosciences), 2 mg L^{-1} hemin (Sigma), 0.1 mg L^{-1} resazurin (Sigma), 8 g L^{-1} Na_2CO_3 (Amresco), and 1 g L^{-1} cysteine-HCl (MP Biomedicals). Importantly, *C. perfringens* did not grow in this medium unless supplemented with a carbohydrate. Variations on the established MM recipe were attempted to optimize baseline *C. perfringens* growth using previously described *C. perfringens* media (Roberton and Stanley, 1982; Stanley et al., 1986) as well as a defined amino acid medium (Riha and Solberg, 1971) with no success. Therefore, the most minimized medium was selected for growth studies to limit any potential confounding effects.

Clostridium perfringens *Growth Cultures*

Mucin Monosaccharide Growth Cultures *Clostridium perfringens* CP1 strain was grown in MM using each of the 6 primary mucin monosaccharides (GalNAc; GlcNAc; Fuc; Gal; Man; *N*-acetyl-D-neuraminic acid or sialic acid, Neu5Ac) or using additional carbohydrates commonly found in the poultry diet (D-glucose, **Glc**; D-xylose, **Xyl**; D-arabinose, **Ara**; and L-rhamnose, **Rha**) as the sole carbon source. Each monosaccharide was dissolved in deionized distilled water at 1% (w/v) and autoclaved prior to addition to the growth medium. Broth culture was prepared with the Hungate method (Bryant, 1972), maintaining anaerobic conditions within glass sample tubes, for monosaccharides at a 1:1 (v/v) ratio with 2 × MM to provide a final carbohydrate concentration of 0.5%. Briefly, media were autoclaved for 30 min and set to cool under a constant flow of anaerobic grade carbon dioxide (**CO_2**) gas (CD 4.0 AN-K). While remaining under CO_2 gas, the additions of 8 g Na_2CO_3 and 1 g cysteine were made once the medium cooled. Sterile screw topped Hungate glass tubes were placed under constant anaerobic CO_2 gas and the anaerobic medium was then transferred with a glass pipette connected to constant CO_2 gas to ensure anaerobic transfer of medium, as is consistent with the Hungate method. The tubes were quickly capped and only opened under a stream of CO_2 for inoculations. Each monosaccharide culture (made in 3 replicates) was inoculated with a separate overnight starter culture grown in Columbia (Difco) to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Overnight cultures were centrifuged at 5000 × g for 5 min and washed with anaerobic 2 × MM before inoculation. Each broth culture containing different monosaccharide additions were incubated anaerobically at 37°C and OD_{600} was measured every 5 h for a total of 30 h.

Mucin and Mucus Preparation The project was approved by the Agriculture and Agri-Food Canada (AAFC) Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (LeRDC Animal Use Protocol #1615). Chickens culled by the producer following industry standards were obtained from 3 different broiler farms in southwestern Alberta late in the production cycle, and birds were immediately transported to a large animal necropsy facility located at AAFC LeRDC and processed within 2 h of death. To obtain mucus, the caudal-ventral abdomen was opened and the intestinal tract was removed from the abdomen cavity, sections from the duodenum, jejunum, and ileum were excised from the intestinal tract, and individual sections were longitudinally opened. Mucus was gently removed from the intestinal segments using a sterile scalpel tip, and was pooled from all the birds. Care was exercised to exclude as much digesta and external debris (small rocks, etc.) from the mucus as possible.

Chicken Mucus and Pig Gastric Mucin Growth Cultures Freeze dried mucus collected from pooled duodenal, jejunal, and ileal sections from broiler chickens were suspended at 2% (w/v) in PBS buffer at pH 7.4 and sterilized by autoclaving for 30 min. Pig gastric mucin (**PGM**; Sigma) was also prepared using the same methods. Broth cultures containing PGM or chicken mucus were prepared by additions at a 1:1 (v/v) ratio with 2 × MM. Cultures were prepared in triplicate aerobically within glass culture tubes with loose fitting caps and then placed in an airtight sealed anaerobic jar. Each jar had oxygen removed by vacuum and was filled with anaerobic grade CO_2 gas (CD 4.0 AN-K). These cultures could not be monitored by OD_{600} due to the

turbidity of the solution. Serial dilutions of each broth culture were plated (100 μL) on supplemented brain heart infusion agar in 3 replicates and incubated 24 h at 37°C anaerobically using anaerobic jars filled with anaerobic grade CO_2 gas (CD 4.0 AN-K). Time points were taken every 4 h. Enumeration of bacterial cells were completed by quantifying the number of CFUs per dilution plate, then converting to CFU mL^{-1} of media.

Chemical Release of Mucin O-Glycans

Sodium Hypochlorite O-Glycan Extraction Using a protocol previously designed for PGM (Song et al., 2016), mucin *O*-glycans were released from chicken intestinal mucus with sodium hypochlorite (**NaClO**). Freeze-dried crude intestinal mucus from farm-reared broiler chickens was dissolved into 50 mL 18 MΩ cm^{-1} ultrapure H_2O at 2% (w/v) solution. While stirring at room temperature, 25 mL of 6% NaClO, or household bleach that does not include a polymer addition was introduced to the mucus suspension. The bleach solution was mixed for 20 min at room temperature. Slowly, 0.75 mL of concentrated formic acid was added until the solution was well mixed, approximately 3 to 5 min. Preliminary work using this protocol suggested longer incubation times resulted in glycan degradation of the released product; therefore, shortened incubations were favorable for chicken mucus samples. The acidified mucus solution was then clarified by centrifugation at 13,000 × g for 60 min at room temperature and dried at 45°C, under vacuum. The dried product was suspended in 15 mL 18 MΩ cm^{-1} ultrapure H_2O and filtered through a 0.45 μm syringe filter (Millipore). The volume was increased to 50 mL with 18 MΩ cm^{-1} ultrapure H_2O and the pH adjusted to 7.6 using sodium hydroxide (NaOH). Bleach was added to this mixture (6.6 mL) and incubated overnight at room temperature while stirring. Formic acid was added (0.2 mL) to the mixture until well mixed and the sample was dried once again under vacuum. Using the minimal volume of 18 MΩ cm^{-1} ultrapure H_2O required for solubility, the dried product was suspended and aliquoted into 1.5 mL microcentrifuge tubes and centrifuged at 16,900 × g for 10 min. Supernatants were pooled and added to a Sephadex G-25 fine resin desalting column (2.5 × 100 cm, 500 mL) using a flow rate of 4 mL min^{-1}. Fractions were collected and carbohydrates detected using thin layer chromatography and a sulfuric acid assay (Albalasmeh et al., 2013). Fractions that contained carbohydrates were pooled, snap frozen in liquid nitrogen, and lyophilized. Dried *O*-glycans were then stored at −20°C in glass bottles until required.

Ammonia-catalyzed β-Elimination O-Glycan Extraction Release of chicken mucin *O*-glycans was performed according to microscale non-reductive techniques previously described for bovine mucins (Huang et al., 2001), with modifications. Freeze-dried crude intestinal mucus (500 mg) from farm-reared broiler chickens was placed into a screw cap Erlenmeyer flask. Samples were reduced using 5 g $(NH_4)_2CO_3$ and 50 mL 28% NH_4OH and incubated on silver beads at 60°C for 45 h. The flask was cooled in a −20°C freezer for 30 min to release the pressure built over incubation. The solution was centrifuged at 17,000 × g for 10 min to remove protein from solution, and the supernatant was dried under vacuum. Dried material was then washed with 18 MΩ cm^{-1} ultrapure H_2O 3 times, drying under vacuum each time. The final product was suspended in 5 mL 18 MΩ cm^{-1} ultrapure H_2O and then centrifuged to remove residual particulates. The supernatant, which contains the extracted *O*-glycans, was desalted using graphitized carbon solid phase extraction cartridges (EnviCarb; 250 mg). The solid phase extraction column was washed with water and centrifuged at 200 × g for 2 min. Sample was added and centrifuged again. To elute neutral glycans in the sample, 4 additions of 20% methanol in 18 MΩ cm^{-1} ultrapure H_2O was used, and charged glycans were eluted using 4 additions of 50% acetonitrile, 0.1% trifluoroacetic acid in 18 MΩ cm^{-1} ultrapure H_2O. Eluted glycans were pooled and lyophilized. Dried *O*-glycans were then stored at −20°C in a glass bottle until required.

Proteolysis and Alkaline β-Elimination O-Glycan Extraction Mucin *O*-glycans from crude preparations of chicken mucus were also prepared using reductive β-elimination according to previous work using PGM (Martens et al., 2008) that was adapted from Manzi et al., (2000). This method has been shown to be effective for the chemical analysis of chicken mucin *O*-glycan structures (Struwe et al., 2015); however, growth studies using chicken intestinal mucin *O*-glycans produced with this method had not been previously performed. Dried sample was suspended at 2.5% (w/v) in 100 mM Tris buffer, pH 7.4 in a screw top glass bottle and autoclaved to increase the solubility of the mucus. The solution was cooled to 65°C and proteinase K (VWR, Cat#97,062–238) was added. Hydrolyzed mucin proteins were removed from the solution by centrifugation at 21,000 × g for 30 min at 4°C and the supernatant was reduced using 0.1 M NaOH and 1 M sodium borohydride ($NaBH_4$). The final solution pH was decreased to 7.0 with hydrochloric acid and centrifuged once more. The supernatant was sterile filtered using a 0.22 μm filter (Millipore) and filtrate was dialyzed using a 1 kDa cutoff against 18 MΩ cm^{-1} ultrapure H_2O. The sample was then flash-frozen in liquid nitrogen, and lyophilized and stored in a glass bottle at −20°C.

Clostridium perfringens *Growth Cultures in the Presence of Extracted Mucin O-Glycans* Chicken mucin *O*-glycans extracted using the above methods were resuspended as needed at 2% (w/v) in PBS at pH 7.4. A total of 3 technical replicates of each broth culture were prepared with the Hungate method for each prepared mucin *O*-glycan solution at a 1:1 (v/v) ratio with 2 × MM and incubated at 37°C. Growth profiles of *C. perfringens* on mucin *O*-glycans were monitored using OD_{600} in parallel with viable plate counts to quantify bacteria, presented as average CFUs

mL^{-1} of culture. The OD_{600} was measured every 3 h for 33 h, and CFU mL^{-1} were determined every 4 h for 24 h. CFU enumerations were completed on each broth culture using supplemented brain heart infusion (BHIS) agar plating as described above.

Clostridium perfringens *Culture Post-growth Analysis*

Clostridium perfringens cultures grown on PGM, chicken mucus, or chicken mucin *O*-glycans were centrifuged 5000 × g for 5 min. Media containing extracted mucin *O*-glycans were sterile filtered using a 0.2 μM filter (Millipore), whereas media containing mucus or PGM could not be sterile filtered due to the physical properties of the sample and thus were autoclaved. Sterile samples were then lyophilized, and the resultant spent media glycans were analyzed by the following methods: gas chromatography with flame ionization detection (**GC-FID**), high-performance anion exchange chromatography coupled with pulsed amperometric detection (**HPAEC-PAD**), and LC-MS.

Gas Chromatography with Flame Ionization Detection To determine the monosaccharide composition of chicken intestinal mucus and mucin *O*-glycans used for *C. perfringens* growth studies, gas chromatography (**GC**) with flame ionization detection (**FID**) was used to quantitate acid hydrolyzed, alditol-converted samples. Each sample (2 to 4 mg) from 3 replicate growth cultures was incubated with 200 μL of 2 M trifluoroacetic acid at 121°C for 2 h, dried under vacuum at 40°C, and then washed with isopropanol 3 times. The released monosaccharides were then converted into their volatile derivatives for GC-FID analysis in a 2-step process. First, the carbohydrates were converted into alditol acetates by reduction with $NaBH_4$. Hydrolyzed samples were reduced by 200 μL $NaBH_4$ (10 mg mL^{-1} $NaBH_4$ in 1 M NH_4OH) overnight. The reaction was neutralized with glacial acetic acid, then washed with methanol and dried under vacuum 3 times. Reduced monosaccharides were *O*-acetylated by incubating with 250 μL of acetic anhydride at 50°C. Samples were purified by phase separation by adding 0.2 M Na_2CO_3 and dichloromethane. The organic layer was concentrated at 40°C under nitrogen and the dried material resuspended using 200 μL of dichloromethane. The resulting solution was transferred to a GC auto sampler vial containing a 250 to 300 μL micro insert and injected into gas chromatograph (Hewlett Packard 5890) with a polar capillary GC column (Sigma, SP2330). Sample retention was visualized with a flame ionization detector.

High-Performance Anion Exchange Chromatography Coupled with Pulsed Amperometric Detection High-performance anion exchange chromatography runs were performed with a Dionex ICS-3000 chromatography system equipped with an auto-sampler and pulsed amperometric detection. Ten microliters of diluted glycan hydrolysate was injected onto an analytical CarboPac PA20 column and eluted at 0.4 mL min^{-1} flow rate with a stepwise sodium acetate (NaOAc) gradient (0–1′: 0 mM; 1–18′: 250–850 mM; 18–20′: 850 mM; 20–30′: 850–0 mM) in 100 mM NaOH. The elution was monitored with a pulsed amperometric detection detector. Data were collected using the Chromeleon chromatography management system. *O*-glycan standards (Tailford et al., 2015) were run in parallel.

Liquid Chromatography—Mass Spectrometry Ammonia-catalyzed β-eliminated *O*-glycans (Huang et al., 2001) incubated with *C. perfringens*, in triplicate, were reduced using 1 M $NaBH_4$ in 50 mM NH_4OH (2 h, 60°C) post-incubation to avoid chromatographic resolution of α/β-anomers. After neutralization with acetic acid, reduced *O*-glycans were subsequently desalted using graphitized carbon solid phase extractioncartridges (EnviCarb; 250 mg) essentially as described by Packer et al. (1998). *O*-glycan samples were analyzed by HPLC-quadrupole-time-of-flight (qTOF) MS using a method optimized for the analysis of milk oligosaccharides (Vicaretti et al., 2018). MassHunter's (Agilent Technologies) find-by-formula algorithm was used to search total ion chromatograms for ions with *m/z* values consistent with glycan compositions previously identified in chicken intestinal tissues (Struwe et al., 2015). Peak areas for all *O*-glycans with mass errors of 10 ppm or less and find-by-formula scores above 90 were recorded and are reported as a percentage of the total glycans detected in each sample. Total sialic acid quantitation was performed as reported by Wylie and Zandberg (2018), with several modifications. Briefly, $[^2H]_3$6′-sialyllactose (containing a $[^2H]_3$-labeled acetamido group, installed by sequentially de-*N*-acetylating Neu5Ac with neat hydrazine (Bergfeld et al., 2017) followed by re-*N*-acylation with $[^2H]_6$ acetic anhydride) and $[^{13}C]_2$KDN (from Prof. Andrew Bennet; Simon Fraser University) were added to lyophilized mucin samples to a final concentration of 2,000 ppb, prior to hydrolysis in 500 μL 2 M acetic acid at 80°C for 3 h. Samples were cooled on ice, centrifuged (Spectrafuge 24D microcentrifuge, Mandel; Guelph, ON, Canada) at 12,000 × *g* for 15 min, and the supernatant was collected and dried on a Savant SPD121P SpeedVac concentrator connected to a Savant RVT5105 refrigerated vapor trap (Thermo Fisher Scientific; Waltham, MA, USA). Sialic acids were labelled with 4,5-dimethylbenzen-1,2-diamine (DMBA) at 24 mM in 40 mM trifluoroacetic acid at 4°C for 16 h, dried, then dissolved in 200 μL 18 MΩ cm^{-1} H_2O and purified by solid-phase extraction on Strata C18-E cartridges (Phenomenex; Torrance, CA, USA). The aqueous 50% ACN eluate was dried *in vacuo* and dissolved in 100 μL aqueous 30% MeOH prior to analysis. External calibration curves for DMBA-labelled Neu5Ac, Neu5Gc, and KDN were prepared concurrently with the mucin samples from stock standard solutions at 8 levels covering 50 ppb to 10,000 ppb and were analyzed immediately prior to the samples. Extracted ion chromatograms for analyte *m/z* values were extracted with a ±15.00 ppm mass accuracy limit and ±0.500 min retention time window, based on their

retention times from the calibration curves. Quantitation was performed using Quantitative Analysis (Agilent) by normalizing analyte peak area to that of $[^2H]_3$Neu5Ac to account for hydrolysis efficiency, and $[^{13}C]_2$-KDN as a global internal standard.

Assessment of C. perfringens *Enzymes Involved in Mucolysis*

ATCC13124 sialidases NanH (CPF_0985), NanI (CPF_0721), and NanJ (CPF_0532) sequences were obtained from NCBI, and a BLASTn search was performed for *C. perfringens* (tax ID: 1502) genomes across all annotated strains. Whole genome sequences for *C. perfringens* strains ATCC13124, 13, SM101, FORC_003, JP55, JP838, FORC_025, LLY_N11, CBA7123, EHE-NE18, JXJA17, NCTC13170, NCTC2837, Del1, CP15, and F262 were acquired from the NCBI genome database, and protein sequences for NanH, NanI, and NanJ were aligned in Geneious (Geneious 11.1.4, Biomatters Ltd.) using MUSCLE (Edgar, 2004). NanH, NanI, and NanJ protein sequences from ATCC13124 and Del1 strains were compiled and used as query sequences for SACCHARIS (Jones et al., 2018). Sequences of characterized GH33s were retrieved and accession numbers were remotely extracted from the CAZy database. Best-fit model selection using the sequence alignment was performed using ProtTest (Darriba et al., 2011) and FastTree (Price et al., 2010) to generate the tree.

RESULTS

Select Mucin Monosaccharides are Required for C. perfringens *Growth*

A defined medium was optimized from Deplancke et al. (2002) in order to examine the growth profiles of *C. perfringens* CP1 on monosaccharides and *O*-glycans. This MM did not support growth in the absence of additional carbohydrate. Using each of the 6 primary mucin monosaccharides found in chickens (GalNAc, GlcNAc, Fuc, Gal, Man, and Neu5Ac) as sole carbon sources for anaerobic bacterial growth cultures, growth rates were monitored at OD_{600} over 30 h (Figure 1A). Common dietary carbohydrates (Glc, Xyl, Ara, and Rha) were also used as limiting carbohydrates to determine if *C. perfringens* CP1 displayed any specialization for the metabolism of carbohydrates which are not components of the mucus layer. Columbia broth and Glc were used as positive controls, and both were shown to reach peak OD_{600} of 0.94 and 0.80 within the first 20 h, respectively. Gal, Neu5Ac, GlcNAc, and Man also supported growth of the bacterium with peak OD_{600} of 0.71, 0.59, 0.74, and 0.69, respectively over the 30 h incubation. Bacterial growth on Man, Glc, Gal, and GlcNAc displayed similar growth profiles and each had a lag phase of approximately 5 h,

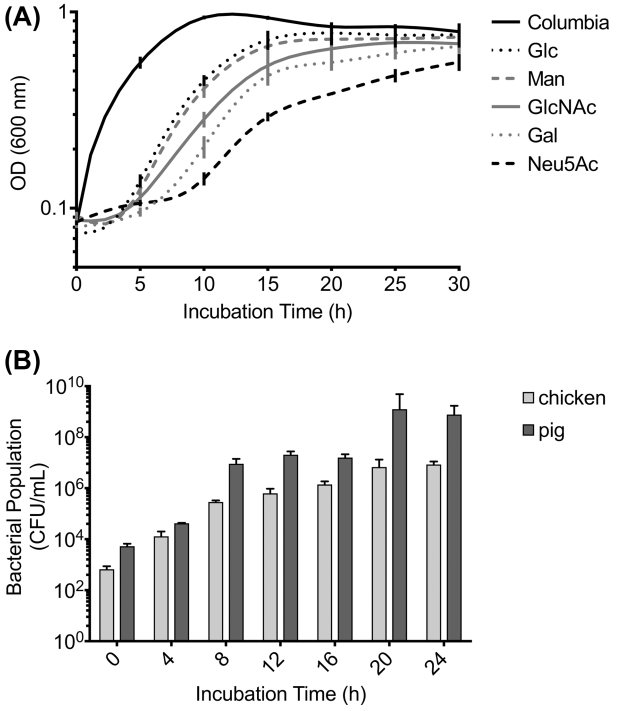

Figure 1. *Clostridium perfringens* utilization of intestinal mucus and mucus monosaccharides. (A) Bacterial growth profiles were measured by OD_{600} when using pure monosaccharides as a sole carbon source in minimalized medium (**MM**). Curved lines were drawn using locally weighted scatterplot smoothing (LOWLESS) in GraphPad Prism (GraphPad Software). Error bars represent SEM for 3 biological replicates. (B) *Clostridium perfringens* CP1 growth on crude chicken intestinal mucus (light grey) and pig gastric mucin (**PGM**) (Type II) (dark grey) was measured by enumerating CFUs per mL of culture. Error bars represent SEM for 3 biological replicates.

with growth appearing to plateau within the 30 h incubation except for Gal-containing medium. Growth on Neu5Ac-containing medium had the longest lag phase of 10 h and demonstrated a linear growth rate that did not reach lag phase during the course of the experiment. The monosaccharides Fuc, GalNAc, Xyl, Ara, and Rha did not display any increases in OD_{600} above baseline (Figure 1A).

Defined Monosaccharides are Released During C. perfringens *Growth on Mucins*

To determine if *C. perfringens* CP1 could be cultured in vitro on a more complex chicken mucin substrate, crude mucus was collected from the small intestine of culled farm broilers. Because of the turbidity and viscosity of native mucus, growth could not be evaluated by optical density; therefore, growth rates were determined by viable CFU counting. *Clostridium perfringens* RS42, Type A was previously reported to grow on PGM (Stanley et al., 1986), and therefore, this substrate was used as a control. Both crude cull bird mucus and PGM supported growth of *C. perfringens* CP1 (Figure 1B); *C. perfringens* CP1 growth reached a maximum average of 8.5×10^6 CFU mL^{-1} at 24 h on cull mucus and 1.2×10^{10} CFU mL^{-1} at 20 h on PGM. CFU mL^{-1} increased

4 orders of magnitude for cull bird mucus and seven orders of magnitude for PGM, supporting the observation that *C. perfringens* can metabolize the monosaccharide components of broiler chicken intestinal mucus glycans in vitro. The higher density growth of *C. perfringens* CP1 on PGM likely results from chemical and protease pretreaments increasing the accessibility of glycan substrates.

The spent medium containing PGM and crude cull bird mucus were analyzed by GC-FID to determine if monosaccharides or small oligosaccharides were released as a byproduct of carbohydrate metabolism during growth (Figure 2). Molar compositions of individual monosaccharides, relative to the total carbohydrate detected in sample, revealed detectable changes following growth (Figure 2A and B). Analysis of PGM samples revealed few changes after incubation with *C. perfringens* CP1 (Figure 2A). Of the 5 monosaccharides analyzed, only Fuc, Gal, and Glc showed statistically significant changes; the relative amount of Fuc increased by approximately 10.9%; whereas both Gal and Glc compositions saw reductions post-incubation of approximately 9.5% and 8.1%, respectively. Cull bird mucus samples, when analyzed by GC-FID, demonstrated an overall pattern of monosaccharide compositional changes similar to that of PGM (Figure 2B). Only 2 monosaccharide compositions changed significantly when incubated with *C. perfringens* CP1; Gal was identified at a level approximately 18.9% less after bacterial incubation, whereas GalN levels increased by approximately 10.7%. As the processing of samples for analysis by GC-FID allows for detection of solely deacetylated monosaccharides, it is assumed that the detection of GlcN and GalN are the direct result from the presence of GlcNAc and GalNAc in the original sample.

For more insight into the structure of oligosaccharide products from a physiologically relevant carbohydrate source, HPAEC-PAD was performed on crude cull bird mucus both in the presence and absence of *C. perfringens* CP1 incubation. As each peak in the chromatogram represents an intact oligosaccharide purified from the sample, complex mixtures of glycans were released from both substrates. Notably, 2 major oligosaccharide peaks present in a region of the chromatograms corresponding to anionic oligosaccharides (Figure 2C) were observed to have disappeared after 24 h bacterial incubation of the cull mucus samples, suggesting a possible removal of negatively charged monosaccharides (e.g., Neu5Ac) or modifications (e.g., sulfate) by *C. perfringens*.

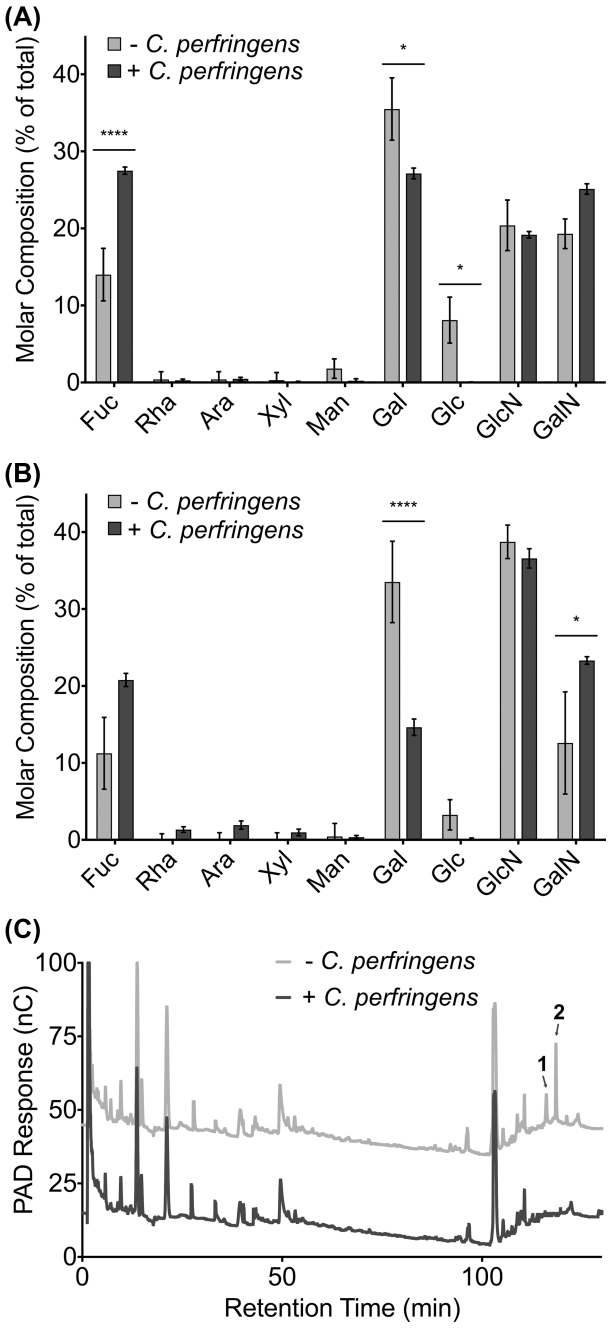

Figure 2. Intestinal mucin *O*-glycan modification by *C. perfringens*. Mucin substrates from pig gastric mucin (**PGM**) (Type II) (A) or culled farm-reared broiler chicken intestinal mucus (B) were used as the sole carbon source for bacterial growth with (dark grey) and without (light grey) *C. perfringens* CP1, and spent bacterial culture was analyzed by gas chromatography with flame ionization detection. Molar composition is calculated as a % of relative abundance. Any statistical significance between control and *C. perfringens* conditions are indicated as $^{****}(P < 0.0001)$ or $^{*}(P < 0.05)$. (C) High-performance anion exchange chromatography coupled with pulsed amperometric detection analysis of culled farm-reared broiler chicken intestinal mucus with *C. perfringens* CP1 (dark grey) and growth medium without bacteria (light grey). Each peak represents an independent population of glycan(s), with 2 distinct peaks highlighted. For all panels, error bars represent SEM for 3 biological replicates.

Clostridium perfringens *can Metabolize Chicken Mucin O-Glycans Extracted Using Ammonia-based Methods*

There are limited chemical methods available for purifying mucin *O*-glycans at yields and purity suitable

for microbiological growth studies (Tierney et al., 2007; Alemka et al., 2010). Therefore, 3 different extraction methods were conducted in order to obtain *O*-glycans from broiler chicken intestinal mucus: ammonia-catalyzed β-elimination (adapted from (Huang et al., 2001)), NaClO ("bleach") oxidation (Song et al., 2016), and proteolysis followed by $NaBH_4$/NaOH ("sodium borohydride") treatment, i.e., reductive β-elimination (Manzi et al., 2000; Martens et al., 2008). Ammonia-catalyzed elimination and bleach oxidation have not been previously performed with crude chicken intestinal mucus, although the sodium borohydride reduction was modified from previous studies (Alemka et al., 2010). Significantly, each *O*-glycan isolation method generates unique product chemistry at the reducing end of the glycan, which may impact its subsequent metabolism. Indeed, in MM supplemented with extracted *O*-glycans, only cull bird mucus that was treated with ammonia supported growth of *C. perfringens* CP1 (Supplemental Figure S1A). CFUs were also quantified over 24 h from 6 biological replicates of *C. perfringens* CP1 grown on ammonia-extracted broiler chicken intestinal mucin *O*-glycans in MM, showing an increase of 2 orders of magnitude over the timeline of the experiment (Supplemental Figure S1B).

Clostridium perfringens *Preferentially Degrades Sialic Acid from Extracted Cull Broiler Chicken Mucin*

As *C. perfringens* CP1 was found to utilize ammonia-extracted mucin *O*-glycans for metabolism and growth in minimalized conditions, spent supernatants from the 33 h cultures were analyzed by GC-FID and HPAEC-PAD to determine whether *C. perfringens* CP1 is capable of modifying the structure of the 3 different *O*-glycan substrates extracted from chicken intestinal mucins. Compositional analysis of these samples by GC-FID showed no significant change in the compositional ratios of these glycans (Figure 3A). Despite a lack of large-scale changes in monosaccharide composition, HPAEC-PAD of the *O*-glycans revealed the presence of several major differences in peak profiles (Figure 3B; Supplemental Table S1). For example, 2 large peaks (peaks 1 and 2) were observed in the *C. perfringens* CP1-treated samples that were not present in the negative control. Additionally, multiple glycans (peaks 3 to 7) disappeared following treatment with *C. perfringens* CP1 in the region consistent with anionic oligosaccharides. This suggested that glycans bearing Neu5Ac or sulfate moieties, carbohydrate species that cannot be detected by GC-FID, were being modified by *C. perfringens* CP1.

It was determined that more chemically-informative insights into the changes in glycan structures were necessary to identify the direct *O*-glycan products following *C. perfringens* growth. Accordingly, high-performance liquid chromatography-mass spectrometry (**HPLC-MS**) was performed on the ammonia-extracted spent supernatants. Glycan structures were identified based on the m/z values for intestinal mucus-borne *O*-glycans previously identified in broiler chickens ((Struwe et al., 2015); Supplemental Table S2). Analysis of the intact *O*-glycans indicated that all Neu5Ac-containing glycans and a number of charged species were completely eliminated by the bacteria to below the detection limit of HPLC-MS. Although direct comparisons between different glycan species is not possible without absolute quantitation, the decrease in sialylated and sulfated glycans corresponded with statistically significant increases in several neutral species treated with *C. perfringens* (Figure 4A). In order to test whether the Neu5Ac (and related analogues Neu5Gc and KDN) cleaved from *O*-glycans were metabolized

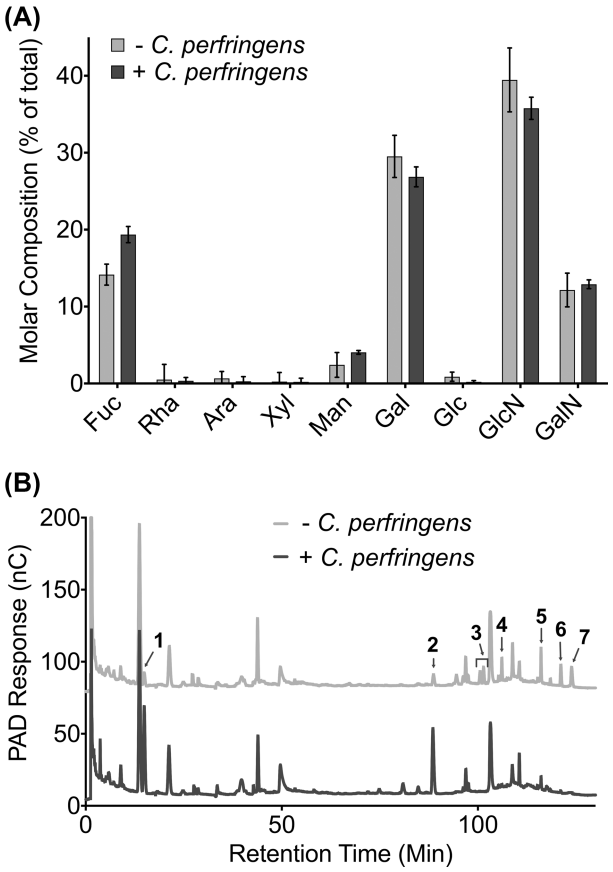

Figure 3. *Clostridium perfringens* modification of ammonia-extracted chicken mucin *O*-glycans. (A) Gas chromatography with flame ionization detection analysis of growth medium with (dark grey) and without (light grey) *C. perfringens* CP1 bacterial culture following ammonia-catalyzed extraction of *O*-glycans from chicken mucin. Molar composition is calculated as a percentage of relative abundance. No statistically significant differences between control and *C. perfringens* conditions were observed for each mucin monosaccharide. Error bars represent the SEM for 3 biological replicates. (B) High-performance anion exchange chromatography coupled with pulsed amperometric detection analysis of ammonia-extracted chicken mucin *O*-glycans with *C. perfringens* CP1 (dark grey) and growth medium without bacteria (light grey). Each peak represents an independent population of glycan(s). Six distinct peaks and 1 peak region have been highlighted.

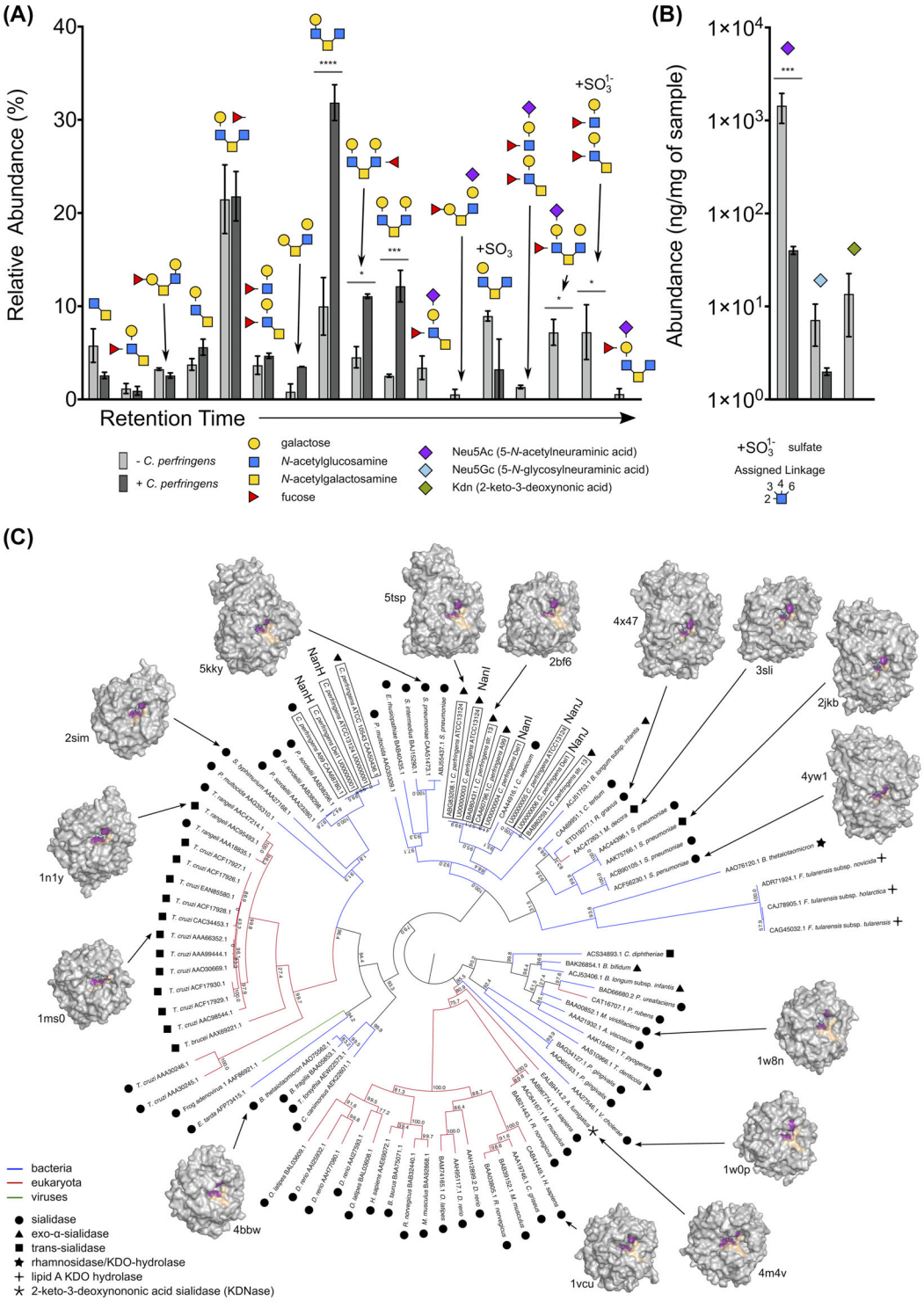

Figure 4. *Clostridium perfringens* preferentially modifies *N*-acetyl-D-neuraminic acid (**Neu5Ac**)-containing *O*-glycans in vitro. (A) Reducing *O*-glycans extracted from intestinal mucus using ammonia-catalyzed β-elimination from culled birds were added to culture media in the absence or presence of *C.* perfringens for 33 h after which they were reduced, desalted and analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS); each condition was tested in triplicate. Absolute MS detector responses for each glycan are reported as a percentage of the total signal from all glycans detected in each sample. Relative peak areas are reported with error bars representing the SEM. Any statistical significance between control and *C. perfringens* conditions are indicated as $^{****}(P < 0.0001)$, $^{***}(P < 0.001)$, or $^{*}(P < 0.05)$. One sulfate- and all Neu5Ac-containing *O*-glycans detected in the *C. perfringens* sample were at levels below that of the method detection limit. Monosaccharides are represented by standard symbol nomenclature (Varki et al., 2015), and assigned saccharide linkages are as indicated. (B) HPLC-MS quantitation of Neu5Ac, Neu5Gc, and KDN present after the incubation of intestinal *O*-glycans with *C. perfringens*. (C) A phylogenetic tree of characterized GH33 (n = 84) was plotted using 6 seeded query sequences. Boxed GenBank accession IDs highlight sequences from *C. perfringens* (Table S3), and query enzymes NanH, NanI, and NanJ are labeled. Coloured lines represent the taxonomic source of the protein sequence, as labelled. The CAZy database annotated function, as per protein name, is represented by symbols as shown. GH33 with known 3D protein structures were mapped onto the tree and are indicated by their PDB ID. Representative structures were chosen for those proteins with multiple known structures. Rendered surface models are shown (grey) with highlighted conserved catalytic (purple) and carboxylate interaction (wheat) residues. Bound products are represented as sticks (carbon = teal, nitrogen = blue, oxygen = red).

by *C. perfringens* under these assay conditions, the abundances of several sialic acid species were directly quantitated (Figure 4B). A statistically significant 1 Log reduction in Neu5Ac was observed following *C. perfringens* incubation, as well as the complete elimination of KDN, a deaminated sialic acid. Less of the diet-derived Neu5Gc was also observed in the *C. perfringens*-treated samples.

Clostridium perfringens *Sialidases are Closely Related to Characterized Sialidases from Pathogenic Organisms*

Sialidases have been previously identified in *C. perfringens* and have demonstrated activity for binding and cleaving Neu5Ac from substrates (Shimizu et al., 2002; Boraston et al., 2007; Newstead et al., 2008; Li and McClane, 2014; Li et al., 2017). Furthermore, sialidases have been hypothesized to contribute to virulence and pathogenicity of *C. perfringens* in chickens affected by NE (Li et al., 2016). For these reasons, the relatedness of *C. perfringens* CP1 sialidases (i.e., GH33-family enzymes) with other known GH33s was examined. Due to the genome for *C. perfringens* CP1 not being available, protein sequences for the homologous NanH, NanI, and NanJ from *C. perfringens* ATCC13124 and Del1 strains were embedded into a phylogenetic tree of characterized GH33s using SACCHARIS ((Jones et al., 2018); (Figure 4C)). Importantly, bacterial species for which GH33s have been characterized are predominantly animal zoonotic pathogens (Lombard et al., 2014), which suggests that they may share a common role in pathogenicity (Corfield, 1992; Corfield et al., 1993; Juge et al., 2016). Some eukaryotic sequences are involved in glycan remodeling (Monti et al., 2010), whereas others may contribute to pathogenesis, such as trans-sialidases in *Trypanosoma* (Colli, 1993; Buschiazzo et al., 2002). A total of 34 GH33 proteins were extracted (Supplemental Table S3; 44 bacterial, 39 eukaryotic, and 1 viral GH33) and aligned. As expected, the query sequences aligned most closely with homologous sequences from other *C. perfringens* strains. Nan I and NanJ partition into 2 closely related clades of co-clusters containing "*exo*"-acting sialidases (i.e., active on the terminal Neu5Ac of *O*-glycans), which suggests Nan I and J have a paralogous evolutionary history resulting from gene duplication. These 2 enzymes share a common ancestral sequence with a group of sialadases from distantly-related bacterial animal pathogens, including: *Pasteurella multocida*, a member of the Bovine Respiratory Disease complex; and *Erysipelothrix rhusiopathiae* FUJISAWA, a pathogen that is responsible for erysipelas in pigs and chickens. NanH displays a different evolutionary history. Although it also is characterized as an *exo*-acting sialidase in other *C. perfringens* strains (Lombard et al., 2014), NanH is distantly related to NanI and NanJ, and is indeed structurally very different, lacking ancillary domains and secretion signals present in both NanI and NanJ (Li et al., 2016). Furthermore, NanH branches from a group of eukaryotic sialidases and trans-sialidases (Figure 4C) suggesting there was a horizontal gene transfer event in the history of these GH33s. Protein sequences for GH33s from the genomes of 16 different *C. perfringens* strains were compared by MUSCLE (Edgar, 2004). Demonstrated protein identities of 97.8%, 98.6%, and 97.4% for NanH, NanI, and NanJ, respectively, were calculated. The sequence variation primarily resided in predicted loop regions. The majority of protein residues, including those involved in substrate recognition and catalysis (Newstead et al., 2008), are highly conserved between *C. perfringens* strains. Future research will be required to determine if there is a differential role for NanH, NanI, and NanJ in chicken mucus *O*-glycan modification and intestinal colonization by *C. perfringens* CP1.

DISCUSSION

Clostridium perfringens is an opportunistic enteric pathogen primarily responsible for NE in poultry, and is thought to degrade chicken intestinal mucus as part of the progression of disease (Prescott et al., 2016). Yet, the mechanistic role of *C. perfringens* CP1 in chicken mucin modification, and the metabolism of intact mucin glycans and free mucin monosaccharides had not been reported. In this study, *C. perfringens* CP1 was found to preferentially metabolize monosaccharides that are common in mucin glycans (GalNAc, GlcNAc, Fuc, Gal, Man, and Neu5Ac), with the exceptions of Fuc and GalNAc, whereas sugars potentially encountered in dietary sources (Xyl, Ara, Rha) did not support bacterial growth. Indeed, mucolytic enzymes that release GlcNAc (i.e., NagH, NagI, NagJ, and NagK) and Neu5Ac (i.e., NanA, NanE) from mucin *O*-glycans have been found in *C. perfringens* strains, whereas the appropriate enzymes required for Fuc (e.g., Fcl pathway) and GalNAc (e.g., Aga pathway) metabolism are lacking (Almagro-Moreno and Boyd, 2009; Ravcheev and Thiele, 2017).

The ability of *C. perfringens* to degrade broiler chicken intestinal mucus is considered the most important criteria in the development of NE in poultry (Prescott et al., 2016). Mucin monosaccharide metabolism is dependent upon the ability of a bacterium to release monosaccharides from complex mucin oligosaccharides or scavenge the depolymerization products of other resident bacteria. Interestingly, at least 23 mucolytic enzymes have been characterized or predicted to be involved in mucus modification by *C. perfringens* including: 18 glycoside hydrolases, which hydrolyze glycosidic bonds (Davies and Henrissat, 1995), among them 3 α-L-fucosidases (Fan et al., 2016), and a novel endo-α-GalNAcase (Ashida et al., 2008). Additionally, 3 M60-like zinc metalloproteases (Zmps), which hydrolyze peptides but require recognition of carbohydrates for proteolysis (Noach et al., 2017); and

3 putative sulfatases that could desulfate mucin glycans (Berteau et al., 2006) have also been described. In this regard, the inability of *C. perfringens* CP1 to grow on Fuc and GalNAc (Figure 1) suggests that the metabolism of all monosaccharides in mucus is not necessary for colonization, however, removal of these monosaccharides may be important to enabling complete saccharification of chicken *O*-glycans. The majority of mucolytic glycoside hydrolases in *C. perfringens* are secreted into the extracellular milieu (Ashida et al., 2008) where their activities would facilitate disruption of the mucus layer. Notably, these enzymes are complex multi-modular proteins; specific ancillary modules promote enzyme complex formation and adherence to carbohydrate receptors (Boraston et al., 2007; Adams et al., 2008; Ficko-Blean et al., 2009; Ficko-Blean et al., 2012; Ficko-Blean and Boraston, 2012b; Grondin et al., 2017; Noach et al., 2017), which would facilitate disruption of the mucus layer. Growth studies on more native structures such as extracted *O*-glycans and mucus may help illuminate these relationships.

Clostridium perfringens *Growth on Chicken O-Glycans and Intestinal Mucus*

For the first time, we have observed that broiler chicken intestinal mucus is a substrate metabolized by *C. perfringens* CP1. Furthermore, when the spent supernatants of the ammonia-extracted chicken mucus growth cultures were analyzed by HPAEC-PAD, there was almost a complete reduction in the amount of glycans that elute after 110 min (Figure 3B); and importantly, glycans that elute within this region are commonly anionic and includes sulfated and sialylated glycan structures. Broiler chicken *O*-glycans are known to be enriched in Neu5Ac and sulfated residues (Struwe et al., 2015), whereas charged mucin *O*-glycans comprise less than a third of the total glycan population in PGM and human gastric mucins (Karlsson et al., 1997; Rossez et al., 2012). The rate of mucin degradation is known to depend on the efficiency of removing the terminal residues of colonic mucin oligosaccharide chains (Corfield et al., 1993). The nearly complete elimination of charged glycans in chicken mucus post bacterial growth observed here (Figure 2C), suggests that clearance, and potentially metabolism, of charged mucins may be a key step in the colonization of *C. perfringens* CP1 in vivo.

To evaluate the growth proficiency of *C. perfringens* CP1 on extracted *O*-glycans and optimize an in vitro growth method for screening mucolytic activity, 3 different reactions were performed on crude chicken intestinal mucus: classical alkaline β-elimination (i.e., sodium borohydride) (Manzi et al., 2000; Martens et al., 2008), oxidation (i.e., bleach) (Song et al., 2016), and ammonia-catalyzed, non-reductive β-elimination (i.e., ammonia) (Huang et al., 2001). These reactions generate unique chemistries at the reducing end of the glycan. Of the 3 *O*-glycan products, only the ammonia method supported growth of *C. perfringens* CP1 (Supplemental Figure S1). Ammonia-catalyzed reactions resulted in the highest yield of *O*-glycans, though different glycan purification strategies were used. The reductive β-elimination and oxidation methods both utilize a highly alkaline-based reaction medium which risk excessive salt contamination (Huang et al., 2001) and uncontrollable "peeling reactions" (Huang et al., 2001; Song et al., 2016). It may be possible that due to the differential cleanup methods residual bleach or alkaline reagents may interfere with bacterial growth. Although *Bacteroides thetaiotaomicron* had previously been shown to metabolize extracted glycans from PGM by alkaline β-elimination (Martens et al., 2008), the differences in glycan composition between PGM and crude chicken intestinal mucus, including the increase of sialo- and sulfomucins, may have interfered with the extraction method. Whereas ammonia-based extraction methods may still be at risk for salt contamination and "peeling reactions" resulting in a loss of terminal glycans, these effects should be minimized, as evidenced by the highest relative yield and diversity of *O*-glycans extracted. Thus, ammonia-catalyzed elimination may be more favorable to use for future bacterial mucin *O*-glycan growth studies.

Clostridium perfringens *Utilization of Neu5Ac-containing Mucin O-Glycans*

Analysis of the spent ammonia-extracted glycan *C. perfringens* growth supernatants by GC-FID did not discern any significant differences in glycan structure (Figure 3A). However, HPAEC-PAD revealed that the *O*-glycan species, which elute in range of the chromatogram associated with negatively charged oligosaccharides, were depleted (Figure 3B, peaks 3 to 7). Importantly, Neu5Ac and sulfated monosaccharides are not detectable by GC-FID. This suggests that *C. perfringens* CP1 can remove charged groups (e.g., Neu5Ac or sulfate), which is consistent with 2 possible and non-exclusive outcomes: the bacterium may release Neu5Ac to be used as a carbon source, or may remove these terminal anionic residues to expose underlying *O*-glycan carbohydrates. Quantitative LC-MS analysis confirmed that all Neu5Ac-containing glycans and a number of sulfated species were completely eliminated by the bacterium (Figure 4A) and there were decreases in total Neu5Ac and KDN following incubation with *C. perfringens* CP1 (Figure 4B). Removing Neu5Ac or sulfate groups from *O*-glycans to gain access to underlying monosaccharides is a common microbial strategy, and consistent with an *exo*-mode of glycan degradation for dismantling and metabolism of mucus carbohydrates (Tsai et al., 1992; Mougous et al., 2002; Rho et al., 2005; Ng et al., 2013; Huang et al., 2015; Tailford et al., 2015; Sicard et al., 2017). *Clostridium*

perfringens was the first bacterial species identified to have neuraminidase activity (Nees et al., 1976), and this process is well-documented across a variety of strains (Walters et al., 1999). Metabolism of Neu5Ac (Figure 1A) and the depletion of total Neu5Ac and KDN (Figure 4B) may be one of the contributing factors promoting host specificity of this pathogen as it does not appear to be selective for Neu5Gc; chickens are unable to naturally biosynthesize Neu5Gc, obtaining it solely from the diet. Detailed studies of the specificity of *C. perfringens* neuraminidases isolated from different hosts would help reveal such interactions. In contrast, sulfatase activity in *Clostridia* is rare (Sardiello et al., 2005; Berteau et al., 2006), and the role of *C. perfringens* sulfatases in mucin *O*-glycan modification and degradation remains to be described.

Clostridium perfringens is the etiological agent of several human intestinal diseases, including hemorrhagic necrotizing gastroenteritis (Petit et al., 1999), acute food poisoning and antibiotic-associated diarrhea (Rood and Cole, 1991). In domestic livestock, it is responsible for a wide range of enteric diseases (Niilo, 1980), including NE in poultry (Prescott et al., 2016). The role of bacterial sialidases in pathogenicity has been postulated for many years (Corfield, 1992), and sialidases have been characterized for a number of intestinal and respiratory pathogens (Figure 4C). A similar mucin-degrading strategy has been shown in *Ruminococcus gnavus*, a bacterial pathogen known to incite inflammatory bowel diseases (Crost et al., 2016). Furthermore, bacterial sialidases have been shown to promote *in vivo* growth and colonization of the human or animal intestinal tract (Lewis and Lewis, 2012). Prominent examples of bacteria capable of utilizing Neu5Ac for disease pathogenesis include *Streptococcus pneumoniae* (Kahya et al., 2017), *Vibrio cholera* (Almagro-Moreno and Boyd, 2009; Almagro-Moreno et al., 2015), and *Salmonella enterica* (Almagro-Moreno and Boyd, 2010). The presence of host mucin sulfation and sialylation may work cooperatively to inhibit bacterial adhesion to the mucus layer and sequential translocation to the epithelium (Al-Saedi et al., 2017; Hasnain et al., 2017). Sialylation and overall anionic charge density of intestinal mucin *O*-glycans is much greater in chickens than reported in humans (Struwe et al., 2015). The increase of sialomucins and sulfomucins within the intestinal mucus layer of chickens has been shown to attenuate the binding and colonization of *C. jejuni*, a human pathogenic bacterium that is an abundant member of the chicken intestinal microbiota (Alemka et al., 2010; Struwe et al., 2015). Exploring differences in the sialomucin and sulfomucin glycan composition and acidification may also uncover why some bacterial species are pathogenic in one animal host but commensal in another. Future studies are required to identify how bacterial Neu5Ac utilization in the intestinal tract may circumvent this phenomenon.

CONCLUSION

This study has demonstrated that *C. perfringens* CP1 utilizes components of mucin as a nutrient source and has specialized mechanisms for the metabolism of *O*-glycans and mucus in chickens. Significantly, *C. perfringens* was shown to both remove and metabolize Neu5Ac, a terminal residue in chicken intestinal mucin *O*-glycans (Struwe et al., 2015) from complex substrates. These events have been linked to important roles in the colonization of enteric pathogens and intestinal inflammation (Huang et al., 2015). Evaluation of specific *C. perfringens* mucolytic enzymes deployed to dismantle chicken intestinal mucin in vitro will help illuminate the mechanism by which mucin is actively degraded by *C. perfringens* CP1. Understanding the interaction between *C. perfringens* CP1 and chicken mucus will also help define the relationship between colonization and the onset of NE. This information could inform the development of novel interventions that may prevent the early stages of NE and improve the performance of poultry.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Figure S1. *Clostridium perfringens* growth profiles on broiler chicken intestinal mucin 979 O-glycans from 3 extraction methods.
Table S1. HPAEC-PAD peak areas for culled chicken intestinal mucus O-990 glycans.
Table S2. *Clostridium perfringens* modification of ammonia-extracted chicken mucin O-glycans—list of detected O-glycans.
Table S3. GH33 family members from a tree of characterized enzymes.

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