GLYCOMIC ANALYSIS OF THE *IN VITRO* MODIFICATION AND UTILIZATION OF CHICKEN INTESTINAL MUCIN *O*-GLYCAN CARBOHYDRATES BY THE ENTERIC POULTRY PATHOGEN *CLOSTRIDIUM PERFRINGENS*

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Dedication

I would like to dedicate this body of work to my mother.

Abstract

Clostridium perfringens is the etiological agent of necrotic enteritis (NE) in poultry. NE occurs within the small intestine and is characterized by intestinal mucus depletion and tissue inflammation. This disease costs the global poultry industry an estimated USD $2-6 billion per year. The small intestine mucus glycans of birds contain a large abundance of sialic acid (Neu5Ac) and sulfate, which may impede colonization by enteric avian bacterial pathogens. *C. perfringens* was shown here to liberate and metabolize Neu5Ac from extracted broiler chicken mucin *O*-glycans. By correlating these findings with carbohydrate active enzymes active on mucin *O*-glycans and the core metabolic enzymes present within *C. perfringens*, a biochemical basis for these relationships can be established. These new findings suggest that Neu5Ac utilization is significant for mucus colonization by *C. perfringens* and may provide *C. perfringens* with a competitive advantage in the avian intestine

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AAFC-AID	Agriculture and Agri-Food Canada - Animal Industry Division
AGP	antimicrobial growth promoter
Ara	D-arabinose
Asn	asparagine
BHI	brain heart infusion
BHIS	supplemented brain heart infusion
BIG	bacterial immunoglobulin-like
CAZome	collection of carbohydrate-active enzymes encoded by the genome of an organism
CAZyme	carbohydrate-active enzyme
CBM	carbohydrate-binding module
CE	carbohydrate esterase
CFG	Consortium for Functional Glycomics
CFU	colony forming unit
CL2	containment level 2
CPMM	*Clostridium perfringens* minimalized medium
CSB	Columbia sheep blood
ddH2O	distilled deionized H2O
DTT	dithiothreitol
FBF	find-by-formula
FIVAR	found in various architectures
Fuc	L-fucose
Gal	D-galactose
GalNAc	*N*-acetyl-D-galactosamine
GC	gas chromatography
GC-FID	gas chromatography - flame ionization detector
GH	glycoside hydrolase
GIT	gastrointestinal tract
Glc	D-glucose
GlcNAc	*N*-acetyl-D-glucosamine
GMO	genetically modified organism
HPAEC-PAD	high-performance anion-exchange chromatography coupled with pulsed amperometric detection
IML	inner mucus layer
LC-MS	liquid chromatography - mass spectrometry
LCU	livestock containment unit
LeRDC	Lethbridge Research and Development Centre
Man	D-mannose
MM	minimalized medium
NE	nectrotic enteritis
Neu5Ac	*N*-acetylneuraminic acid (sialic acid)
OD	optical density
OML	outer mucus layer
PBS	phosphate-buffered saline
PGM	pig/porcine gastric mucin

PHAC	Public Health Agency of Canada
PKD	polycystic kidney disease
PLC	phospholipase C
PUL	polysaccharide-utilization loci
qTOF	quadrupole time of flight
Rha	L-rhamnose
RVT	Registered Vascular Technologist
SEM	standard error of the mean
Ser	serine
SPE	solid phase extraction
TFA	trifluoroacetic acid
Thr	threonine
TLC	thin layer chromatography
TSC	tryptose-sulfite-cycloserine
UC	ulcerative colitis
UV	ultraviolet
Xyl	D-xylose
YE	yeast extract
Zmp	zinc metalloprotease

Chapter 1

The molecular basis of necrotic enteritis in chickens and implications for the poultry farming industry

1.1 Poultry farming at a glance

Poultry production is a prominent agricultural industry in Canada. In 2017, the Canadian chicken and egg industry was valued at $4.4 billion, of which chicken products accounted for $2.5 billion (1). In 2017, there were 2,836 regulated chicken producers in Canada and over 6.9 million chicks exported to 28 countries, which totaled over $23.3 million (1). Livestock market information (Agriculture and Agri-Food Canada - Animal Industry Division (AAFC-AID)) and Statistics Canada have reported a decrease in the consumption of red meat (pork and beef) animal protein in the Canadian diet since 2008 (2), which correlates with a steady increase of consumer demand for chicken and global chicken production (3,4). Although this forecasts a bright economic picture for the industry, it is not without its production challenges as the economics of bird production have very small margins. For example, increasing on-farm intensification must not compromise bird health, food safety, or product quality. Towards this end, understanding the mechanisms of infectious disease in poultry flocks and developing interventions to mitigate detrimental outcomes (i.e. morbidity and mortality) are primary concerns for the industry.

The global poultry industry has traditionally relied on the use of in-feed antimicrobial growth promoters (AGPs) administered at non-therapeutic concentrations to enhance the growth performance of livestock and prevent enteric diseases, such as necrotic enteritis (NE) caused by *Clostridium perfringens* and coccidiosis (5). Although a common agricultural practice since the early 1950s, the mechanism(s) of AGP action still remain(s) unclear. Use of germ-free animals to identify the effects of AGP originally suggested that antimicrobials likely act by remodeling intestinal microflora diversity and relative abundance, which provide host benefits (6,7). New

evidence suggests, however, that AGPs may work in a more complex manner, as modulators of both the immune system and of the intestinal bacterial communities in a cooperative fashion (8).

The success of AGPs in poultry production led to their widespread use, a practice that is believed to have contributed to the emergence of antimicrobial resistance in zoonotic bacterial pathogens (3). The potential for transmission of resistance genes from bacteria present in food animals to bacteria that cause human disease within environment, food, or farm reservoirs has become a public health concern (9). In Canada, the prevalence of antimicrobial resistant food-borne pathogens sourced from chicken products was noticeably high in 2016 (Public Health Agency of Canada, PHAC (10)). Alarmingly, most *Escherichia coli*, *Campylobacter spp*., and *Salmonella spp.* isolates recovered from retail chicken had shown resistance to multiple antimicrobials; and concomitantly, the contamination rates of *E. coli* (96%), *Campylobacter* spp. (25%), and *Salmonella* spp. (34%) were also higher in Canada in 2016 (5,10).

To help mitigate selection for antibiotic resistant bacteria, legislated reductions and changes to poultry production practices were implemented, including limiting use of AGPs (2014) (11). The trend of antimicrobial use in broiler chickens for poultry production in Canada between 2014 and 2016 had therefore shifted toward more disease prevention (69% to 89%) and less for growth promotion (less than 3%) (10). This shift in practice was almost immediately followed by a dramatic increase of associated health problems of broiler flocks, including *C. perfringens* incited NE (12).

1.2 Necrotic enteritis in poultry

First described in 1961, NE occurs within the small intestine of chickens infected with a pathogenic strain of *C. perfringens*, and is characterized by intestinal mucus depletion and tissue inflammation (13,14). NE has been noted as the most common and clinically relevant poultry

disease incited by a bacterium. The resurgence of NE in poultry in the post-AGP era costs the global poultry industry an estimated USD $2-6 billion per year ($0.05 per bird) due to high mortality (i.e. clinical disease) and morbidities leading to reduced harvest weights (i.e. subclinical disease; (15-17)).

Clinical forms of the disease are characterized by the sudden increase of flock mortality, with upwards to 1% losses per day (18). Premonitory signs of clinical NE, including depression, dehydration, decreased feed consumption, diarrhea, and ruffled feathers can be observed; however, clinical NE may also present asymptomatically in broilers (12,13). Necropsies of chickens with clinical NE show a discoloration of mucosal surfaces and cellular debris in the intestinal lumen. Lesions can also be detected within the small intestine and other organs such as the caeca, liver, and kidney (12). The subclinical form of NE, which is primarily asymptomatic, confers no peak mortality, is more prevalent and may persist undetected for prolonged periods in broiler flocks, is most detrimental to the poultry industry (17). Chronic intestinal mucosal damage leads to decreases in bird performance due to poor digestion and nutrient absorption, resulting in reduced weight gain and increased feed-conversion ratios (12). Thus, subclinical NE is responsible for the greatest economic losses in poultry production because of its severe effect on growth rates (17).

Chicken NE is a complex disease involving multifactorial predisposing factors which include: stressors, e.g. overcrowding or physical handling; nutrition, physical, and environmental changes, e.g. ammonia levels in the litter; and the composition of the intestinal microbiota (19-23). The host immune status can also greatly influence NE onset and severity especially when maternal antibodies dissipate within the bloodstream around 3-weeks-of-age. This is considered the peak risk period for NE infection (19). In addition, previous viral or bacterial infections have also been noted to largely influence the susceptibility of birds to NE during this peak phase (19). One prominent NE co-infection, coccidiosis, is caused by parasitic apicomplexans from the

Eimeria genus. Damage to the epithelium by coccidia is believed to be a major predisposing factor for NE (19,24). Predisposing factors and co-infections are believed to promote *C. perfringens* adherence and invasion to different regions of the intestinal mucosa (consisting of the intestinal mucus, epithelium, and lamina propria) in chickens (25,26). One prospective study evaluated the impact of AGP withdrawal, with focus on the rate of *C. perfringens* NE occurrence, in 1.5 million birds across eight commercial broiler farms in Quebec, Canada (27). Unfortunately, this AGP-free program was not a favorable strategy, as the study concluded that there are reductions in bird performance and higher risks of gastrointestinal tract (GIT) disorders within AGP-free broiler flocks.

Developing alternatives to AGPs to prevent NE in chickens is an active area of research and many reviews on the subject are available (18,28,29). Antimicrobial molecules such as small ribosomal synthesized antimicrobial peptides naturally produced by bacteria, termed bacteriocins, and bacteriophages targeting specific bacterial species in the GIT had both been identified as promising candidates for new antimicrobials but were deemed problematic (28,30). As well, the use of engineered bacteria to competitively exclude *C. perfringens* has also been proposed, but consumer resistance to the use of genetically modified organisms (GMOs) in food restricts these options (28). Phytonutrients including herbs and essential oils, hyper-immune antibodies for immune modulation, and probiotics and prebiotics have also been proposed with limited success (31).

The potential for vaccination has also been investigated as a strategy against NE. Live attenuated vaccines were thought to be superior because they often have the ability to be orally administered and early studies have shown the potential of vaccination with live strains (32), yet only partial protection against infection was achieved. Protein-based vaccines which include inactivated bacterial toxins or crude culture supernatants (inactivated or not), were determined to be safer and more characterized compared to live attenuated vaccines (33). Previous work had

demonstrated only limited and intermittent protection when chickens were vaccinated with crude culture supernatants of *C. perfringens* (34,35). The discovery of NetB (36), an essential virulence factor in *C. perfringens*, opened new avenues for the development of vaccines against poultry NE using inactivated bacterial toxins, but only offered partial protection when tested (37). It has therefore become apparent that a deeper understanding of NE pathogenesis will be required to develop effective, antimicrobial-free, on-farm mitigation strategies.

1.3 *Clostridium perfringens*: the etiological agent of necrotic enteritis

C. perfringens is a Gram-positive anaerobic spore-forming, rod-shaped, motile bacterium with type IV pili (38,39). As a species they can produce more than 15 different toxins; however, individual strains only produce a subset of them. *C. perfringens* strains are classified by toxinotype (A through E) based on the ability to produce one or more of the four toxins α, β, ϵ, or l (13,40). Some strains of *C. perfringens* are opportunistic pathogens in human beings (41,42). *C. perfringens* is the second leading cause of death resulting from foodborne infections and third most frequent source of foodborne illness for human beings (43). Thus, *C. perfringens* infections place a large economic burden on public health care systems (44,45) in addition to the poultry industry.

Poultry NE is caused by *C. perfringens* toxinotype A strains, which produce phospholipase C (Cp-PLC or α toxin) and the pore-forming NE toxin (NetB), among other toxins (13). Positive identification of *netB* gene locus within the genome of the majority of *C. perfringens* strains isolated from NE birds suggests this toxin plays a significant role in disease pathogenesis (46-48). The NetB toxin gene is carried on a 42 kb plasmid-encoded pathogenicity locus (NELoc-1), which also encodes 36 accessory genes, each of which seem to play a critical role in NE onset in addition to the toxin in chicken models (49). Numerous studies have

confirmed the presence of NetB as a key factor to incite disease in chickens and turkeys (50,51). In contrast to these results, the genomes of a few strains of NE causing *C. perfringens* isolated from poultry do not encode *netB* (52,53), and more interestingly the prevalence of *netB* in broiler flocks may be limited to geographical areas (54). The question therefore arises as to whether these NetB-negative strains produce alternative toxins for NE or if toxin production is coupled with other yet to be described virulence factors.

The pathogenesis of NE by *C. perfringens* follows classical bacterial infection models, whereby different stages must be completed to progress infection. These involve: colonization at the site of disease, proliferation, acquisition of nutrients to supply rapid proliferation, damage to the host, and transmission (55). *C. perfringens* commonly resides in the intestines of animals, including chickens (40). Invasion of the small intestine mucus layer is an essential prerequisite for nutrient acquisition and the development of NE (13). At this stage adherence to the surface of intestinal cellular structures and subsequent release of toxins allow bacterial access to the deeper regions of the submucosa (55,56). Once established at the innermost areas of the epithelium, chronic destruction of enteric tissue commences. The pore-forming toxin NetB and other *C. perfringens* toxinotype A toxins, such as Cp-PLC, affect membrane phospholipids and have been shown to be necessary for NE lesion induction (13). Intestinal gross lesions due to *C. perfringens* NE vary in both extent and severity, and in some cases lesions may affect the entire small intestine (57). Despite the significance of *C. perfringens* as a bacterial pathogen, many of the factors that contribute to Clostridial disease, including NE, remain to be elucidated.

1.4 The intestinal mucus barrier

The avian (58) and mammalian (59) intestines contain approximately 10^{11}- 10^{14} commensal bacteria, representing upwards of 1000 different species collectively termed the

microbiota. The small intestine is categorized based on three anatomical sections (duodenum, jejunum, and ileum) and empties into the large intestine or colon. The small intestine is the primary site for nutrient absorption and has a distinct cellular morphology of finger-like projections (microvilli) on the apical surface of each epithelial cell (60). Interspersed between epithelial cells are goblet cells which secrete a protective layer of mucus that migrates out toward the lumen (Figure 1.1A). The intestinal mucus layer covers and protects the intestinal epithelial cells from direct contact with the intestinal microbiota and also serves as a protective lubricant against physical damage from digesta (61). The mucus layer is continuous throughout the intestine, consisting of either the tightly adherent layer, which is anchored to the epithelial cells, or loosely adherent layer, which is secreted into the lumen (61). The colon has much thicker mucus layer, compared to the small intestine and lacks villi that are associated with nutrient absorption in the small intestine (Figure 1.1B).

Interactions between the intestinal microbiota and the host occur at the mucus interface. The intestinal mucus layer is effective at excluding pathogenic microorganisms. The inner-most region (a.k.a. "tightly-adherent" layer) of the intestinal mucus barrier is believed to be devoid of bacteria (62); whereas, the loose outermost regions function as a physio-chemical barrier and as a substrate for mucus consuming (i.e. mucolytic) microorganisms (63). Thus, some bacteria can selectively colonize the outermost regions of the mucus while the tightly adherent layer remains relatively sterile. Invading bacteria can also be expelled from the mucus layer in a matter of hours through peristalsis and mucus sloughing (61). In human beings, it has been shown that variation in intestinal mucus thickness is hypothesized to be correlated with microbial density which increases toward the colon (63). The abundance of bacteria is highest in the large intestine where colonization of the epithelium is challenged by a thick mucus layer (~700 μm) and more rapid mucus turnover, whereas in the small intestine mucus thickness is much less (100-400 μm) (60). This is likely true for avian intestinal mucus thickness as well, as mucin glycoprotein

concentration increases longitudinally along the small intestine (64), correlating with an increase in the relative abundance of bacteria toward the caeca; blind pouches located between the small intestine and colon that have rather slow passage rate and a dense microbiota (65,66).

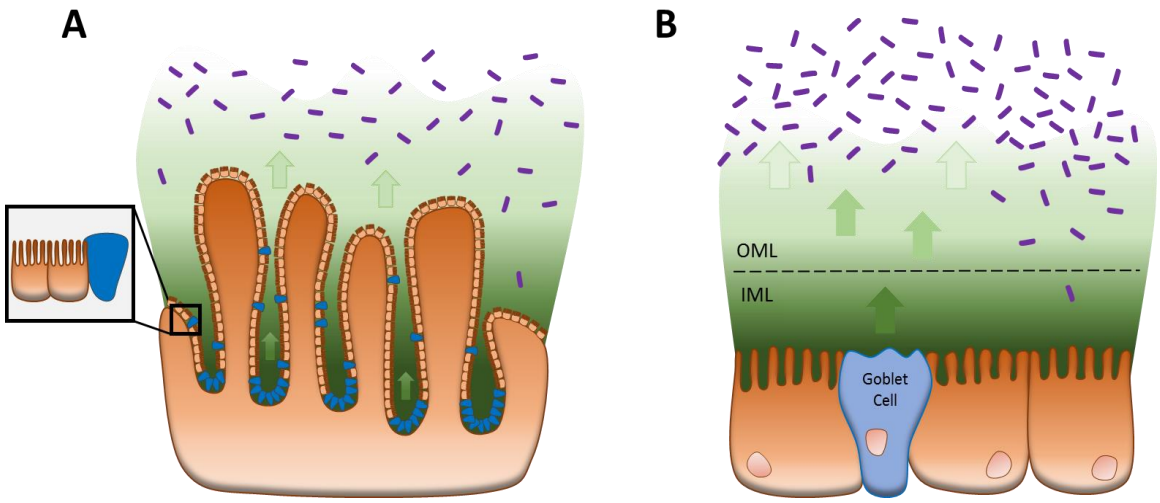


Figure 1.1: Intestinal mucus barrier. The mucus layer of the human and avian intestinal tract is continually maintained by goblet cells (shown in blue) which produce glycoproteins called mucins. The small intestine (A) differs from that of the large intestine (B) by presenting a uniform mucus layer that is less viscous, has decreased thickness, and contains more neutral mucins. The large intestine possesses a thicker mucus layer comprised predominately of acidomucins structured as a loosely adherent outer mucus layer (OML) and a tightly bound inner mucus layer (IML). Commensal bacteria (shown in purple) utilize the outermost regions of this mucus, harvesting carbon sources from the mucins that are not packed tightly, whereas the innermost regions are believed to be devoid of bacteria (62).

The composition of intestinal mucus is variable throughout the intestines and can dynamically respond to changes in microbiota richness and diversity (63). Intestinal mucus is composed of complex glycoproteins called mucins, which contain glycan-rich domains that provide preferential binding sites or substrates for mucolytic bacteria (67). Some enteric pathogens have evolved host-specific strategies for disrupting and utilizing intestinal mucins as a nutrient source (60,68,69). In this regard, mucin utilization can confer a competitive advantage within a complex microecosystem. The direct process by which mucus is degraded and utilized by the enteric bacteria is largely unknown for many enteric pathogens, including *C. perfringens*.

1.5 Mucin glycans

Intestinal mucus is comprised primarily of water, but also contains salts, lipids, cholesterol, glycoproteins, as well as immune-active or host defense agents such as immunoglobulins, lysozymes, host defense peptides, among others (70). The main structural components of intestinal mucus are mucins, which endow it with viscous, gel-like properties. Mucins are predominantly carbohydrate (up to 80% by dry weight), which can be in the form of an array of diverse linear or branched glycan decorations (71,72). Intestinal mucins are dominated by *O*-linked glycans conjugated to free hydroxyl groups of serine (Ser) or threonine (Thr). *N*-linked mucin glycans, whereby the glycan is appended to asparagine (Asn) residues in mucins are also present (Figure 1.2) (71). The complex mucin glycosylations are arranged in a "bottle brush" configuration from the protein core (70). Intestinal mucins have been difficult to characterize, because of their heterogeneity in size (from 200-500 kDa (70) to 2.7-7 MDa (73,74)), high degree of structural complexity (75,76), and loss of viscoelastic properties *in vitro* (77). Core *O*-glycosylation structures have; however, been identified for both human (78) and chicken (79)

intestinal mucins. These core structures present remarkable structural diversity upon which further glycosylation can occur (Figure 1.2). Small modifications of extended core structures have

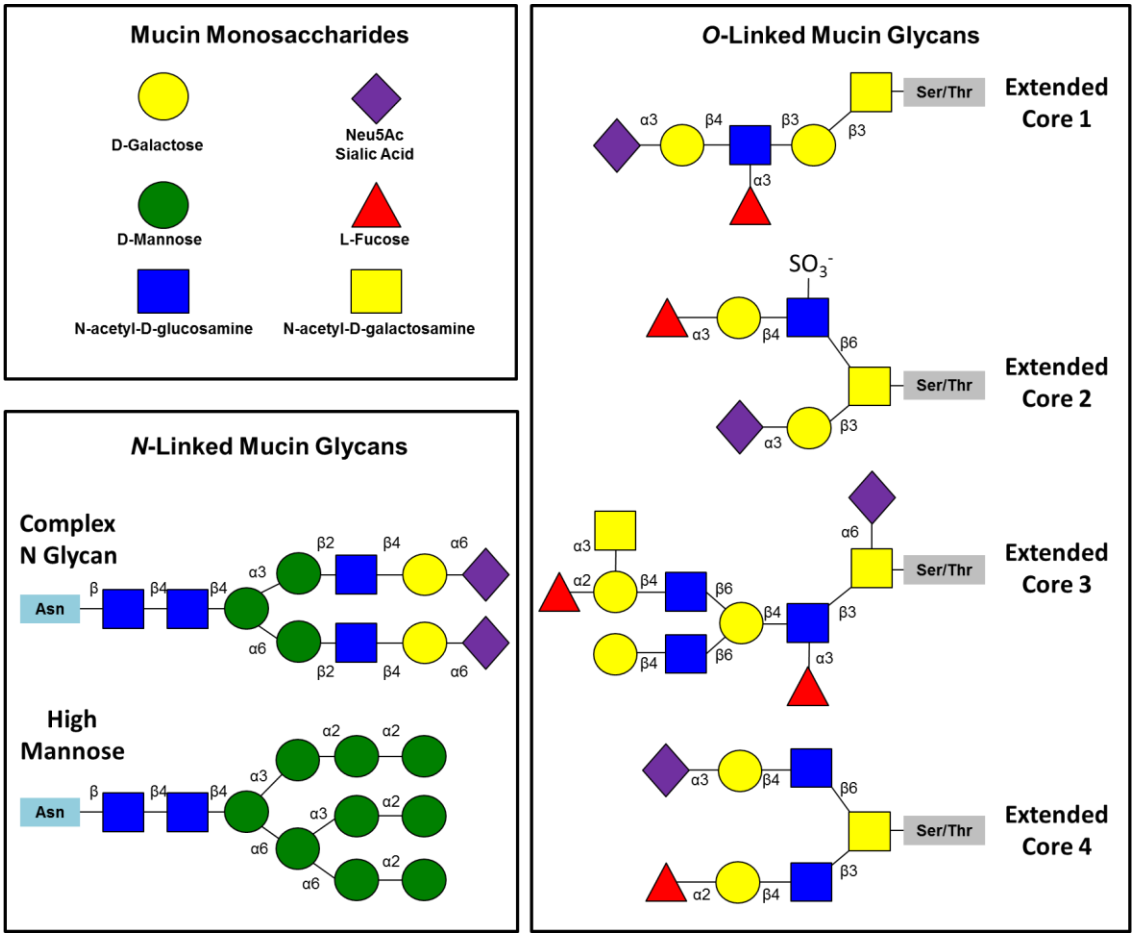


Figure 1.2: *N*- and *O*-linked extended core glycan structures of intestinal mucins. Schematic representation of human intestinal mucin glycan extended core structures (76,80) based upon nomenclature committee Consortium for Functional Glycomics (CFG) (81). Glycosidic linkage is shown here in a condensed notation where C1 of neutral monosaccharides are not assigned, and the C2 of Neu5Ac residues also lack linkage assignments.

been observed in chickens, which differ from human small intestinal core structures, such as an overall decrease of terminal L-fucose (Fuc), and a substantial increase in *N*-acetylneuraminic acid (Neu5Ac, sialic acid) and sulfates; however, most core mucin glycans are observed across species (79).

The oligosaccharide composition of mucins contains variable sequences, branches, and glycosidic linkages of six primary monosaccharides: Fuc, Neu5Ac, D-galactose (Gal), D-mannose (Man), *N*-acetyl-D-galactosamine (GalNAc), *N*-acetyl-D-glucosamine (GlcNAc), and terminal sulfation (82). Mucin *O*-glycans containing terminal Neu5Ac or sulfates, termed sulfo- or sialomucins have been shown to serve an important role in higher-order mucin structure as they increase mucus viscosity (83) and limit bacterial degradation and adhesion (84,85). Intestinal mucus extracted from human (78) and mice (86) demonstrate a distinct acidic gradient comprised of highly charged sulfomucins and sialomucins along the intestinal tract with increasing concentrations in the colon. This mucin gradient seems to correlate with the increased abundance of bacterial populations toward the colon and may confer additional protection from bacterial invasion (83). Interestingly, this charge gradient is not conserved across species. In broiler chickens, sulfo- and sialomucin densities differ from human intestinal mucin *O*-glycans (78,79) and display a greater number of sulfo- and sialomucins relative to neutral mucins in both the small and large intestine (79). In addition, several mucin *O*-glycan structures were identified in broiler chickens that were not previously identified in human beings, further suggesting that there is species diversity in the glyco-structures of mucus (79).

The oligosaccharide composition of mucins has remarkable structural diversity, yet its role in the maintenance of gut health is still largely unknown. Much progress has been made with respect to the ultrastructure of poultry intestinal mucin (79). Recent studies have suggested that chicken mucins may be responsible for preventing colonization by bacterial pathogens and that enrichment of Neu5Ac and sulfate residues may prevent mucus colonization by *Campylobacter*

jejuni (79). In contrast, *C. perfringens* has been demonstrated to thrive and cause disease in this heavily-sulfated and sialylated environment; however, the molecular basis of this interaction remains to be determined. In this regard, structural information on mucin structure before, during, and following *C. perfringens* colonization would be informative.

1.6 Intestinal mucin utilization by enteric pathogens

The role of mucins in preventing colonization of bacterial pathogens, such as *Yersinia enterocolitica* and *Shigella flexneri* in humans (71) and *C. jejuni* in chickens (87), has been established. Some bacterial pathogens have acquired the ability to degrade intestinal mucus, including: *Vibrio cholerae* (59), *Bacteroides* spp. (68,88-90), and *Ruminococcus gnavus* (91). Given the structural diversity and complexity of intestinal mucins, this process relies upon genome encoded collections of carbohydrate-active enzymes (CAZymes) (55,91-94) within mucolytic bacteria.

Mucin-degrading enzymes of gut bacteria have been well-studied in Bacteroidetes. Transcriptomic analysis of *Bacteroides thetaiotaomicron* demonstrated that specific polysaccharide-utilization loci (PULs), including genes coding for putative glycoside hydrolases (GHs) such as α-fucosidase, endo-β-*N*-acetylglucosaminidase, endo-β-galactosidase and α-mannosidase, were up-regulated when the bacterium was grown on mucin *O*-glycans (68). Furthermore, analysis of *Bacteroides fragilis* genome confirmed that *Bacteroides* spp. contained a much larger number of genes encoding mucolytic enzymes compared to other sequenced intestinal bacteria, suggesting mucin degradation is a complex enzyme-mediated process (95), and relies on the specific binding, degradation, and transport of mucin carbohydrates (93). One explanation why mucin *O*-glycans are so structurally complex in intestinal mucus may be to deter its degradation by the intestinal microbiota (69). Martens *et al*., suggested that some intestinal

bacteria have adapted as mucin specialists, perhaps by optimizing enzymatic strategies for *O*-glycan deconstruction; focusing on the most abundant, and therefore most economical to harvest, carbohydrates and linkages that are present (69).

The ability of *C. perfringens* to penetrate the mucus barrier in broiler chickens may be the most important aspect for the onset of NE (55). Preliminary data suggests that *C. perfringens* possesses elaborate metabolic arsenals (Figure 1.3) finely-tuned to dismantle mucin glycans (Figure 1.4; Table 1.1). This system contains over 50 predicted GHs, some of which are known virulence factors, unique glycoproteases, and sulfatases (Figure 1.3) (96-110). In support of this hypothesis, *C. perfringens* has been shown to modify mucins in the intestine of pigs (111) and grow on pig gastric mucin (PGM) as a sole carbon source (112); deglycosylation models using *C. perfringens* CAZymes neuraminidase and α-N-acetylgalactosaminidase on submaxillary mucins also confirmed that enzymatic degradation of mucin glycans chemical modifies its architecture, resulting in a loss of its viscoelastic properties (113); animal models validated the ability of *C. perfringens* to affect mucus ultrastructure during NE (114); whole genome sequencing analyses has revealed that there is strain specificity in the NE-causing isolates of *C. perfringens* (115,116); and different isolates of NetB producing strains have variable genomic loci that could contribute to alternative pathogenesis mechanisms in NE causing strains of *C. perfringens* (116).

Understanding the complex interaction between dietary and mucus-derived glycans and *C. perfringens* during the onset of NE will help illuminate new aspects of how the intestinal microbiota contributes to health and disease. Expanding our knowledge of the roles of CAZymes and other mucolytic enzymes in the etiology of subclinical and clinical NE in chickens is critical for developing innovative therapeutic approaches. For example, glycoproteases (97) and sialidases (117) have been identified in many bacterial pathogens, including *Vibrio cholera* (85) and *Ruminococcus gnavus* (118), which enable for mucin degradation. Mucolytic enzymes could therefore represent key enzyme targets for inhibition with small molecules in *C. perfringens*.

Additionally, the impact of additional factors which are present in animal agriculture, such as stress and diet on intestinal mucin structure must also be considered. In this regard, a cooperative approach to best-practices and mucolytic enzyme inhibitors may provide non-GMO and non-antibiotic interventions for NE, and contribute to the sustainability, including profitability of the poultry sector in Alberta.

With this regard, it is hypothesized that *C. perfringens* uses a complex arsenal of mucolytic enzymes, including CAZymes, to deconstruct and metabolize the released monosaccharides from intestinal mucin *O*-glycans. To test this, mucin *O*-glycans will be extracted from native state broiler chicken intestinal mucus and the ability of *C. perfringens* to deconstruct the mucin glycan substrate and metabolize released carbohydrates will be analyzed using analytical techniques, such as chromatography and mass-spectrometry. To date, the use of native state mucus has not been attempted for the release and purification of intestinal mucin *O*-glycans, and the ability of *C. perfringens* to utilize intestinal mucus as a sole carbon source is also unknown. These newly established protocols and findings will serve as a foundation upon which further insight toward mucin utilization strategies by enteric pathogens may be uncovered.

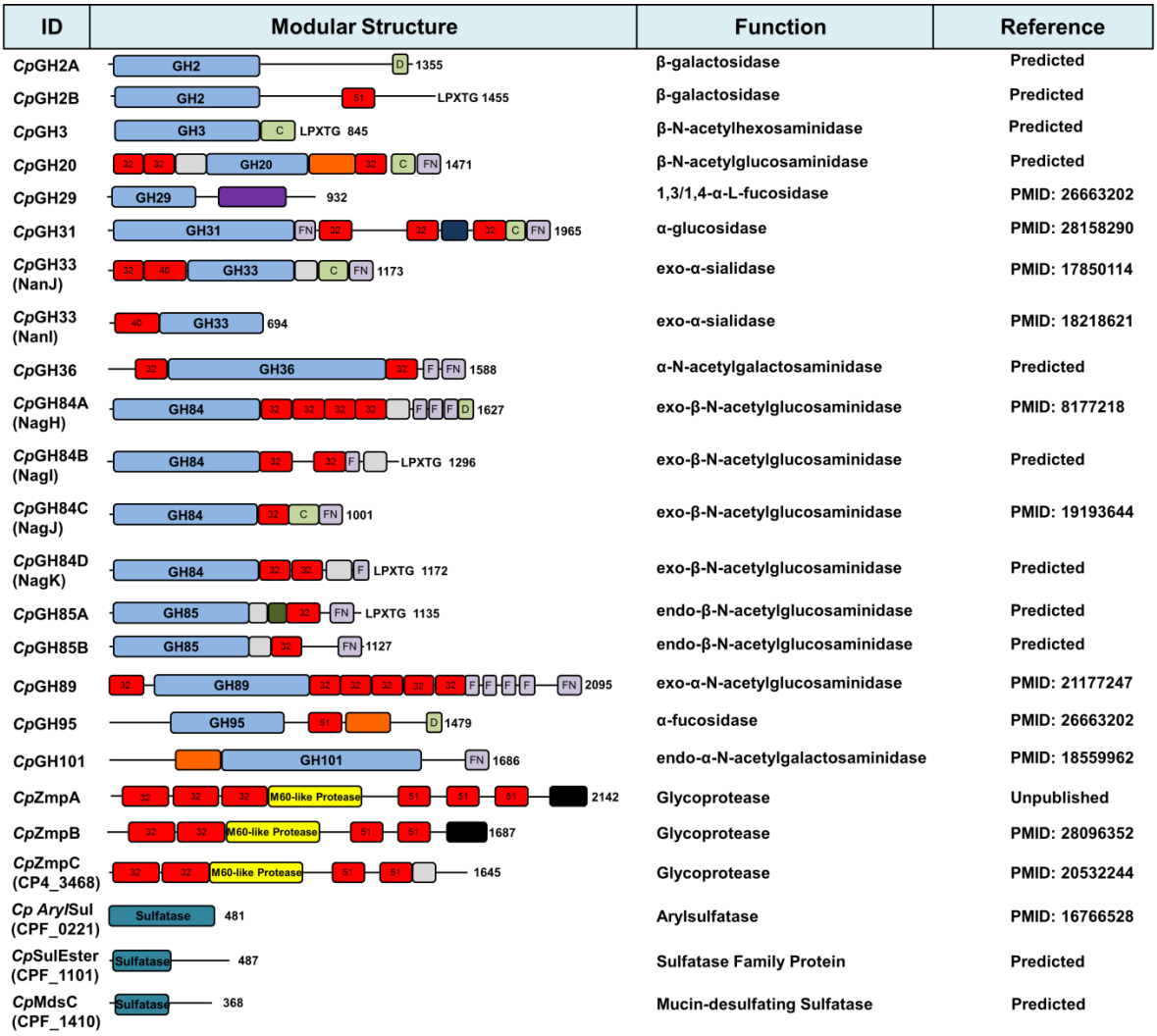


Figure 1.3: Molecular basis of *O*-glycan metabolism by *C. perfringens*. Deconstruction of broiler chicken intestinal mucus is predicted to be carried out by a collection of mucolytic enzymes in *C. perfringens*. Modular structures of *C. perfringens* mucolytic enzymes include: 18 glycoside hydrolases (GHs) (light blue), 3 glycoproteases (M60-like protease) (yellow), and 3 sulfatases (teal). Ancillary domains such as carbohydrate-binding modules (CBMs) (red) have their CAZy family classification designated, cohesins (C) and dockerins (D) (light green), FIVAR modules (F) and fibronectin-type III modules (FN) (light purple), ConA-like (orange), BIG (navy blue), PKD (dark green), CalXβ (dark purple), internalin (black) and modules of unknown

function (grey) are also included in modular schematics. Number denotes length of enzyme in amino acids and protein schematics are drawn to scale.

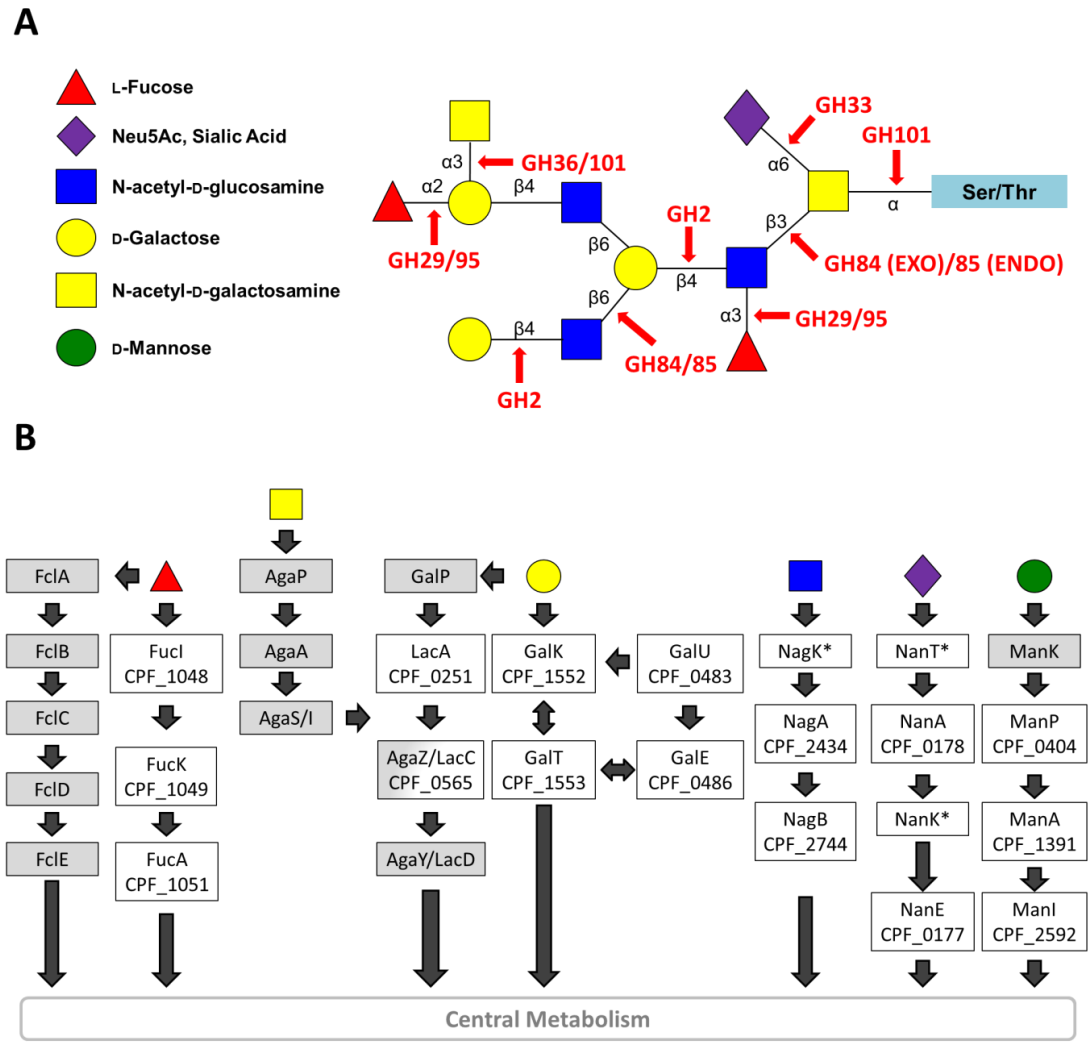


Figure 1.4: Enzymes involved in mucin glycan degradation by *C. perfringens*. (A) Extended core 3 mucin *O*-linked glycan showing sites of proposed GH action (red arrows) by *C. perfringens*. The following enzymes are highlighted: GH36/101, endo-α-N-acetylgalactosamine; GH33, neuraminidase; GH84/85, β-N-acetyl-glucosaminidase; GH2, β-galactosidase; and GH29/95, α-fucosidase. (B) Predicted pathways for utilization of the released mucin monosaccharides for *C. perfringens* strain ATCC13125, or strain 13 denoted by (*). Genes absent from *C. perfringens* are shown in grey. See Table 1.1 for additional information.

Table 1.1: Mucin monosaccharide metabolism pathways identified from *C. perfringens* strains 13 and ATCC13124

Pathway	Protein Name	Strain ID	GenBank ID	Gene Name
GlcNAc	NagK	13	BAB80985.1	CPE1279
	NagA	ATCC 13124	ABG82467.1	CPF_2434
		13	BAB81882	CPE2176
	NagB	ATCC 13124	ABG84042	CPF_2744
		13	BAB82140	CPE2434
Neu5Ac	NanT	13	BAB79892	CPE0186
	NanA	ATCC 13124	ABG84330	CPF_0178
		13	BAB79891	CPE0185
	NanK	13	BAB79894	CPE0188
	NanE	ATCC 13124	ABG84369	CPF_0177
		13	BAB79890	CPE0184
Fuc	FucI	ATCC 13124	ABG83873	CPF_1048
		13	BAB80024	CPE0318
	FucK	ATCC 13124	ABG83979	CPF_1049
		13	BAB80023	CPE0317
	FucA	ATCC 13124	ABG82250	CPF_1051
		13	BAB80025	CPE0319
Gal	GalK	ATCC 13124	ABG84966	CPF_1552
		13	BAB81051	CPE1345
	LacA	ATCC 13124	ABG84539	CPF_0251
		13	BAB80032	CPE0326
	LacB	13	BAB80033	CPE0327
	FruK (LacC)	ATCC 13124	ABG84376	CPF_0565
	LacC (FruB)	13	BAB80034	CPE0328
	GalT	ATCC 13124	ABG84320	CPF_1553
		13	BAB81052	CPE1346
	GalU	ATCC 13124	ABG83366	CPF_0483
		ATCC 13124	ABG82996	CPF_0487
	GalE	ATCC 13124	ABG83942	CPF_0486
		ATCC 13124	ABG84560	CPF_0282
		13	BAB79992	CPE0286
		13	BAB80215	CPE0509

(Table 1.1 continued)

Pathway	Protein Name	Strain ID	GenBank ID	Gene Name
Man	GlcK	13	BAB79787	CPE0081
	ManP IIA	ATCC 13124	ABG83824	CPF_0404
	ManP IIB	ATCC 13124	ABG84240	CPF_0818
	ManP IIB	ATCC 13124	ABG84444	CPF_0401
	ManP IIC	ATCC 13124	ABG82794	CPF_0819
	ManP IIC	ATCC 13124	ABG82202	CPF_0402
	ManP IID	ATCC 13124	ABG84907	CPF_0820
	ManP IID	ATCC 13124	ABG84128	CPF_0403
	ManA	ATCC 13124	ABG83961	CPF_1391
		13	BAB80893	CPE1187
	ManI	ATCC 13124	ABG85003	CPF_2592

Note: Gene information for known mucin monosaccharide catabolism denoted according to UniProt search.

Chapter 2

Compositional analysis of mucin *O*-glycans extracted from broiler chicken small intestinal mucus

2.1 Abstract

Physical separation of the microbiota from the host epithelium of avians and mammals within the intestines relies largely on a viscous mucus layer primarily composed of mucin glycoproteins. Intestinal mucin *O*-glycans have remarkable structural diversity between different mammalian species. Analytical approaches that have characterized the differential composition and structure of mucin glycosylation have been largely limited to human and mouse intestinal mucus. Many of the common protocols result in modified glycan structures, such as desialylation or deacetylation, which compromises the accuracy of mucin *O*-glycan characterization. Reported here is a reliable native-state mucin extraction method from broiler chicken mucus which generates intact mucin glycans for analyses. In combination with a low-resolution, quantitative analytical method, gas chromatography, characterized differential structural fingerprints were shown to exist between porcine and chicken mucin *O*-glycans, chickens raised with variations in feeding schedules, and chickens raised in an animal facility and on-farm. This approach may be suitable for larger throughput investigations of dynamic changes in mucus, resulting from effects such as diet, stress, infectious disease, and variations between individual birds and flock populations. Expanding upon current techniques to assess *O*-glycan compositional variability will provide improved analytical power to thoroughly evaluate the relationship between environmental variables and to inform production practices for improved intestinal health for poultry.

2.2 Introduction

The intestinal tract is a specialized organ that harbors trillions of diverse microorganisms, referred to as the intestinal microbiota. The first line of defense at the interface between the intestinal microbiota and the host epithelium is the intestinal mucus barrier (119). Intestinal mucus is largely comprised of glycoproteins called mucins, which are produced by a specialized epithelial cell, called goblet cells (120). When mucins are secreted by goblet cells, they are packaged into a condensed, water-depleted granule. Upon release from the cell, the relatively low local concentration of calcium ions causes mucins to unfold and occupy up to 100 times more volume, a process termed "mucin swelling" (77). Secreted mucins contribute to the mucus barrier by generating a fibrous-like mesh network of diverse mucin molecules which entangle and become cross-linked through hydrogen and disulfide bonds in a randomized fashion (121). This network of interactions provides a gel-like consistency to the mucus. Maintaining the integrity of the mucus network for study *in vitro* has proven difficult as many classical mucin purification methods use chaotropic (e.g. guanidinium chloride) and require reducing agents (e.g. dithiothreitol, DTT), which result in irreversible denaturation of mucin (77). This may explain why purified mucins do not replicate the viscoelastic properties of native mucus (122), and the use of commercially available porcine gastric mucins (PGM) has previously produced inconsistent and confounding experimental data (123).

Mucin proteins are heavily glycosylated and the structure of these glycans can vary between species. For example, sheep mucins contain only one carbohydrate structure (124) while in human beings (125) and chickens (79), many different carbohydrate chains may exist on the same molecule. Oligosaccharides comprise up to 80% of the mucin molecular mass and contribute to the viscoelastic properties of intestinal mucus (72,126). Regions of the mucin protein are often rich in *O*-glycosylations at serine or threonine residues with core oligosaccharides that all possess an *N*-acetyl-D-galactosamine (GalNAc) at their reducing end

(Figure 2.S1). Alternatively, asparagine residues of mucin proteins can also be *N*-glycosylated through a *N*-acetyl-D-glucosamine (GlcNAc); however, the abundance is much lower, compared to *O*-glycans (121).

O-linked mucin glycans, compared to *N*-linkages via asparagine residues, are more difficult to analyze because there are no Ser/Thr consensus sequences known, no universal enzymes available for the release of *O*-glycans, and they display high levels of glycan heterogeneity (127). Core mucin *O*-glycan extended structures can be extensively glycosylated and branched, and consist primarily of the monosaccharides: GalNAc, GlcNAc, D-galactose (Gal), L-fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) (Figure 1.2) (72). A noticeable difference between *N*- and *O*-linked glycans is the abundance of the monosaccharide D-mannose (Man) for *N*-linked mucin oligosaccharides and not for *O*-glycans. The oligosaccharide compositions of mature mucins display remarkable structural diversity which depends on linkage, branching and stereochemistry (75,79,127)**.**

The composition and structure of broiler chicken intestinal mucin *O*-glycans has not been extensively studied. However, new evidence suggests that avian mucins are distinct from human mucins in that they contain an increased density of sulfated (sulfomucins) or sialylated (sialomucins) at their terminal ends, which confers a net negative charge to the mucus (72). In addition, the role of glycan variability in poultry health and performance is also not well understood. For example, a number of intestinal bacteria have been shown to forage on host mucin glycans (93). Some of these bacteria assist in the turnover of sloughed glycans; some are pathogens, and the deconstruction and fermentation of mucin glycans facilitates mucus penetration and colonization (93,128). How the chicken intestinal microbiota contributes to mucus integrity remains to be elucidated. Analytical approaches that have characterized the differential composition and structure of mucin glycosylation have been largely limited to human and murine intestinal mucus. Many of the common protocols result in modified glycan structures,

such as desialylation or deacetylation, which compromises the accuracy of mucin *O*-glycan characterization (127). Reported here is a reliable native-state mucin intestinal extraction method from broiler chicken mucus that should generate more intact glycans for analyses. We have coupled this extraction method with a low-resolution, quantitative analytical method to characterize the differential structural fingerprints that exist between porcine and chicken mucin *O*-glycans; chickens raised with variations in feeding schedules; and chickens raised in a an animal facility and on-farm. This approach may be suitable for larger throughput investigations of dynamic changes in mucus structure, resulting from effects such as diet, stress, infectious disease, and variations between individual birds and flock populations. These insights may help inform best-practices to improve enteric health and poultry production outcomes.

2.3 Materials and Methods

2.3.1 Ethics statement

Samples obtained from chickens were carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. Before commencement, projects were reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (LeRDC Animal Use Protocols 1615 and 1622).

2.3.1.1 Production bird sampling.

Broiler chickens were obtained from three different poultry farms in southern Alberta. Each farm collection ranged from 6-20 culled birds. Culled broiler chickens were also collected from a local abattoir. These samples were denoted as Production Samples, as the birds were in transport to the poultry production line. Production Samples ranged from 80-110 culled birds per

collection period and represent the primary source used for this study. Once collected, culled birds were transported to a large animal necropsy facility located at Agriculture and Agri-Food Canada Lethbridge Research and Development Centre (LeRDC) where all animal surgical procedures were completed according to approved guidelines of the LeRDC Animal Care Committee and Biosafety Committee.

2.3.1.2 Livestock containment unit-reared birds.

Mucus carbohydrate composition was evaluated from chickens of a broiler meat breed (Ross 308). Broiler chicks were obtained from Sunrise hatchery via a local broiler farmer and reared within the livestock containment unit (LCU) at the LeRDC. All broiler chicks were placed on a standard 20% protein starter diet. At 31 days, birds were humanely euthanized and mucus was collected from the duodenum. Five birds had access to feed prior to necropsy (Treatment A, control). In order to provide mucus samples with reduced dietary carbohydrate contamination, five birds had their feed restricted for 8 hours prior to mucus collection (Treatment B). All animal work was pre-approved by the LeRDC Animal Care Committee and Biosafety Committee (animal use protocol 1622).

2.3.2 Mucus extractions from intestinal tissue

Necropsies and intestine collections were carried out with aseptic technique according to standard surgical technique (i.e. Alberta practitioners and Registered Vascular Technologist (RVTs); University of Alberta Faculty of Veterinary Medicine). Each bird was dissected by cutting the abdomen using a Y incision toward the anal region. Fully intact intestines were removed from the abdomen cavity and placed in a sterile 90 mL screw cap collection cup and

sealed. Intestine samples were then transported on ice to a microbiology facility where intestinal mucus collections were completed.

Intestinal mucus sampling did not occur in chickens that appeared ill, had fluid in the abdominal area, or had discolored (darkened) intestines. Mucus was collected from intestinal sections of the duodenum, jejunum, and ileum by longitudinally excising each intestine length-wise and gently scraping mucus away from the intestinal tract using a sterile scalpel tip. Measures were taken to exclude as much contaminant digesta and external debris (small rocks, etc.) from the mucus. Due to the high amount of contaminants in the culled bird intestinal mucus and the lack of loose mucus within the small intestine, simpler methods for mucus collection, such as aspiration techniques used previously for mice (62), could not be utilized. Mucus scrapings were pooled from the small intestines across multiple chickens and suspended in ice-cold 1X phosphate buffered saline (NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.44 g; KH_2PO_4, 0.24 g per 1 L) at pH 7.4 in the minimal volume required for the mucus to solubilize. The mucus solution was stirred for 1 hour at ambient temperature, then autoclaved according to standard liquid sterilization cycle parameters, and dialyzed for two days against 4 L distilled water using 0.5-1 kDa dialysis tubing at 4 °C. The 4 L distilled water was changed after 4 hours and then again twice a day. Following dialysis, the sample was transferred into a 50 mL tube, flash frozen in liquid nitrogen, freeze-dried, and stored in a sealed glass bottle at -20 °C for long-term storage.

2.3.3 Extraction of *O*-glycan by alkaline β-elimination.

Freeze-dried chicken mucus and commercially available porcine gastric mucin (PGM, Sigma) was reduced through alkaline β-elimination reaction as described previously by Martens *et al.* (69) and Manzi *et al*. (129). 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 mucus solubility. The

solution was cooled to 65°C and 0.1 mg mL^{-1} proteinase K (VWR, Cat# 97062-238) was added. The mucin proteins were removed from the sample by differential centrifugation at 21,000 xg for 30 minutes at 4 °C and the resulting supernatant was reduced using 0.1 M sodium hydroxide (NaOH) and 1 M sodium borohydride ($NaBH_4$). The final pH was decreased to 7.0 with hydrochloric acid (HCl) and clarified by centrifugation as noted above. The resulting supernatant was filtered using a 0.22 μM filter (Millipore) and the filtrate was dialyzed using 1 kDa dialysis tubing against deionized distilled water. The sample was then freeze-dried and stored in a glass bottle at -20°C.

2.3.4 Visualization of mucin *O*-glycans

2.3.4.1 High-Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection

HPAEC-PAD was performed with a Dionex ICS-3000 chromatography system (Thermo Scientific) equipped with an auto-sampler as well as a pulsed amperometric (PAD) detector. 10 μL of 1:100 diluted acid hydrolyzed sample was injected onto an analytical (3 X 150 mm) CarboPac PA20 IC column (Thermo Scientific Cat# 060142) and eluted at a flow rate of 0.4 mL min^{-1} with a sodium acetate gradient (0 to 1 min: 0 mM, 1 to 18 min: 250-850 mM, 18 to 20 min: 850 mM, 20 to 30 min: 850-0 mM) in a constant background of 100 mM NaOH. The elution was monitored with a PAD detector (standard quadratic waveform). Data were collected using the Chromeleon® chromatography management system (Dionex) via a Chromeleon® server (Dionex). HPAEC-PAD was employed to profile different glycans within chicken mucus, as well identify the carbohydrate composition of chicken mucin *O*-glycans based on known carbohydrate standards (93).

2.3.4.2 Gas Chromatography with Flame Ionization Detection

Glycans to be analyzed with GC-FID were prepared as described by Blakeney, *et al.*, (130) with changes to the hydrolysis and reduction method. Sampled glycans were first hydrolyzed using trifluoroacetic acid (TFA, Sigma). 2-4 mg of dried carbohydrate was incubated in glass vials with 200 µL 2 M TFA at 121°C for 2 hours. The acid mixture was dried under vacuum at a 40 °C, and then washed with isopropanol three times. The hydrolyzed sample was then reduced with $NaBH_4$, dried and incubated overnight at room temperature in 200 µL $NaBH_4$ (10 mg/mL $NaBH_4$ in 1 M NH_4OH). The reaction was quenched using borohydride and glacial acetic acid. The glacial acetic acid converts borohydride to boric acid, thus a methanol: acetic acid mixture was then added which is removed by acid methanol as methyl borate. The reaction mixture was then dried to completion in a speed-vac followed by three wash steps with methanol. Next, released monosaccharides were *O*-acetylated by the addition of 250 µL of acetic anhydride, the aqueous layer of the reaction mixture taken by the addition of 0.2 M sodium carbonate and dichloromethane, and then dried on a speed-vac to a volume of 200 µL. The resulting solution was transferred to a GC auto-sampler vial containing a 250-300 micro insert and injected into a gas chromatograph (Hewlett Packard 5890) and separated with a polar capillary GC column (Sigma, Cat# SP2330). Samples were then detected using FID and peak area was integrated for each monosaccharide in the sample via GC ChemStation software Rev. A. 10.02 (Aligent Technologies, 2003) using known carbohydrate standards.

2.4 Results

2.4.1 Chemical isolation of broiler chicken intestinal mucin *O*-glycans

Previous techniques to harvest intestinal mucus from broiler chickens included steps that disrupt mucin-mucin disulfide bonds (88). To avoid this, new techniques were developed to help preserve the mucin native-state. Culled broiler chickens were collected from regional poultry producers in southern Alberta and intestines were harvested using a standard necropsy technique. Intestinal mucus was collected and visible contaminants such as digesta and debris (*e.g.* small rocks) were excluded. The resulting mucus scrapings were pooled from each intestine. Mucus scrapings were suspended in buffer, autoclaved, dialyzed against distilled water and freeze-dried for long-term storage. Dried chicken mucus samples were reduced by alkaline β-elimination reaction as described previously (68,129). HPAEC-PAD was used to analyze differences in mucin *O*-glycan composition between the chemically-reduced intestinal mucus (cull mucus) and PGM (Sigma) (Figure 2.1). Several compositional differences were observed in PGM samples that were absent in mucus extracted from culled chickens (Figure 2.1, peaks 1 and 2), indicating that chicken and porcine mucin *O*-glycans differ in their oligosaccharide structure.

2.4.2 Intestinal mucin *O*-glycan compositional analysis

To quantitate the chemical differences between the mucin *O*-glycans extracted from PGM and cull mucus, the crude samples were acid hydrolyzed, chemically reduced and converted to their alditol derivatives to enable detection of individual monosaccharides in the sample using GC (Figure 2.2). Fuc, Gal, GlcNAc, and GalNAc were detected, which is consistent with previously determined mucin monosaccharides (Figure 2.2A). In addition, rhamnose (Rha), arabinose (Ara), xylose (Xyl), and Glc were also detected in both PGM and Cull samples, which suggests that dietary carbohydrates are also present in the samples (Figure 2.2A). Chromatogram peak areas were integrated and PGM and cull mucin *O*-glycans were quantified based on the amount of each monosaccharide in a set of known standards. The molar composition (%) of each

monosaccharide was calculated relative to the total amount of calculated carbohydrate in sample (Figure 2.2B). For both PGM and cull mucin *O*-glycans, Gal accounted for at least a third of the total carbohydrates detected in sample. Fuc was the second most abundant monosaccharide for PGM *O*-glycans, representing 27% of the total carbohydrates, whereas Fuc only accounted for 16% of the monosaccharides in cull *O*-glycans. *N*-acetylated monosaccharides were present in PGM and cull *O*-glycans in equal amounts, with an average of 22% GlcNAc and an average 11% for GalNAc (Figure 2.2B).

2.4.3 Mucin *O*-glycan composition across different rearing conditions

To determine if different rearing conditions had an effect on mucin carbohydrate composition, mucus was extracted from the intestines of LCU-reared broiler chicks (total = 10) were subjected to differential diet intake to quantify the degree of dietary contamination within intestinal mucus samples. Half of the chickens received a standard diet for the full extent of the experiment (Treatment A) and half had food withheld for 8 hours prior to extraction (Treatment B). Mucus was extracted from LCU-reared broiler chickens, and samples were analyzed by GC-FID. The abundance of Fuc, Rha, Man, Gal, and *N*- acetylated sugars GlcNAc and GalNAc were found to be similar for chickens with either diet treatment (Figure 2.3A). Peak areas for Ara, Xyl, and Glc were depleted approximately 7-fold, 6-fold, and 16-fold, respectively, in mucus samples from Treatment B chickens (Figure 2.3A).

Relative molar composition was calculated for both LCU-reared and farm-reared broiler chicken samples (Figure 2.3B). Molar composition was denoted by relative percentage and allowed for more precise comparisons of each monosaccharide in the sample. Minimal differences were observed in the relative molar compositions of Fuc, Rha, Ara, Xyl, Man, and Gal when comparing chickens reared in farm and animal facilities. Farm reared broiler chickens

exhibited lower abundance of *N*-acetylated monosaccharides when compared to broilers raised within a LCU, with decreases of 9% and 20% for GlcNAc and GalNAc, respectively relative to the total carbohydrate detected in sample. Glc was found to be much more abundant in Treatment A broiler chicken mucus compared to Treatment B broilers, which may reflect reduced levels of dietary starch and cellulose in these samples (Figure 2.3B).

2.5 Discussion

Previous work using intestinal mucus extracted from human beings (125), murine models (86), and chickens (79) demonstrated that mucin glycans are structurally diverse. Comparing *O*-glycan structure between samples is further complicated by the inherent variability in bird populations, housing environment, mucus preparation methods and extraction chemistries. To address these shortcomings, I have developed a simple method for determining the compositional variability of mucus that could be adapted to a variety of species and enables the analysis of semi-

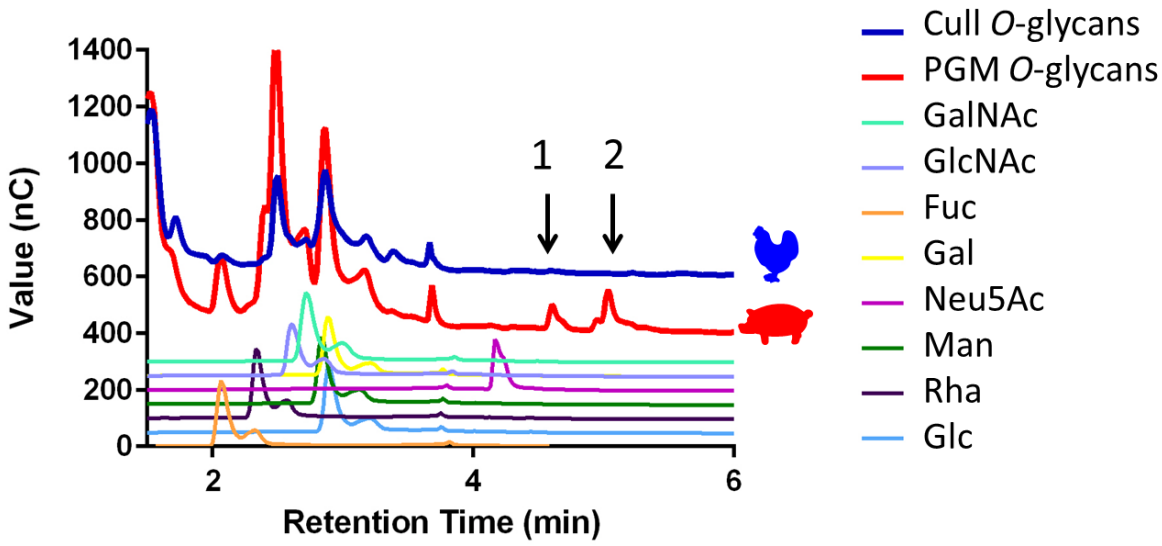
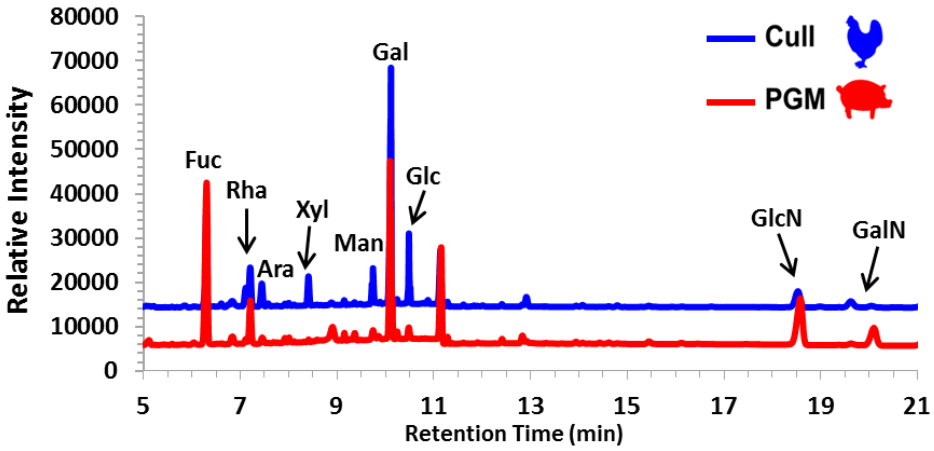


Figure 2.1: Differential mucin *O*-glycan compositional profiling between chicken and pig. HPAEC-PAD analysis of acid hydrolysed mucin *O*-glycans extracted with alkaline β-elimination (sodium borohydride) from crude preparations of cull broiler chicken intestinal mucus and porcine gastric mucins (PGM, Sigma). Arrows (1 and 2) indicate two different monosaccharide appearances between pig and chicken mucin *O*-glycans.

A

	Peak Area Counts								
	Fuc	Rha	Ara	Xyl	Man	Gal	Glc	GlcN	GalN
CULL	66524	9945	6637	1550	7725	166865	66430	44966	15500
PGM	99739	2287	3335	900	4326	122657	8844	44507	17782



B

	Relative Molar Composition (%)								
	Fuc	Rha	Ara	Xyl	Man	Gal	Glc	GlcN	GalN
CULL	15.928	1.923	1.187	0.353	1.811	40.845	7.457	20.577	9.918
PGM	26.862	0.497	0.671	0.230	1.141	33.772	1.117	22.910	12.799

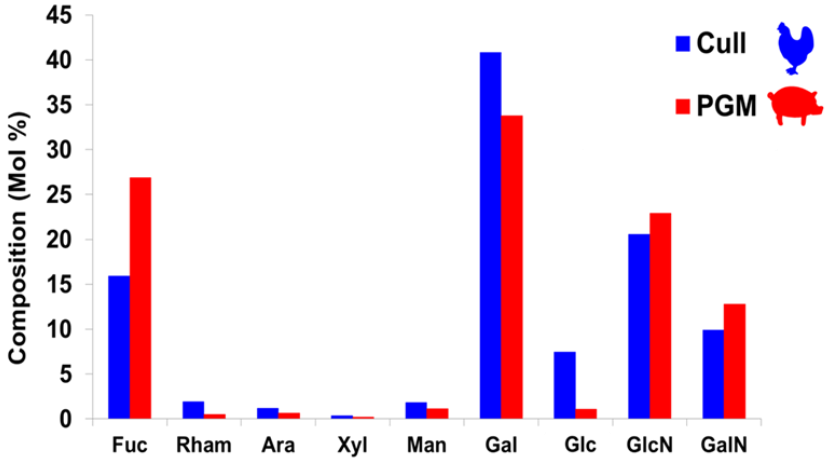
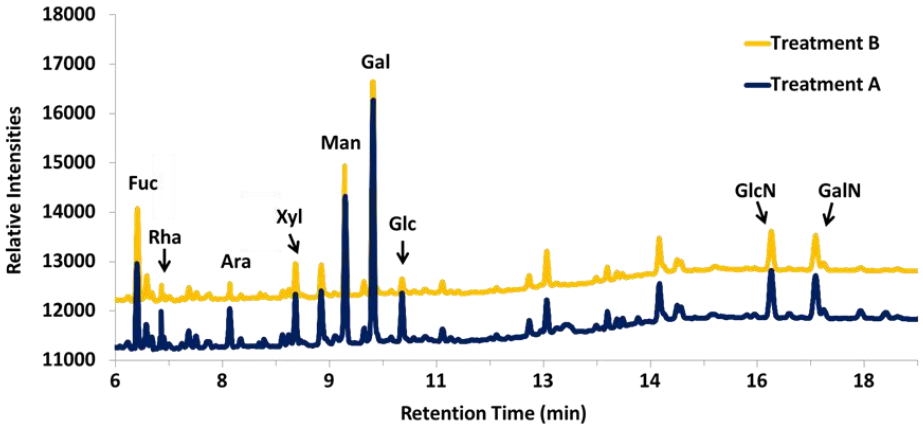


Figure 2.2: Compositional variability in mucin *O*-glycans. (A) GC-FID of alkaline β-elimination (sodium borohydride) reduced intestinal mucin *O*-glycans from broiler chickens reared at a poultry farm or porcine gastric mucin (PGM, Sigma) (B) The relative molar composition of each detected carbohydrate from farm reared broiler chicken intestinal mucus samples and PGM were calculated using three replicates. The percent abundance for each monosaccharide is relative to the total carbohydrate detected in sample.

A

	Peak Area Counts								
	Fuc	Rha	Ara	Xyl	Man	Gal	Glc	GlcN	GalN
Treatment A	2075	1261	2912	2049	3396	8312	13199	4186	4111
Treatment B	2570	1343	393	331	2815	4744	826	3221	3292



B

	Relative Molar Composition (%)								
	Fuc	Rha	Ara	Xyl	Man	Gal	Glc	GlcN	GalN
FARM	9.6796	3.038	6.802	6.247	10.49	26.936	17.44	15.743	3.623
Treatment A	9.4186	2.632	3.612	4.337	6.146	17.865	12.04	24.832	19.121
Treatment B	11.081	4.836	1.23	1.385	9.462	24.547	2.155	23.865	21.44

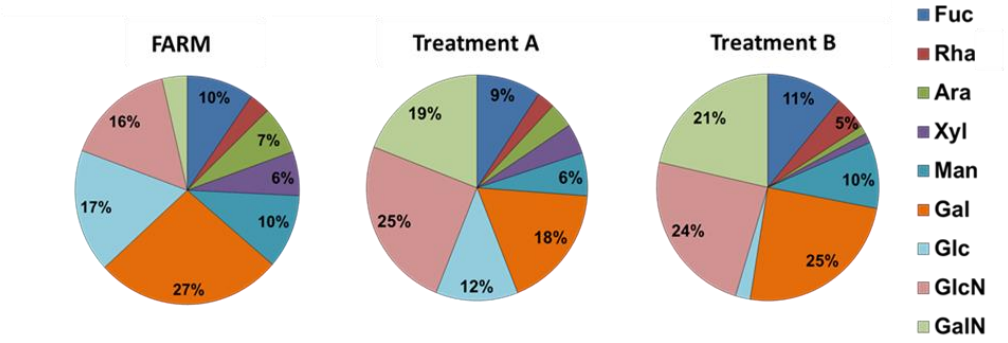


Figure 2.3: Effect of diet and rearing conditions on mucin *O*-glycan composition of broiler chickens. (A) GC-FID of alkaline β-elimination (sodium borohydride) reduced mucin *O*-glycans from broiler chickens reared at a poultry farm (i.e. farm-reared), or at an animal facility at LeRDC (LCU-reared). LCU-reared chickens were allowed to consume a standard 20% protein starter grain-based diet *ad libitum*, or had their diet withdrawn for 8 hr prior to intestine harvest. The confounding carbohydrates due to digesta contamination (predominately Glc) were ameliorated with the 8 hr feed withdrawal period. (B) The relative molar composition of each detected carbohydrate from farm-reared or LCU-reared broiler chicken intestinal mucus samples were calculated. The percent abundance for each monosaccharide is relative to the total carbohydrate detected in sample. Percentages of less than 5% have no denoted number within the pie chart.

purified, native-state mucin. The majority of mucin purifications use a common preparation method (131). The protocols often use high salt concentrations, protease treatments, and chaotropic (e.g. guanidinium chloride) or reducing agents (e.g DTT), which have been shown to alter mucin protein structure (132) and disrupt interactions between glycans (77). These manipulations disrupt the gel-like properties of mucin that are present *in vivo* (70,123,133). For example, commercially available PGM (Sigma) is widely-used but also prepared using chemical disruption methods and there has been a reported loss in its gelation properties (133).

GC-FID analysis identified an unexpectedly low relative abundance of GalNAc for both PGM and chicken intestinal mucus (Figure 2.2). This may have resulted from a confounding effect commonly reported for reductive β-elimination of *O*-glycans. Although reductive β-elimination is viewed as the most reliable release method (127), it requires high concentrations of reducing agents, such as $NaBr_4$ in potassium or sodium hydroxide, to release and reduce mucin *O*-glycans simultaneously (127). This is known to affect glycan yield and purity from mucin samples (134). Additionally, the conversion of reducing end sugars to their respective alditol acetate is also necessary to protect the glycan from peeling reactions (sequential degradation of the released glycans), which are promoted by the alkaline reaction medium (134). Therefore, the depletion of GalNAc in both PGM and chicken intestinal mucus sample (Figure 2.2) could likely be due to unexpected peeling reactions unaccounted for. Important also to note with the use of reductive β-elimination method, is the partial release of *N*-linked glycans (127), which may explain the abundance of mannose in the samples.

The extraction methods presented here aimed to preserve native-state chicken intestinal mucins for further compositional analysis. Previous work using liquid chromatography combined with mass spectrometry (LC-MS) has determined that mucin *O*-glycans from chicken intestines contain core 1, 2, 3, and 4 structures, with core 3 and core 4 accounting for the majority of structures (79) (Figure 2.S1). GC-FID analysis of chicken mucin *O*-glycans demonstrated that, of

the detectable carbohydrate in the sample, over 40% was Gal and over 20% was GlcNAc (Figure 2.2). This data is consistent with what is known for the structural basis of chicken intestinal mucin *O*-glycans, as *O*-GalNAc cores 3 and 4 are the dominant structures and both are composed predominantly of Gal and GlcNAc. The abundance of Fuc in PGM and broiler chicken intestinal mucin glycans was also expected, with the amounts of Fuc in PGM samples higher than those of chicken mucins (Figure 2.2). Gas chromatography-mass spectrometry (GC-MS) of PGM has previously demonstrated a high degree of diverse fucosylated mucin *O*-glycans, especially from the body or corpus region of the stomach (135), whereas fucosylated mucin *O*-glycan structures were previously identified within the intestinal tract of chickens but not to the same degree (79).

Culled broiler chicken mucus demonstrated a seven-fold difference in glucose (7.5%) compared to the commercially available PGM (1.1%) (Figure 2.2). Nearly 20% of the total monosaccharide composition for fully-fed birds was comprised of Glc. Minor nutrient deprivation reduced the relative abundance of Glc six-fold within the mucin *O*-glycan, and increased detection of Gal and GalNAc (Figure 2.3B). Since the compositional analyses are represented as relative to total carbohydrate content, it is possible that the amount of Glc in the samples is artificially inflated by dietary saccharides, such as starch and cellulose which are both rich in Glc and abundant in grain-based feeds. In such a case, mucus from nutrient deprived birds represents a more accurate sample for profiling monosaccharide composition. Furthermore, when animal facility-reared chickens were compared to farm-reared broilers, the detectable levels of amino sugars GalNAc and GlcNAc were much higher. In this regard, increased detection of GalNAc and GlcNAc can be directly related to the total abundance of mucin glycans present, consistent with previous data (76); and that intestinal mucin expression increases when food is withheld (64).

Based upon what was observed in diet controlled, LCU-reared birds, the low abundance of GalNAc detected in farm-reared broiler chicken mucin *O*-glycans correlates with a lower abundance of purified mucin glycans. This could result from several possibilities, which may not

be mutually exclusive. First, higher amounts of dietary carbohydrate would decrease the relative abundance of mucin monosaccharides (i.e. Glc, Gal, and Ara are highest in the farm sample). Second, production stress could result in an overall reduction in mucus abundance, which would increase the confounding effects of dietary carbohydrates (Figure 2.3) (136-138). In this regard, the LCU conditions provided less stressful environment: adequate space (only 4 chickens were maintained together in a single cage), less noise, a cleaner environment, and other comforts, such as air conditioning. Additionally, the mucus from the farm was collected from culled birds, which may have been suffering stress related to health and welfare complications. Prolonged stress in rats has been shown to induce *O*-glycan structural changes which result in hindered intestinal barrier integrity (139). The dynamic properties of intestinal mucus allows for constant adaptation to the changing stimuli. Production stress may also give mucolytic bacteria a competitive advantage, as dietary carbohydrates become limited, further depleting the mucus layer in those animal hosts (64). Notably, the methods presented in this study were limited to neutral mucins only, and complimentary analysis of acidic mucins may provide further insight the effects of diet and stress on mucus structure.

2.6 Conclusion

Developing effective methods for evaluating the effects of production conditions on *O*-glycan structure and mucus barrier integrity will help in the generation of best-practices. For example, variation in glycan composition of intestinal mucus greatly affects host susceptibility to infection and disease (140). The use of native-state mucus preparations, demonstrated in this study, allow for compositional analyses of mucin *O*-glycans that can differentiate between farm reared and LCU reared broiler chickens, and chickens that have been deprived of feed for 8 hours. Suitably, this approach can be used for studying differences in mucin *O*-glycosylation between

species. Additionally, it was established that withholding feed (when possible) is more favorable to study baseline glycan compositions as background signals from dietary carbohydrates are less prevalent. Finally, these methods are also suitable for larger throughput investigations of dynamic changes in mucus, such as diet, stress, infectious disease, and variability between populations and even individuals. Further investigation using the experimental tools described here will enable more precise analytical assessments of the impact of environmental factors on *O*-glycan variability and help inform production practices for improved intestinal health.

2.7 Supplemental Information

Figure 2.S1

A

O-glycan Core	Structure
Core 1	Gal β1-3 GalNAc α Ser/Thr
Core 2	GlcNAc β1-6 (Gal β1-3) GalNAc α Ser/Thr
Core 3	GlcNAc β1-3 GalNAc α Ser/Thr
Core 4	GlcNAc β1-6 (GlcNAc β1-3) GalNAc α Ser/Thr
Core 5	GalNAc α1-3 GalNAc α Ser/Thr
Core 6	GlcNAc β1-6 GalNAc α Ser/Thr
Core 7	GalNAc α1-6 GalNAc α Ser/Thr
Core 8	Gal α1-3 GalNAc α Ser/Thr

B

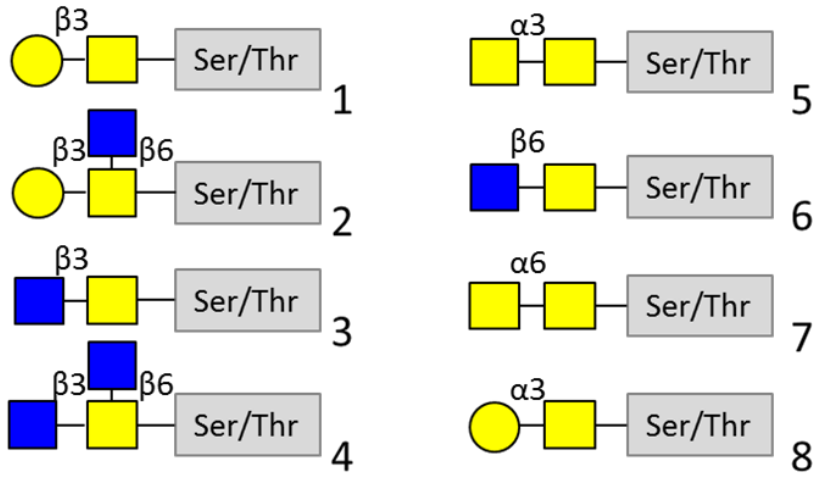


Figure 2.S1: Core mucin *O*-glycans. Schematic representation of intestinal mucin *O*-linked glycan core structures based upon nomenclature committee Consortium for Functional Glycomics (CFG) (81).

Chapter 3

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

3.1 Abstract

Clostridium perfringens is a Gram-positive opportunistic pathogen that is the principle 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 poultry *O*-linked glycans varies from other species, and is enriched in sulfated monosaccharides and *N*-acetylneuraminic acid (Neu5Ac, sialic acid). These modifications increase the overall negative charge of mucins and are believed to impede colonization by avian 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 many mucolytic enzymes specialized for the modification of avian mucins. To further investigate this relationship and provide insight into the structure of small intestine chicken mucins, *O*-glycans from *Gallus gallus domesticus* duodenum, jejunum, and ileum mucus were extracted and characterized using liquid and gas chromatography, and mass spectrometry. Chicken mucin monosaccharides contain L-fucose (Fuc), D-mannose (Man), D-galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc), *N*-acetyl-D-glucosamine (GlcNAc), and *N*-acetylneuraminic acid (Neu5Ac, Sialic acid). Using these monosaccharides as sole carbon sources, *C. perfringens* CP1 was shown to grow on Neu5Ac, Man, Gal, and GlcNAc but not on Fuc and GalNAc. We have also shown *C. perfringens* grows

on different native-state preparations of mucins and mucus, including porcine mucins, chicken mucus, and chicken mucin. Finally, 24 hour anaerobic incubation of chicken *O*-glycans with *C. perfringens* determined that there is preferential removal of Neu5Ac. These observations are interpreted in the context of the predicted metabolic potential of *C. perfringens* and the characterized mucolytic enzymes encoded with its CAZome.

3.2 Introduction

The intestines of poultry harbour trillions of bacteria and other microorganisms which collectively are termed the intestinal microbiota. Chicken intestines are predominately colonized by three prokaryote phyla: Firmicutes, Bacteroidetes, and Proteobacteria (>90%) (66). A major function of the intestinal microbiota is the metabolism of dietary fibre into short chain fatty acids that are absorbed by the host as an energy source (66). In addition to dietary glycans, select populations of intestinal bacteria can also metabolize host secreted glycans, 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 serves to protect the host epithelia from pathogenic bacteria (60). 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 (71). Mucins are densely *O*-glycosylated at serine or threonine residues, or *N*-glycosylated at asparagine residues (70). *O*-linked glycans are more abundant than *N*-linked glycans in the intestinal tract mucins and are complex in nature, consisting of variable sequence and branching of Fuc, Neu5Ac, Gal, GalNAc, and GlcNAc. In contrast, *N*-linked glycans consist predominately of repeating Man and GlcNAc (76,80). Mucin glycans account for over 80% of the total mucin

molecule by weight and provide an abundant energy source for mucolytic intestinal bacteria (66,70).

Mucin glycans degraded by mucolytic bacteria in the intestinal microbiota are continually replenished by secreted gel-forming mucins to maintain barrier integrity (Figure 1.1) (60). This relationship is dynamic. For example, increased mucin secretion has been linked to the onset of enteric infection (141) and in response to immune modulation (142) in murine models. Newly shed mucins infuse into the intestinal mucus matrix and are stabilized by the formation of disulfide bonds. Further modifications, such as increasing the density of negatively charged carbohydrates (i.e. 'acidification') can also greatly alter mucus viscosity (60). Thus, secreted mucin structural chemistry affects the main functional components of mucus, and defects in mucin *O*-glycosylation compromise the fidelity of mucus formation (143), resulting in an increased susceptibility to infection (140).

Mucin glycans are compositionally and structurally complex. Liquid chromatography-mass spectrometry (LC-MS) of a single mucin type that is expressed in the human colon, Muc2, revealed the presence of >100 different oligosaccharides linked to the mucin protein core (60,64). Furthermore, across species different mucin *O*-glycans emerge, as novel structures were identified when chicken intestinal mucins were analyzed against human intestinal mucin *O*-glycans using LC-MS (79). Alteration of glycan structure on a conserved protein backbone enables the dynamic function of mucins within the intestines. This changing glyco-landscape provides an extremely 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), primarily glycoside hydrolases (GHs), to selectively adhere to and modify mucin glycans, respectively (94). Given the structural diversity of mucin glycans, utilization of mucus carbohydrates by intestinal microorganisms is a complex process (92,94).

C. perfringens is a Gram-positive, spore-forming, obligate anaerobe commonly found in the intestines of animals including human beings (13). *C. perfringens* has the ability to produce more than 15 different toxins as well as various enzymes that can cause disease in many animal hosts, including humans (13,40). One such disease is 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 (13). NE 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, release of cytological toxins by *C. perfringens*, such as α toxin and NetB induce epithelial cell death and tissue necrosis (55).

The mechanism by which *C. perfringens* deconstructs broiler chicken intestinal mucus during NE and the role *O*-glycan modification in subclinical and clinical disease have not been elucidated. 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 (79,87). 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 currently available for determining changes in the structure of mucin glycans and the effect of physiological stimuli (e.g. stress) or biological interactions (e.g. mucolytic bacteria). In this study, I evaluate the fidelity of three different chemical *O*-glycan extraction methods for chicken mucus and present a reproducible *in vitro* assay for studying *C. perfringens* mucin utilization and modification. These techniques have allowed for 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.

3.3 Materials and Methods

3.3.1 Bacterial cultures

3.3.1.1 Virulent *C. perfringens*.

Wild-type *C. perfringens* CP1, a strain known to cause NE in avian hosts, was obtained from John Prescott (Ontario Veterinary College, University of Guelph). To ensure the retention of the NetB toxin-plasmid (88kb), quality checks were conducted by PCR using plasmid specific primers, CP4_3449 (netB) forward (GCTGGTGCTGGAATAAATGC) and CP4_3449 (netB) reverse (GCTGGTGCTGGAATAAATGC) as previously described (144). *C. perfringens* cultures were grown on 1.5% Columbia sheep blood (CSB) agar plates, and glycerol stocks were prepared for all NetB positive isolates and stored at -80°C. Prior to all growth studies, the purity of each culture was determined by plating on CSB agar and tryptose-sulfite-cycloserine (TSC) agar plates, the latter of which is a selective medium for *C. perfringens* (145).

3.3.2 *C. perfringens* growth medium.

3.3.2.1 Minimalized 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 growth medium adapted from Deplancke et al. (111) (Table 3.S1). This minimalized medium (MM) was concentrated 2X and contained tryptone but not yeast extract. Importantly, *C. perfringens* did not grow in this medium unless supplemented with a carbohydrate, suggesting the necessity of tryptone for minimal growth requirements (Table 3.S2). Variations on the established MM recipe

were attempted to optimize baseline *C. perfringens* growth using previously described *C. perfringens* media (88,146) as well as a defined amino acid medium (147) with no success; therefore, the most minimized medium was selected for growth studies to remove any potential confounding effects.

3.3.3 C. perfringens growth cultures

3.3.3.1 Mucin monosaccharides.

To determine if *C. perfringens* can utilize the products of mucin degradation, *C. perfringens* CP1 strain was grown in MM using each of the six primary mucin monosaccharides; GalNAc, GlcNAc, Fuc, Gal, Man, and Neu5Ac as the sole carbon source. Growth studies with additional carbohydrates commonly found in the diet (i.e. D-Glc, D-Xylose, Xyl; D-Arabinose, Ara; and L-Rhamnose, Rha) were also conducted to determine if *C. perfringens* CP1 displayed any specialization for the metabolism of carbohydrates which do not comprise the mucus layer. 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 for monosaccharides at a 1:1 (v/v) ratio with 2X MM to provide a final carbohydrate concentration of 0.5% (148). Media were autoclaved for 30 minutes 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 addition of Na_2CO_3, 8 g and cysteine, 1 g were made once the medium cooled. Sterile screw topped Hungate glass tubes were placed under constant anaerobic CO_2 gas and the anaerobic media was then transferred with a glass pipette connected to constant CO_2 gas to ensure anaerobic transfer of media. The tubes were quickly capped and only opened under a stream of CO_2 for inoculations. Each monosaccharide culture (made in three replicates) was inoculated with a separate overnight starter culture grown in Columbia (Difco) to an optical density (OD, 600 nm) of 0.6-0.8. Overnight cultures were centrifuged at 5,000 x g for 5 minutes and washed with

anaerobic 2X MM before inoculation. Each broth culture containing different monosaccharide inclusions were incubated anaerobically at 37 °C and OD (600nm) was measured every 5 hours for a total of 30 hours.

3.3.3.2 Mucin and mucus preparation.

Freeze dried mucus collected from pooled duodenum, jejunum and ileum sections of the small intestine from farm-reared broiler chickens were suspended at 2% (w/v) in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4, per 1 L) buffer at pH 7.4 and sterilized by autoclaving 30 minutes. 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 2X 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 carbon dioxide (CO_2) gas (CD 4.0 AN-K). This process was repeated three times prior to incubation at 37 °C. These cultures could not be monitored by OD (600 nm) due to the turbidity of the solution. Cell viability counts were quantified using colony forming unit (CFU) measurement. Serial dilutions of each broth culture were plated on supplemented brain heart infusion (BHI) agar in three replicates and incubated 24 hours at 37 °C anaerobically using anaerobic jars filled with anaerobic grade carbon dioxide (CO_2) gas (CD 4.0 AN-K). Enumeration of bacterial cells were completed by quantifying the number of CFUs per dilution plate, then converting to CFU per milliliter of media, factoring in the amount plated and its dilution factor as described previously for CFU measurements using anaerobic bacteria (149).

MM agar containing PGM (Sigma, Type II) or extracted chicken mucus was prepared by adding 1% (carbohydrate dry weight) to the final volume, and agar was added at 1.5% (w/v). The growth medium was autoclaved 30 minutes in 900 mL deionized distilled water (ddH_2O), and cooled to 50°C. Sterile filtered (0.2 µm pore) ddH_2O containing sodium carbonate (Na_2CO_3), 8 g L^{-1} and cysteine, 1 g mL^{-1} was added prior to plate pouring. PGM or chicken mucus agar plates were inoculated aerobically and incubated anaerobically within an anaerobic jar using anaerobic grade CO_2 gas as discussed above (Figure 3.S1).

3.3.4 Chemical release of mucin *O*-glycans

3.3.4.1 Sodium hypochlorite *O*-glycan extraction.

Using a protocol previously designed for PGM (150), 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 ddH_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 minutes at room temperature within a well ventilated area. Slowly, 0.75 mL of concentrated formic acid was added until the solution was well mixed, approximately 3-5 minutes. Preliminary work using this protocol completed by Dr. Wesley Zandberg (University of British Columbia, Okanagan, Kelowna, BC, Canada) suggested longer incubation times resulted in glycan degradation of the released product; therefore shortened incubations were favorable for chicken mucus samples. Acid-mucus solution was then clarified by centrifugation at 13,000 xg for 60 minutes at room temperature and dried at 45°C, under vacuum. The dried product was suspended in 15 mL ddH_2O and filtered through a 0.45 µm syringe filter (Millipore). The volume was increased to 50 mL with ddH_2O and the pH changed 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 ddH_2O required for solubility, the dried product was suspended and aliquoted into 1.5 mL microcentrifuge tubes and centrifuged at 16,900 xg for 10 minutes. Supernatants were pooled and added to a desalting column (2.5 x 100 cm, 500 mL) having Sephadex G-25 fine resin and flow rate of 4 mL min^{-1}. Fractions were collected and carbohydrates detected using thin layer chromatography (TLC) and a sulfuric acid assay (151). 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.

3.3.4.2 Ammonia-catalyzed β-elimination *O*-glycan extraction.

Release of chicken mucin *O*-glycans was performed according to microscale nonreductive techniques previously described for bovine mucins (134), 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 ammonium carbonate ($(NH_4)_2CO_3$) and 50 mL ammonium hydroxide 28% (NH_4OH), and incubated on silver beads at 60°C for 45 hours. The flask was cooled in -20°C freezer for 30 minutes to release the pressure built over incubation. The solution was centrifuged at 16,873 xg for 10 minutes to remove protein from solution, and the supernatant was dried under vacuum. Dried material was then washed with 5 mL ddH_2O three times, drying under vacuum each time. The final product was suspended in 5 mL ddH_2O and then centrifuged to remove residual particulates. The supernatant, which contains the extracted *O*-glycans, was desalted using Supelclean LC-18 solid phase extraction (SPE) tubes (3 mL). The SPE column was washed with water and centrifuged at 201 xg for 2 minutes. Sample was added and centrifuged again. To elute neutral glycans in sample, four additions of 20% methanol in ddH_2O was used, and charged glycans were eluted using four additions of 50%

acetonitrile, 0.1% TFA in in ddH_2O. Eluted glycans were pooled and lyophilized. Dried *O*-glycans were then stored at -20°C in a glass bottle until required.

3.3.4.3 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 (68) that was adapted from Manzi, *et al.* (129). This method has been shown to be effective for the chemical analysis of chicken mucin *O*-glycan structures (79); 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#97062-238) was added. Hydrolyzed mucin proteins were removed from the solution by centrifugation at 21,000 xg for 30 minutes 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 (HCl) 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 ddH_2O. The sample was then lyophilized and stored in a glass bottle at -20°C.

3.3.4.4 Thin layer chromatography and sulfuric acid assay of mucin *O*-glycans.

TLC was used to separate and visualize carbohydrate composition in each eluted fraction when *O*-glycans were desalted. Samples were spotted (total 9 μL; 3 x 3 μL) onto the bottom of the silica plate (Silica gel 60; EMD Millipore Corporation) (152) approximately 1 cm from the base. The samples were dried between each spot addition. Carbohydrates were resolved using a mobile phase of 2:1:1 butanol: water: acetic acid and then developed using an orcinol solution (70:3 acetic acid: sulfuric acid with 1% orcinol), dried, and heated at 100°C for 3-5 minutes.

Every third fraction was also analyzed by UV using a protocol adapted from Albalasmeh *et al.* (151). Each sample was aliquoted (25 µL) into a 96 well microplate (Corning, Cat# 07-200-91), and the UV absorbance at 315nm was measured. Concentrated sulfuric acid (75 µL) was added to each well, after which the microplate was shaken to mix the solutions. The plate was cooled on ice and UV absorbance (315 nm) was determined. The final absorbance was calculated by subtracting the final UV absorbance from the absorbance readings prior to sulfuric acid addition.

3.3.4.5 Preparation of glycans for growth culture

Chicken mucin *O*-glycans were flash frozen in liquid nitrogen and then freeze-dried. The product was resuspended at 2% (w/v) in PBS at pH 7.4. Three 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 2X MM, and incubated at 37 °C. Growth profiles of *C. perfringens* on mucin *O*-glycans were monitored using OD (600 nm) in parallel with viable plate counts to quantify CFUs, presented as average bacteria per milliliter of culture. OD and CFU were measured every 4 hours for 24 and 32 hours, respectively. CFU enumerations were completed on each broth culture using BHIS agar plating as described above.

3.3.5 Post-growth analysis

Glycans in the spent media of *C. perfringens* cultures grown on PGM, chicken mucus, and chicken mucin *O*-glycans were analyzed for structural changes. The cultures were centrifuged 5,000 xg for 5 minutes and the supernatants were treated according to the carbon source used for growth. 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 analyzed by TLC, gas chromatography (GC), and capillary electrophoresis (CE).

3.3.5.1 High-Performance Anion Exchange Chromatography Coupled with Pulsed Amperometric Detection

HPAEC runs were performed with a Dionex ICS-3000 chromatography system equipped with an auto-sampler and PAD. 10 μL 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 PAD detector. Data were collected using the Chromeleon® chromatography management system. *O*-glycan standards (93) were run in parallel.

3.3.5.2 Gas Chromatography with Flame Ionization Detection.

To determine monosaccharide composition of chicken intestinal mucus and mucin *O*-glycan samples used for *C. perfringens* growth studies, GC with flame ionization detection (FID) was used to analyze acid hydrolyzed, alditol converted samples. Each sample (2-4 mg) was incubated with 200 uL 2 M TFA at 121 °C for 2 hours, dried under vacuum at 40 °C, and then washed with isopropanol three times. The released monosaccharides were then converted into their volatile derivatives for GC-FID analysis in a two-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 1M NH_4OH) overnight. The reaction was neutralized with glacial acetic acid, then washed with methanol and dried under vacuum three 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 250-300 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.

3.3.5.3 Liquid Chromatography - Mass Spectrometry

NH_3-catalyzed β-eliminated *O*-glycans (134) incubated with *C. perfringens* were reduced using 1 M $NaBH_4$ in 50 mM NH_4OH (2h, 60 °C) to avoid resolution of α/β-anomers and subsequently desalted using graphitized carbon SPE cartridges exactly as described in (153). *O*-glycan samples were analyzed by HPLC-quadrupole-time-of-flight (qTOF) MS using a method optimized for the analysis of milk oligosaccharides (154). MassHunter's (Agilent Technologies) Find-by-formula (FBF) algorithm was used to search total ion chromatograms for ions with *m/z* values consistent with glycan compositions previously identified in chicken intestinal tissues (79). Peak areas for all *O*-glycans with mass errors of 10 ppm or less and FBF scores above 90 were recorded and are reported as a percentage of the total glycans detected in each sample.

3.4 Results

3.4.1 Mucin monosaccharide growth profiling

To examine the growth profiles of *C. perfringens* CP1 on monosaccharides and *O*-glycans, a growth medium was adapted from a previous study (111) (Table 3.S1, Table 3.S2); this medium did not support growth in the absence of additional carbohydrate. Using each of the six primary mucin monosaccharides (GalNAc, GlcNAc, Fuc, Gal, Man, and Neu5Ac) as sole carbon sources for anaerobic bacterial growth cultures, growth rates were monitored at OD_{600} over 33 hr

(Figure 3.1). Columbia (Difco) 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 hours, 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 33 hour incubation. Man and Glc, and Gal and GlcNAc displayed similar growth profiles. Each had a lag phase (~ 5 hours), and appeared to plateau within the 33 hour incubation with the exception of Gal. Growth on Neu5Ac had the longest lag phase of 10 hours 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 (Figure 3.1).

3.4.2 Crude broiler chicken mucus

To determine if *C. perfringens* CP1 could be cultured 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 counts. *C. perfringens* (denoted RS42, Type A) was previously reported to grow on PGM (146) and therefore, this substrate was used a control. Both crude cull bird mucus and PGM supported growth of *C. perfringens* CP1 at 1% (w/v) final concentration of source mucin substrate (Figure 3.2). *C. perfringens* CP1 growth reached a maximum average of $8.5x10^6$ CFUs at 24 hours on cull mucus and $1.2x10^{10}$ CFUs at 20 hours on PGM. Average CFU mL^{-1} increased 4 orders of magnitude for cull bird mucus and seven orders of magnitude for PGM, which supports the observed ability of *C. perfringens* to metabolize the monosaccharide components of native state broiler chicken intestinal mucin glycans as a carbon source *in vitro*. This is the first account of *C. perfringens* growth on chicken intestinal mucus as a sole carbon source. The higher growth of *C. perfringens* CP1 grown on PGM suggests that the

PGM may present a more accessible glycan structure or contain a more preferable composition to support growth.

The spent medium containing crude cull bird mucus and PGM was analyzed by TLC and GC-FID to determine if monosaccharides or small oligosaccharides were released during growth (Figure 3.3). Defined fragment patterns were not detected by TLC; however, there was a heterogeneous population of small oligosaccharide products present in all biological replicates (Figure 3.3A). Molar compositions of individual monosaccharides, relative to the total carbohydrate detected in sample, revealed detectable changes following growth (Figure 3.3B). After incubation with *C. perfringens* CP1, Fuc levels increased by 7.6% and GalNAc increased by 6.2% in the cull bird mucus sample, whereas the relative amounts of monosaccharides Man, Glc and Gal decreased. In contrast to cull bird mucus, TLC analysis of the PGM sample revealed that *C. perfringens* CP1 generated strong banding patterns, consistent with the release of defined populations of monosaccharides or small oligosaccharides (Figure 3.3C). GC-FID analysis of PGM samples determined that the overall pattern in compositional changes were similar to the

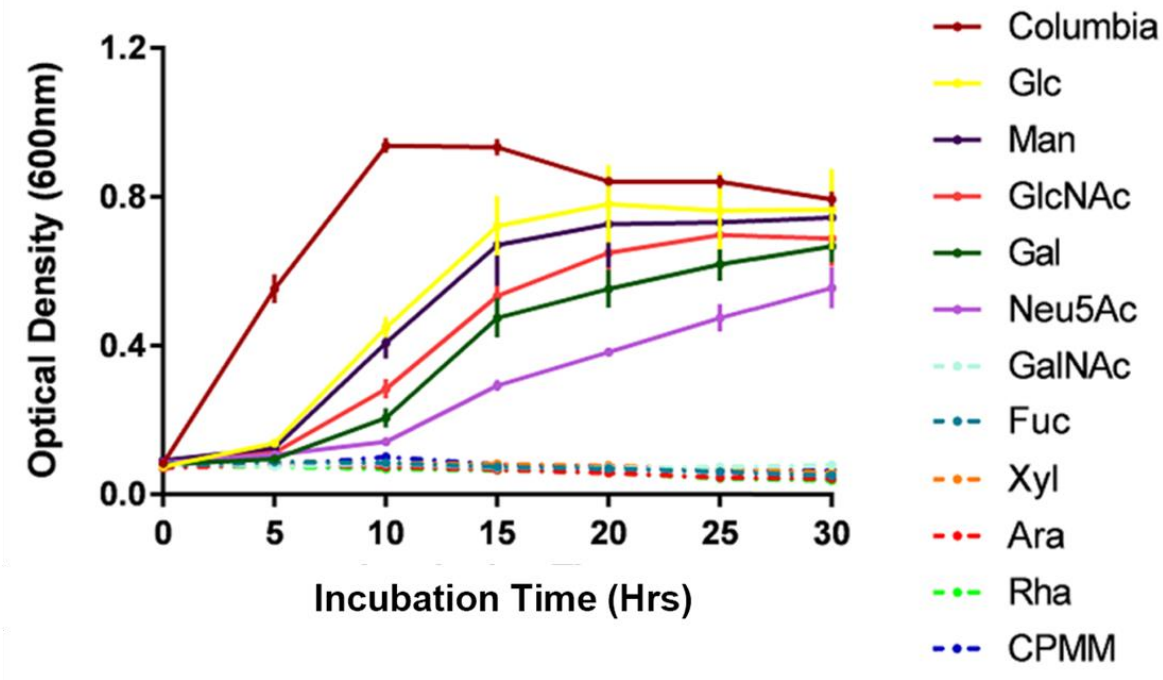


Figure 3.1: *C. perfringens* growth on broiler chicken intestinal mucus components. *C. perfringens* CP1 was incubated anaerobically in minimalized medium which differ by carbon source. Bacterial growth kinetics were measured by optical density (600 nm) when using pure monosaccharides as a sole carbon source. Solid lines indicate carbohydrates that sustain growth; dashed lines indicate no growth. All mucin monosaccharides but Fuc and GalNAc support growth of *C. perfringens*. Columbia broth and Glucose (Glc) were positive controls, and minimalized medium, CPMM, was used as a negative control. Vertical lines represent SEM using three biological replicates.

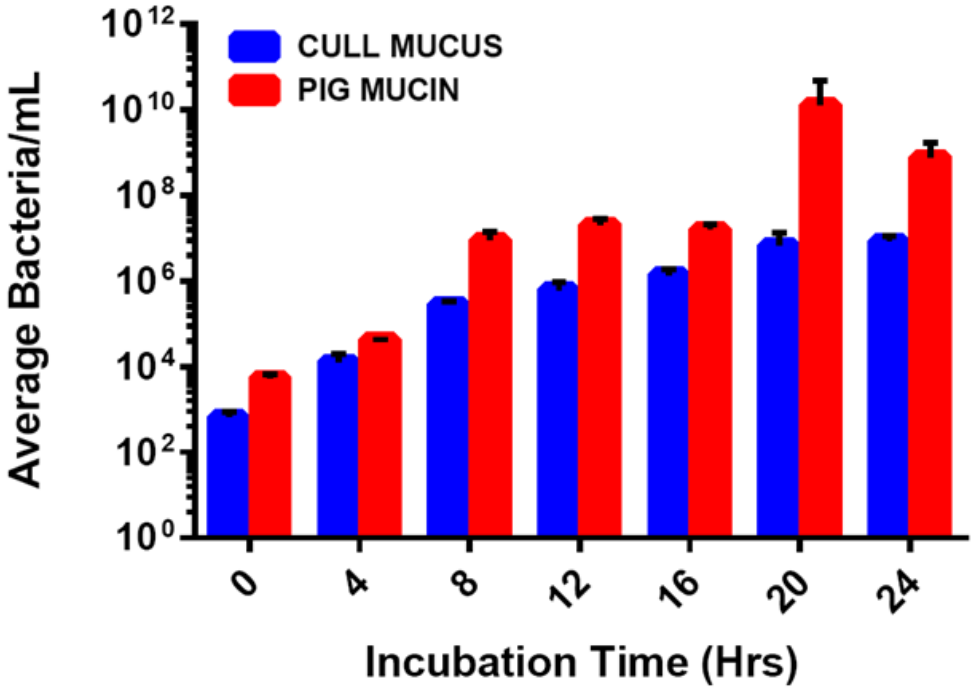


Figure 3.2: *C. perfringens* can utilize complex mucin substrates *in vitro*. *C. perfringens* CP1 growth on semi-purified crude chicken intestinal mucus and PGM (Type II) was measured by enumerating CFUs per milliliter. Vertical lines associated with histogram represent SEM using three biological replicates.

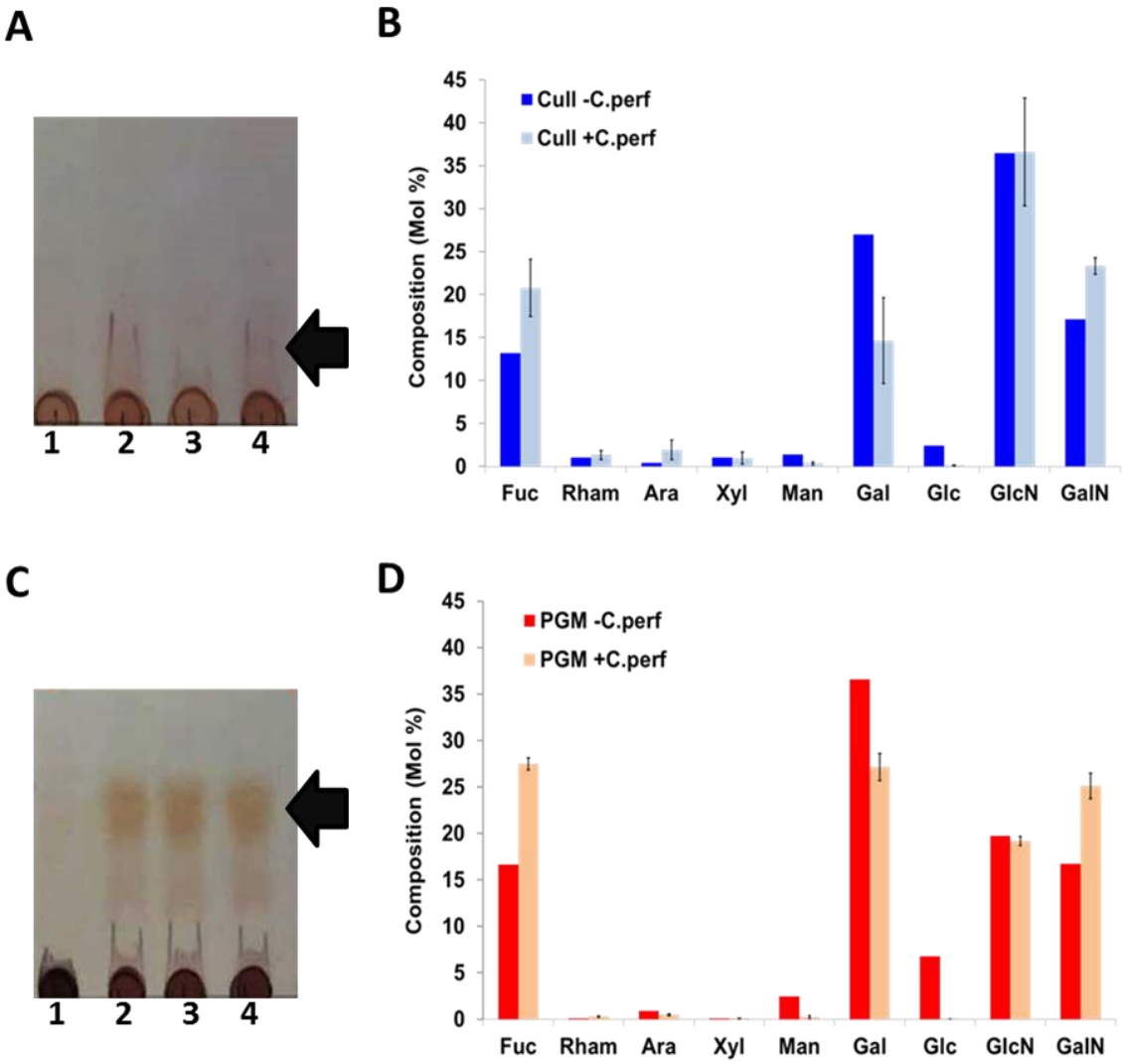


Figure 3.3: Complex substrate modification by *C. perfringens*. Semi-purified intestinal mucus harvested from culled farm-reared broiler chickens (top panel, blue), or crude PGM (Type II) (bottom panel, red) were used as the sole carbon source for bacterial growth with *C. perfringens* CP1 (+C. perf). Spent culture supernatant analysed via TLC (A and C) and GC-FIC (B and D) to observe mucin glycan changes following bacterial utilization, compared to media samples without *C. perfringens* (-C. perf). Captions 1 through 4 on TLCs refer to conditions of growth; where *C. perfringens* is absent (1) or present in media (2-4). Black arrows indicate location of the released small oligosaccharides (A) and other carbohydrates (C) following bacterial addition. Vertical lines associated with histogram indicate SEM using three replicates.

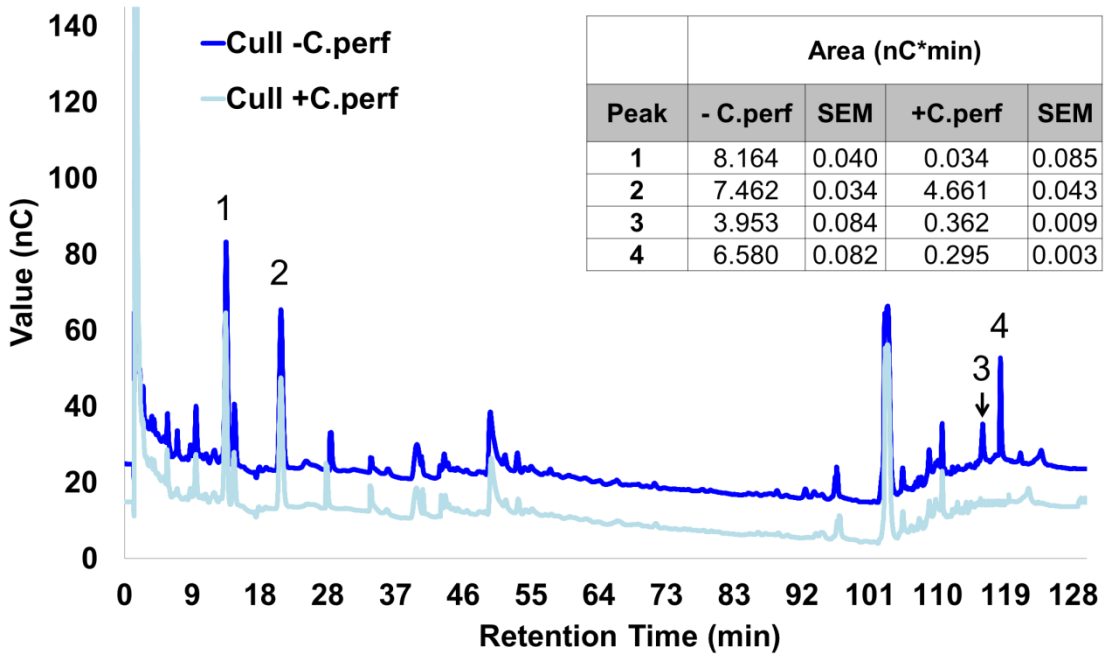
chicken mucus; however, there were differences (Figure 3.3D). Fuc and GalNAc composition increased by 10.9% and 8.4%, respectively; whereas, there was a complete loss of Man and Glc observed (down to 0.2% and 0.0%, respectively) and the quantity of Gal in the sample saw a large decrease (9.5%).

To provide more insight into the structure of oligosaccharide products, HPAEC-PAD was performed on cull bird mucus and PGM samples. Using this technique, each peak in the chromatogram represents an intact oligosaccharide purified from the sample. Complex mixtures of glycans were released from both substrates. Notably, decreases in four major peaks were observed after 24 hr bacterial incubation with cull mucus samples (Figure 3.4). Peaks 1 and 2 decreased by average peak areas of 1.3 and 2.8 nC·min and were collected within a retention time corresponding to neutral glycans. Peaks 3 and 4 had more notable decreases in intensity (~2-fold) and were collected in the region corresponding to anionic oligosaccharides, suggesting there was removal of Neu5Ac or sulfate from these glycans.

3.4.3 Extracted cull broiler chicken mucin *O*-glycans

There are limited chemical methods available for purifying mucin *O*-glycans at yields and purity suitable for microbiological growth studies (87,155). Therefore, three different extraction methods were conducted to extract *O*-glycans from broiler chicken intestinal mucus: ammonia-based elimination (adapted from (134)), NaClO or "Bleach" oxidation (150), and proteolysis coupled to $NaBH_4$/NaOH reduction (68,129). Ammonia elimination and bleach oxidation had not been previously performed with crude chicken intestinal mucus, while the $NaBH_4$/NaOH reduction was modified from (87). Significantly, each method generates unique product chemistry at the reducing end of the glycan, which may impact its metabolism (Figure

3.S2). End products from all three methods were dialyzed for at least 4 days against 4 L of water that was changed every 4-8 hours, and lyophilized to enable equal masses of dry material to be



	Area (nC*min)			
Peak	- C.perf	SEM	+C.perf	SEM
1	8.164	0.040	0.034	0.085
2	7.462	0.034	4.661	0.043
3	3.953	0.084	0.362	0.009
4	6.580	0.082	0.295	0.003

Figure 3.4: Observed changes in broiler chicken intestinal mucin *O*-glycans following *C. perfringens* utilization. HPAEC-PAD analysis of culled farm-reared broiler chicken intestinal mucus with *C. perfringens* CP1 (+C. perf, light blue) compared to growth medium without *C. perfringens* (-C. perf, dark blue) demonstrated a decrease in glycan representation with bacterial inclusion. Each peak represents an independent population of glycan(s). Peak areas are assigned to select peaks (1 through 4). Error is indicated by +/- SEM using three replicates.

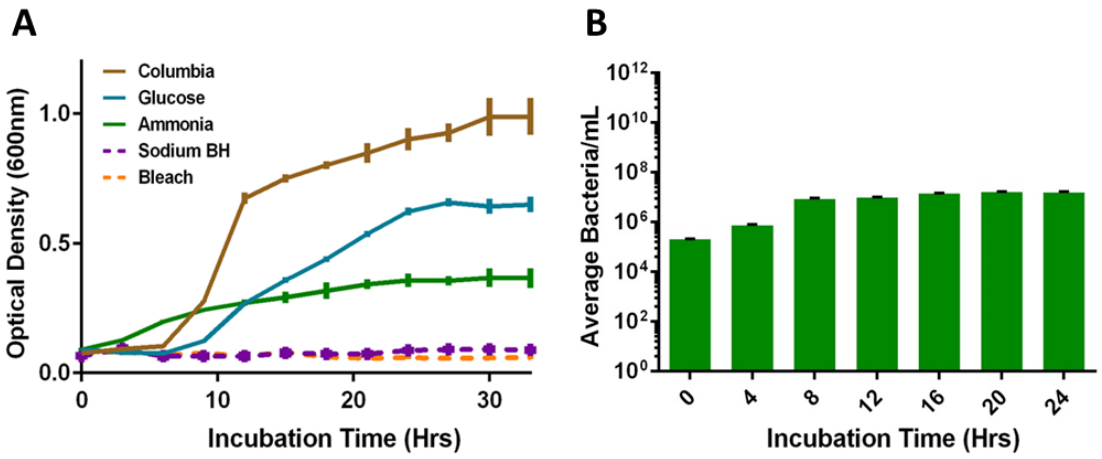


Figure 3.5: *C. perfringens* growth profiling on extracted broiler chicken intestinal mucin *O*-glycans. (A) *C. perfringens* growth on intestinal mucin *O*-glycans extracted by the three different methods is shown in (Figure 3.S2). Ammonia-extracted *O*-glycans (green line) was the only mucin glycan preparation method that supported *C. perfringens* growth. Error is shown as SEM of 3 biological replicates. (B) Colony forming units of *C. perfringens* CP1 grown solely on ammonia extracted mucin *O*-glycans from semi-purified intestinal mucus of farm-reared broilers. Vertical lines indicate SEM using three biological replicates (A) and six biological replicates (B).

used as a sole nutrient in the growth medium. In MM supplemented with extracted *O*-glycans, only cull bird mucus treated with ammonia supported growth of *C. perfringens* CP1 (Figure 3.5A). Viable counts were also quantified over 24 hours from six biological replicates of *C. perfringens* CP1 grown on 1% (v/v) ammonia extracted broiler chicken intestinal mucin *O*-glycans in MM. CFUs showed an increase of two orders of magnitude (Figure 3.5B).

To determine if *C. perfringens* CP1 was capable of modifying the structure of three different substrates, spent supernatants from the culture were analyzed by TLC, GC-FID and HPAEC-PAD. TLC visualization of carbohydrates in the sample demonstrated a disappearance of small oligosaccharides or monosaccharides with *C. perfringens* addition (Figure 3.6A). Compositional analysis of these samples by GC-FID showed little to no change in the compositional ratios of these glycans (Figure 3.6B). The amounts of only Fuc and Man monosaccharides increased following bacterial utilization, increasing by 1.4% and 3.5%, respectively, whereas amounts of Gal, GlcNAc, and GalNAc within growth culture were found to decrease slightly. 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 3.7). For example, two large peaks were observed in the *C. perfringens* CP1 treated sample (~12 min and ~90 min) that were not present in the negative control. Additionally, multiple glycans (peaks 3-7) disappeared following treatment with *C. perfringens* CP1, suggesting that glycans were being modified, but these reactions were not sufficient to support growth of the bacterium comparable with crude substrate.

To provide high resolution insights into the changes in glycan structure following *C. perfringens* CP1 growth, LC-MS was performed on the ammonia-extracted spent supernatants. Freeze-dried bacterial growth culture supernatants +/- *C. perfringens* were reduced with ammonium-based reagents (134) and glycan structures were identified based on m/z values compared to what was generated with broiler chicken *O*-glycans previously (79). Analysis of

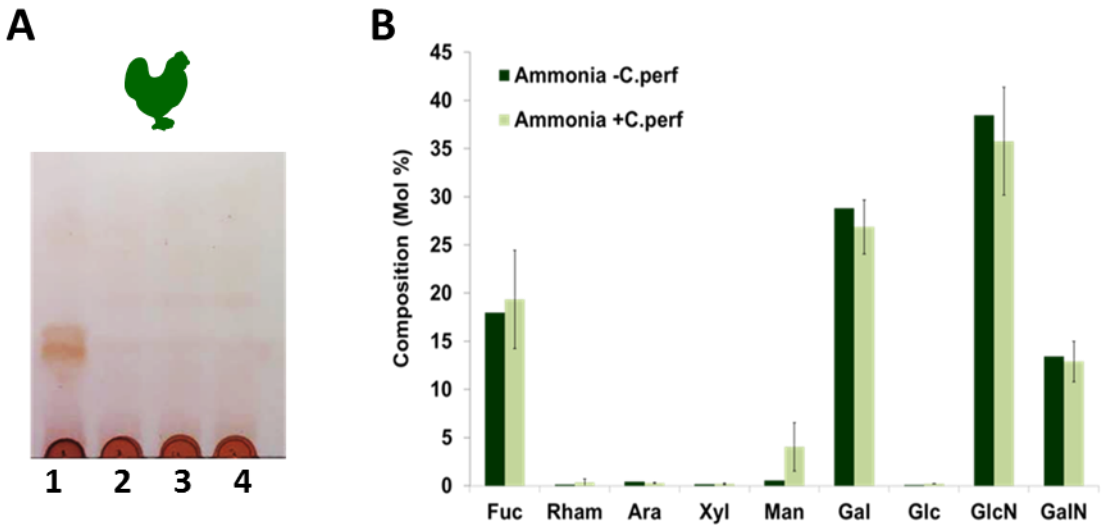
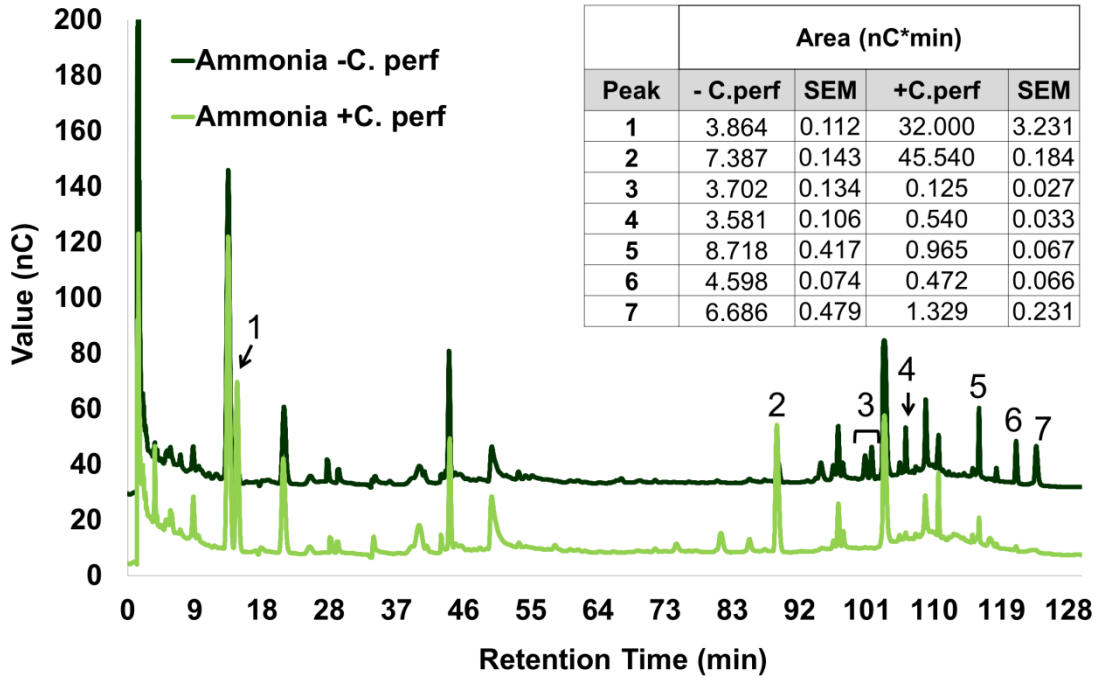


Figure 3.6: Carbohydrate composition of ammonia extracted mucin *O*-glycans following bacterial incubation. Analysis of growth medium with (+C. perf) and without (-C. perf) *C. perfringens* CP1 on ammonia extracted mucin *O*-glycans. TLC (A) demonstrates a disappearance of small oligosaccharides or monosaccharides with *C. perfringens* addition. Captions 1 through 4 on TLC refer to conditions of growth; where *C. perfringens* is absent (1) or present in media (2-4). GC-FID (B) of spent bacterial culture; however, reveals minimal changes across three biological replicates (+C.perf). Vertical lines associated with histogram indicates SEM.



	Area (nC*min)			
Peak	- C.perf	SEM	+C.perf	SEM
1	3.864	0.112	32.000	3.231
2	7.387	0.143	45.540	0.184
3	3.702	0.134	0.125	0.027
4	3.581	0.106	0.540	0.033
5	8.718	0.417	0.965	0.067
6	4.598	0.074	0.472	0.066
7	6.686	0.479	1.329	0.231

Figure 3.7: Ammonia extracted mucin *O*-glycans are modified by *C. perfringens*. HPAEC-PAD analysis of spent growth medium containing ammonia extracted mucin *O*-glucans as the sole carbon source with (+C.perf) or without (-C.perf) *C. perfringens* CP1 addition. *O*-glycans modified (2-7) or generated (1) by C. perfringens are indicated and integrated peak areas shown. Error is indicated by +/- SEM using three replicates.

intact *O*-glycans indicated that all Neu5Ac-containing glycans were completely eliminated by the bacteria with concomitant increases in several neutral species following 33 hour incubation (Figure 3.8). Additionally, it appears that the ammonia extraction method described here may enable the detection of a novel mucin *O*-glycan structure within chicken intestinal mucus (labelled * in Figure 3.8), which has not been described in previous LC-MS structural analysis when NaOH-based reduction methods were used (79).

3.5 Discussion

To examine the growth proficiency of *C. perfringens* CP1 on the components of mucus, the bacterium was cultured with Gal, Man, Neu5Ac, GalNAc, GlcNAc, Xyl, Ara, and Rha, using Glc and Columbia broth as positive controls (Figure 3.1). Of these, Gal, Neu5Ac, Man, and GlcNAc supported growth of *C. perfringens* CP1; whereas, no growth was observed on the monosaccharides Fuc, GalNAc, Xyl, Ara, Rha and the negative control (i.e. no carbohydrate; CPMM). These results suggest that *C. perfringens* preferentially metabolized mucin carbohydrates and not contaminant dietary sugars (Xyl, Ara, Rha). This observation was consistent with *C. perfringens* possessing mucolytic enzymes that release GlcNAc (i.e. NagK, NagA, NagB) and Neu5Ac (i.e. NanA, NanE) from mucin *O*-glycans, and lacking the appropriate enzymes required for Fuc (e.g. Fcl pathway) and GalNAc (e.g. Aga pathway) metabolism (Figure 1.4) (Table 1.1) (85,156). *C. perfringens* CP1 likely selectively metabolizes most, but not all, of the carbohydrates present in chicken intestinal mucus.

The ability for *C. perfringens* to degrade broiler chicken intestinal mucus has been noted as possibly the most important aspect for pathogenesis of poultry NE (55). Mucin monosaccharide metabolism is dependent upon the ability of a bacterium to release the

monosaccharide residues from complex mucin oligosaccharides or scavenge the depolymerization products of other resident bacteria. Interestingly, at least 23 mucolytic enzymes

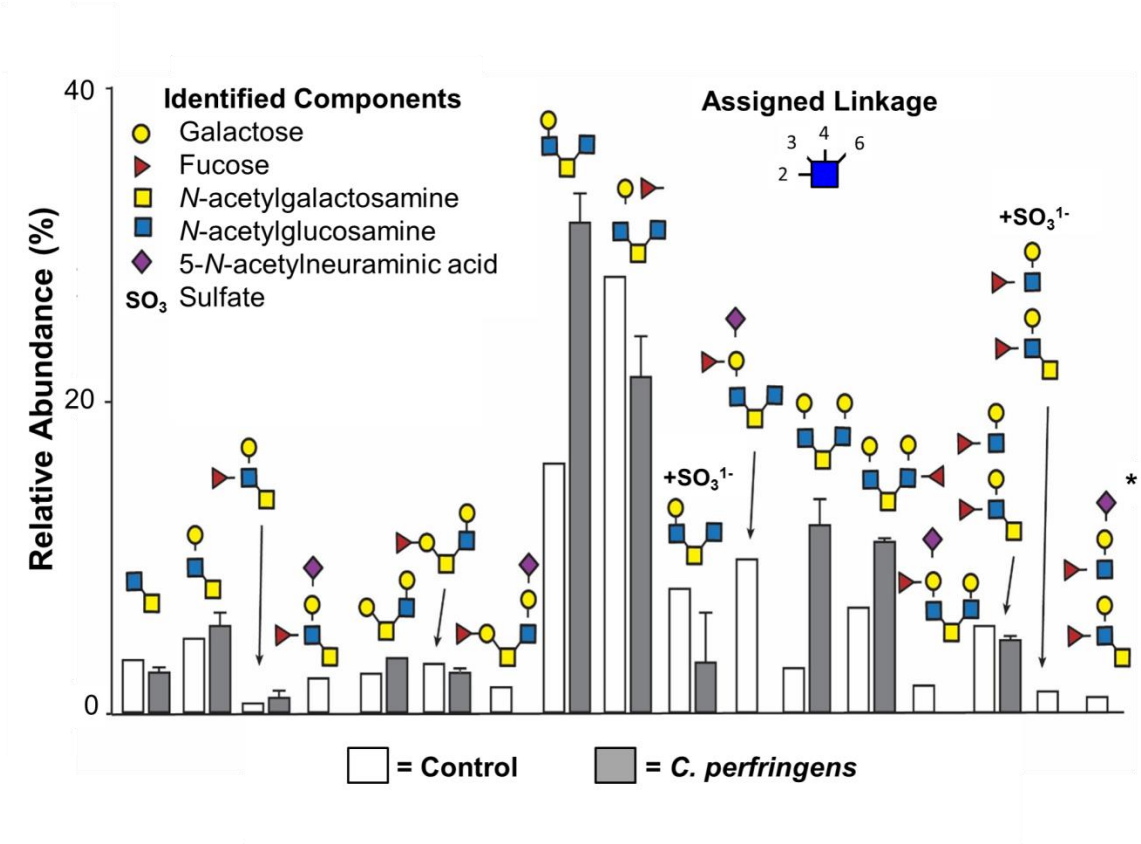


Figure 3.8: *C. perfringens* preferentially modifies Neu5Ac-containing mucin *O*-glycans in vitro. Broiler chicken mucin *O*-glycans extracted using ammonia (NH3) catalyzed β-elimination (Figure 3.S2) were added to medium in the absence (white) or presence (grey) of *C. perfringens* for 33 hr after which they were analyzed by HPLC-MS. Absolute MS detector responses for each glycan are reported as a percentage of the total signal from all glycans detected in each sample, where n = 3 for the samples grown in the presence of bacteria, and n = 1 for the control treatment. Relative peak areas are reported ± the standard error of the mean. The data clearly show that bacterial digestion reduced all Neu5Ac-containing *O*-glycans present in the control sample to levels below that of the method detection limit. The monosaccharide symbols used have been proposed by the CFG (81). Intestinal mucin *O*-glycans that were not previously observed with HPLC-MS (79) are denoted here with (*).

have been characterized or predicted to be involved in mucus modification in the wildtype strain of *C. perfringens* (Figure 1.3). These include 18 GHs, which hydrolyze glycosidic bonds (157); 3 M60-like zinc metalloproteases (Zmps), which hydrolyze peptides but require recognition of carbohydrates for proteolysis (97); and at least 3 sulfatases which could desulfate mucin glycans. Of the GHs, three α-L-fucosidases have been identified in the genome of *C. perfringens* ATCC13124 (158); and a novel endo-α-GalNAcase has been identified in the genome *C. perfringens* Strain 13 (159). In this regard, the inability of *C. perfringens* CP1 to grow on Fuc and GalNAc (Figure 3.1) suggests that the metabolism of all monosaccharides in mucus is not necessary for colonization but they may represent steric impediments to the complete saccharification of other linkages present in chicken *O*-glycans. Notably, the majority of mucolytic GHs in *C. perfringens* are secreted outside the cell (159), and present as complex multi-modular enzymes (97,98,100,107,160), 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.

3.5.1 *C. perfringens* growth on chicken *O*-glycans and intestinal mucus

To determine if *C. perfringens* CP1 could be cultured on more biologically relevant substrates, crude mucus was collected from the small intestine of culled farm broilers and supplied as a sole carbon source. PGM was used as a control (111), and growth was monitored by viable cell counts. Both cull bird mucus and PGM supported growth of *C. perfringens* CP1 (Figure 3.2), which is the first report that crude production broiler chicken intestinal mucus is a metabolizable substrate for *C. perfringens*. GC-FID was used to characterize compositional changes to the *O*-glycans by quantifying changes in relative monosaccharide composition. In the presence of *C. perfringens* CP1, Fuc and GalNAc levels increased in the cull bird mucus sample,

whereas the relative amounts of monosaccharides Man, Glc and Gal decreased (Figure 3.3). When PGM was analyzed, similar patterns were observed (Figure 3.4), suggesting that Fuc and GalNAc may accumulate in post-growth media because *C. perfringens* does not metabolize these sugars.

When the spent supernatants of the chicken mucus growth cultures (+/- *C. perfringens*) were analyzed by HPAEC-PAD, there was almost a complete reduction of glycans that elute after 110 minutes (Figure 3.4); glycans that elute within this region are commonly anionic and would include glycans that are sulfated and sialylated. Broiler chicken *O*-glycans are known to be enriched in Neu5Ac and sulfated residues (79), whereas charged mucin *O*-glycans comprise less than a third of the total glycan population in PGM and human gastric mucins (135,161). The rate of mucin degradation is known to depend on the efficiency of removing the terminal residues of colonic mucin oligosaccharide chains (162). The difference in growth observed between cull mucus and PGM substrate (Figure 3.3) and the observation that there is nearly a complete elimination of charged glycans in cull mucus post growth (Figure 3.4) suggests that removal of charged mucins in cull mucus is a rate-limiting step in mucus deconstruction and metabolism by *C. perfringens* CP1.

To evaluate the growth proficiency of *C. perfringens* CP1 on extracted *O*-glycans and optimize an *in vitro* growth method for screening mucolytic activity, three different reactions were performed on crude chicken intestinal mucus (Figure 3.5A): classical alkaline β-elimination (i.e. sodium borohydride) (68,129), oxidation (i.e. bleach) (150), and ammonia catalyzed elimination (i.e. ammonia) (134). These reactions generate unique chemistries at the reducing end of the glycan (Figure 3.S2). Of the three *O*-glycan products, only the ammonia method supported growth of *C. perfringens* CP1 (Figure 3.5). Ammonia-catalyzed reactions resulted in the highest yield of *O*-glycans, albeit, different clean-up strategies were used. The reductive β-elimination and oxidation methods both utilize a highly alkaline-based reaction medium which risk excessive

salt contamination (134) and uncontrollable 'peeling reactions' (134,150). It may be possible that due to the differential clean up 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 (68), 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. Furthermore, it should be noted that the ammonia glycan release method, when compared to the alkaline β-elimination and oxidation methods, also allow for more precise analysis of mucin *O*-glycans as reducing end resides have been proven to remain unchanged (134). Thus, the ammonia glycan release method may be more favorable to use for future bacterial mucin *O*-glycan growth studies.

Analysis of the spent ammonia extracted glycan growth supernatants by GC-FID did not discern any differences in glycan structure (Figure 3.6) but TLC did indicate that small oligosaccharides or monosaccharides in the ammonia extracted mucin glycan sample had been metabolized by *C. perfringens* CP1. HPAEC-PAD revealed that the *O*-glycan species, which elute in range of the chromatogram associated with negatively charged oligosaccharides, were depleted (Figure 3.7, peaks 3-7). This data revealed that *C. perfringens* CP1 is removing charged groups (e.g. Neu5Ac or sulfate), which is consistent with the bacterium possessing the ability to use Neu5Ac as a carbon source (Figure 3.1) (a carbohydrate that is not detectable by GC-FID); and/or removing these charged groups to expose underlying *O*-glycan carbohydrates.

3.5.2 *C. perfringens* utilization of Neu5Ac-containing mucin *O*-glycans

Analysis of intact chicken intestinal mucin *O*-glycans indicated that all Neu5Ac-containing glycans were completely eliminated by the bacteria with concomitant increases in several neutral species (Figure 3.8). This suggests in addition to removing Neu5Ac from *O*-

glycans to gain access to underlying monosaccharides, a common microbial strategy (93) (163) (164), *C. perfringens* is using liberated Neu5Ac as a nutrient source. In this regard, *C. perfringens* may be specialized among bacterial pathogens. *C. perfringens* was the first bacterial species identified to have neuraminidase activity (165), and this process is well-documented across a variety of strains (166). Metabolism of Neu5Ac is likely one of the contributing factors promoting the broad host spectrum associated with this pathogen.

C. perfringens is the etiological agent of several human diseases, including gas gangrene (40), acute food poisoning and antibiotic-associated diarrhea (167). In domestic livestock, it is responsible for a wide range of enteric diseases (168) including NE in poultry (55). The role of bacterial sialidases in pathogenicity has been postulated for many years (169). The ability of *C. perfringens* CP1 to liberate and utilize Neu5Ac from complex mucin polysaccharides would provide a metabolic advantage. A similar mucin-degrading strategy has been shown in *Ruminococcus gnavus*, a bacterial pathogen known to incite inflammatory bowel diseases (118). Furthermore, bacterial sialidases have been shown to promote *in vivo* growth and colonization of the human or animal intestinal tract (117). Prominent examples of bacteria capable of utilizing Neu5Ac for disease pathogenesis include *Streptococcus pneumoniae* (170), *Vibrio cholera* (85,171), and *Salmonella enterica* (172). The presence of host mucin sulfation and sialyation may work cooperatively to inhibit bacterial adhesion to the mucus layer and sequential translocation to the epithelium (173,174). Sialyation and overall anionic charge density of intestinal mucin *O*-glycans is much greater in chickens than reported in humans (79). The increase of sialo- and sulfomucins within the intestinal mucus layer of chickens has been shown to attenuate the binding and colonization of human pathogen (but chicken commensal) bacterium, *C. jejuni* (79,87). Differences in the acidomucin glycan composition thus, 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.

3.6 Conclusion

This study has demonstrated that *C. perfringens* CP1 utilizes components of mucin as a nutrient, and is specialized for the metabolism of chicken *O*-glycans and mucus. Significantly, *C. perfringens* was shown to both remove and metabolize Neu5Ac, a terminal residue in chicken intestinal mucin *O*-glycans (79) from complex substrates. These events have been linked to important roles in the colonization of enteric pathogens and intestinal inflammation (163). Evaluation of specific *C. perfringens* mucolytic enzymes utilized during chicken intestinal mucin deconstruction *in vitro* 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 be beneficial for defining the relationship between colonization and the onset of NE. This information would be critical for the development of novel interventions that may prevent the early stages of NE and improve the performance of poultry.

3.7 Supplemental Information

Table 3.S1

Table 3.S2

Figure 3.S1

Figure 3.S2

Table 3.S1: *C. perfringens* minimalized medium components as adapted from Deplancke *et al.* (111).

CPMM Recipe	
2X Formula per Litre	g/L
K_2HPO_4	0.46
KH_2PO_4	0.46
$(NH_4)_2SO_4$	0.46
NaCl	0.92
$MgS0_4$-$7H_20$	0.18
$CaCl_2$ $2H_20$	0.08
Tryptone	4
Hemin	0.002
Resazurin	0.0001
Na_2CO_3	8
cysteine-HCl	1

Table 3.S2: *C. perfringens* tryptone dependence in growth medium.

	C. perfringens				*B. thetaiotaomicron*			
Media Type	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4
Trypticase, no YE	-	-	-	-	-	-	-	-
Trypticase, no YE + Glc	++	++	++	-	++++	++++	++++	-
Trypticase, YE	+	+	+	-	-	-	-	-
Tryptone, no YE	-	-	-	-	-	-	-	-
Tryptone, no YE +Glc	+++	+++	+++	-	++++	++++	++++	-
Tryptone, YE	+	+	+	-	-	-	-	-
No Tryptone, No YE +Glc	-	-	-	-	-	-	-	-
Trpticase, YE + Glc	++++	++++	++++	-	++++	++++	++++	-
Tryptone, YE + Glc	++++	++++	++++	-	++++	++++	++++	-
No Tryp. No YE. No Glc	-	-	-	-	-	-	-	-
TYG	++++	++++	++++	-	++++	++++	++++	-

Figure 3.S1: *C. perfringens* CP1 growth on mucus agar. Minimalized medium-agar mixed with 2% (w/v) porcine gastric mucin (Sigma) or crude intestinal mucus harvested from broiler chicken small intestine supported growth of *C. perfringens* CP1 when grown anaerobically for 24 hours.

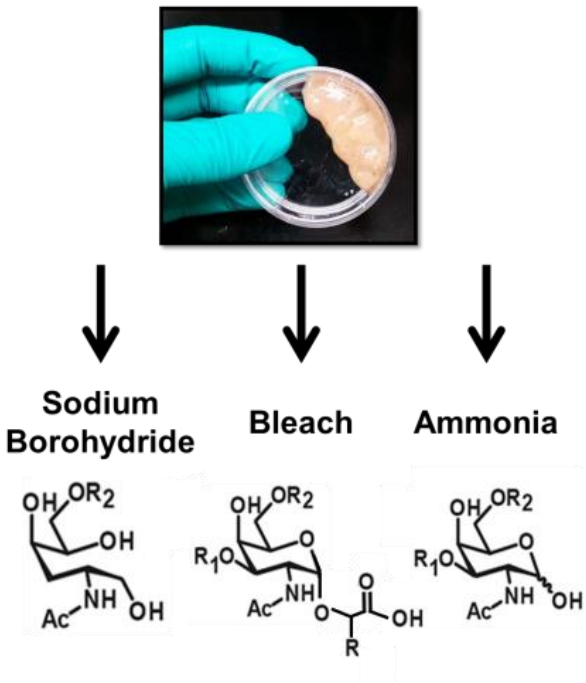


Figure 3.S2: Mucin *O*-glycan reducing end chemistry. Schematic of mucin *O*-glycan extraction methods tested on semi-purified chicken intestinal mucus. Each method used different reducing agents which yield a unique chemistry at the reducing end. The three methods tested used sodium borohydride (129), ammonia (134), or sodium hypochlorite (bleach) (150) as the primary reducing agent in reaction with crude mucus.

Chapter 4

Conclusion and Future Directions

4.1 Conclusion

This study is the first systematic glycomic examination of the *in vitro* utilization of intestinal mucin *O*-glycans by Gram-positive anaerobic opportunistic pathogen, *C. perfringens* CP1. Demonstrated here is a streamlined approach for mucus extraction from broiler chicken intestines which result in a semi-purified substrate. This minimally-processed substrate is more representative of native intestinal mucus when compared to classical methods (88,131) as the material is not fractioned and native disulfide bonds have not been reduced. In addition, this is the first observation of *C. perfringens* mucus utilization without additional modifications or desalting techniques. Furthermore, the ability of the bacterium to metabolize intestinal mucin *O*-glycans extracted from crude mucus using three distinct chemical methods (129,134,150) was also assessed, and modifications to the mucin *O*-glycans and monosaccharide compositional changes following bacterial growth were measured. Significantly, the increase in anionic glycans in broiler chicken intestinal mucin *O*-glycan structures previously proposed to provide shielding or charge-repulsion effects to minimize bacterial pathogen contact (79) were observed to be a favorable substrate for *C. perfringens* CP1 in this study.

Intestinal mucin *O*-glycan composition across species and the mechanism for utilization of these complex glycans by commensal and pathogenic bacterial populations remain ill-defined. Adaptation of model systems, such as the *C. perfringens in vitro* mucin utilization assay demonstrated in this study, will enable investigations at the molecular level into host mucin glycan modification events in the gut. Previous demonstration that mucin glycans can be modified by the host with sialyation and sulfation, to increase the negative charge surface and perturb the adhesion and colonization of bacterial pathogens provided a strong foundation for

understanding the dynamic nature of this barrier and their significance for colonization resistance (143,175). The increase of anionic charge density of the mucus barrier have been noted throughout the alimentary tract as effective for inhibiting pathogenesis for a variety of pathogenic microorganisms (117,162,175). Using an optimized *in vitro* mucin utilization assay, we have successfully demonstrated for the first time that *C. perfringens* uses a mucin glycan acquisition strategy which may provide counter measures for host-driven increases in sialyation and sulfation. Thus, it is likely that the expression of *C. perfringens* sialidases and sulfatases are necessary to circumvent this host adaptation for the onset of mucosal infection.

4.2 Future directions

Many different CAZyme activities have been identified to be important for mucin glycan deconstruction, including sialidases (GH33), *α*-fucosidases (GH29, GH95), *β*-*N*-acetylglucosaminidases (GH84, GH85) *β*-galactosidases (GH2, GH20, GH42), α-*N*-acetylglucosaminidases (GH89), *β*1,4-galactosidases (GH98), α-N-acetylgalactosaminidases (GH101 and GH129) (176), as well as other enzyme classes, such as Zmps (97,177). It has been previously established that *C. perfringens* contain many of these enzyme activities (See Figure 1.3) as well as Zmps and sulfatases. The biological role of many of these mucolytic enzymes in *C. perfringens* CP1; however, remain unclear. Mutagenic and structure-function studies targeting these genes and gene products, respectively, in *C. perfringens* CP1 would help inform how this bacterium interacts with and modifies mucin *O*-glycans. These insights would also be helpful for designing small molecule inhibitors for NE intervention strategies in poultry.

During this project, I hypothesized that metabolism of acidomucin glycans by *C. perfringens* provides a competitive advantage within the chicken intestinal tract. In this regard, the significance of Neu5Ac metabolism *in vitro* and *in vivo* remain promising avenues for future

study. RNASeq was recently used to demonstrate that a sialic acid utilization gene cluster (Nan) was activated in *Ruminococcus gnavus* when cultured with porcine mucins, indicating that Neu5Ac is likely metabolized by this bacterium (118). Interestingly, along with several other bacterial pathogens which colonize the human intestinal tract, including *Vibrio cholera, Yersinia enterocolitica,* and *Salmonella enterica, C. perfringens* also encodes a syntenic Nan cluster (172). Future research to elucidate the role of Neu5Ac metabolism in *C. perfringens* CP1, or the protective contributions of Neu5Ac to the mucus layer could be foundational for designing new therapeutics for animal agriculture and possibly public health.

Veterinary researchers have been studying NE in animal models for many years, and some have successfully reproduced NE in chickens experimentally (24,39,50,178). No study to date, however, has assessed associated mucus carbohydrate modifications during subclinical and clinical NE, or the role of *C. perfringens* CAZymes as virulence factors in disease. The combinatorial approach of animal models with functional glycomics in future studies may provide critical insights for understanding the interaction between *C. perfringens* and intestinal *O*-glycans, and translating this knowledge into effective interventions for poultry production.

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