Identification of O-glycan Structures from Chicken Intestinal Mucins Provides Insight into Campylobacter jejuni Pathogenicity*§

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The Gram-negative bacteria Campylobacter jejuni is the primary bacteria responsible for food poisoning in industrialized countries, and acute diarrheal illness is a leading cause of mortality among children in developing countries. C. jejuni are commensal in chickens. They are particularly abundant in the caecal crypts, and poultry products are commonly infected as a result of cross-contamination during processing. The interactions between C. jejuni and chicken intestinal tissues as well as the pathogenic molecular mechanisms of colonization in humans are unknown, but identifying these factors could provide potential targets to reduce the incidence of campylobacteriosis. Recently, purified chicken intestinal mucin was shown to attenuate adherence and invasion of C. jejuni in the human colorectal adenocarcinoma cell line HCT-8 in vitro, and this effect was attributed to mucin O-glycosylation. Mucins from different regions of the chicken intestine inhibited C. jejuni binding and internalization differentially, with large intestine > small intestine > caecum. Here, we use LC-MS to perform a detailed structural analysis of O-glycans released from mucins purified from chicken large intestine, small intestine, and caecum. The O-glycans identified were abundantly sulfated compared with the human intestines, and sulfate moieties were present throughout the chicken intestinal tract. Interestingly, alpha 1–2 linked fucose residues, which have a high binding affinity to C. jejuni, were identified in the small and large intestines. Additionally, N-glycolyneuraminic/N-acetyleneuraminic acid containing structures present as Sda-like epitopes were identified in large intestine samples but not small intestine or caecum. O-glycan structural characterization of chicken intestinal mucins provides insights into adherence and invasion properties of C. jejuni, and may offer prospective candidate molecules aimed at reducing the incidence of infection. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.044867, 1464–1477, 2015.

Campylobacter jejuni infection is widespread in industrialized countries and is the greatest source of food poisoning worldwide. Infection and the resulting diarrhea are the most common cause of death among young children in developing countries, with an estimated 2.1–2.5 million cases per year in the USA. C. jejuni leads to severe gastroenteritis and is also linked to Guillain-Barré and irritable bowel disease (1, 2). Contaminated poultry meat is the most common source of infection, with up to 88% of poultry products being infected (3). The bacteria are commensal in chickens and exist throughout their intestinal tract. However, they do not invade the epithelium of the gut. Purified mucin glycoproteins from the caecum and small and large intestine have been shown to exhibit inhibitory properties against the bacteria in vitro (4). The commensal relationship between C. jejuni occurrence in chickens and the inability to invade the intestinal tissues is poorly understood. Enhanced understanding of how the properties of the chicken mucosal barrier differ from humans; as well as changes in mucin composition along the chicken intestinal tract, have the potential to explain the commensal behavior of C. jejuni in this species. Such information could also provide leads for the development of agents to limit infection.

Intestinal mucus in vertebrates is a hydrated gel comprised mainly of very high molecular weight and polymeric secreted mucin glycoproteins that are heavily O-glycosylated. O-linked glycans constitute up to 80% of the mucin by weight and represent an abundant potential carbon source for the resident microflora. They also present targets for bacterial adhesion and chemotaxis that may be exploited by both

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1 The abbreviations used are: Fuc, fucose; Hex, hexose; HexNac, N-acetylhexosamine; HexNAcol, reduced N-acetylhexosamine; NANA, Neu5NAc N-acetyleneuraminic acid; NGNA, Neu5Gc N-glycoly- lneuraminic acid.
commensal and pathogenic organisms. Intestinal mucus is a
dynamic barrier layer that is mainly secreted by goblet cells. It
forms a supramucosal layer to protect the gastrointestinal
epithelial cells against infection. A density gradient of vis-
coelastic and highly hydrated mucins polymers exists from
the inner to the outer mucus layer. This stratification is most
clearly demonstrable in the colon, where a relatively tightly
attached mucus layer proximal to the epithelium is normally
dvoid of bacteria, but a less cross-linked more superficial
layer is heavily colonized (5). In humans, it has been shown
that the abundance of bacteria is highest in the large intestine,
where colonization of the mucosal epithelium is contested by
a thick mucus layer (~700 μm) and more rapid mucus turn-
over (6). In the small intestine, however, where bacteria
are much less abundant, the mucus layer is between 100–400
μm thick. O-glycosylation confers the mucus component of
the mucosal barrier with much of the protective properties
required to separate the abundant gut microflora from the
immune system of its host. Defects in mucin glycosylation
lead to severe inflammation and susceptibility to infection,
and the glycans themselves have been shown to be ligands
that can block the binding of microorganisms (7–14). Thus,
secreted gel-forming mucins are key components of intestinal
defense, beyond which bacterial pathogens must normally
penetrate to cause pathology through interaction with epithe-

cells.

Several C. jejuni adhesion proteins required for colonization
of chickens have been identified (15), but the mechanisms by
which the bacteria initiate interactions with carbohydrates on
mucosal surfaces remain unclear. However, many hypotheses
can be made from previous studies where bacterial-carbohy-
drate interactions have been investigated (Table I). Work from
Day et al. demonstrated that glycan array binding of two forms
of the C. jejuni NCTC11168 strain; an original unpassaged
isolate and one with multiple passages having adapted to
laboratory conditions was influenced by temperature and oxi-
gen availability. The different isolates bound structures con-
taining fucose, mannose, terminal galactose, and N-acetyl-
neuraminic acid (7). Additionally, bovine mucins and L-fucose
are known chemoattractants for C. jejuni (8), and L-fucose is a
substrate for C. jejuni growth (13). Glycans from human breast
milk bearing the H(O) blood group antigen (which presents
α1-2-linked fucose) also inhibits infection (12). D-glucose, D-
mannose, and D-fucose, but not the L-sugar equivalents, in-
hibited the binding of C. jejuni to human colonic Caco-2 cells
(16). Muc1, a membrane-bound mucin, is up-regulated during
infection in mice, and knockouts are highly prone to C. jejuni
infection with transepithelial translocation (11). These studies
have provided considerable insight into C. jejuni behavior, but
the specific relationship between the bacteria and mucin gly-
cans in vivo remains unidentified.

Recently, Alemka et al. demonstrated that adherence and
internalization of C. jejuni of the human intestinal cell line
HCT-8 was inhibited by mucins purified from chicken caecum
and small and large intestines in vitro (4). In this study, the
strongest inhibition of binding and invasion was found with
O-glycosylated mucins from the large intestine followed by
the small intestine and caecum, respectively. Following incu-
bation with small and large intestinal mucins, C. jejuni showed
a 6.5-fold decrease in binding to HCT-8 cells and a ~1,000-
fold and ~150-fold decrease in internalization, respectively.
Mucin from the chicken caecum only exhibited a fivefold
reduction in internalization. The presence of chicken mucin
had no effect in bacterial viability. When mucin O-glycans
from each chicken intestinal section were oxidized with so-
dium metaperiodate, binding and internalization by HCT-8
cells returned to levels observed in the untreated controls.

These results illustrate that the O-glycans expressed on the
secreted intestinal mucins of chickens can inhibit colonization
regioselectively. A detailed structural characterization of
chicken intestinal mucin glycans from these different sites
may provide insights into what makes this species resistant
to the pathology caused by this organism in humans. There-
fore, we now report the mucin O-glycan structures from

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**TABLE I**

| Source         | Type                        | Observations                                                                 | Ref. |
|----------------|-----------------------------|------------------------------------------------------------------------------|------|
| Glycan array   | mannose Lewis a, Lewis b    | Bacterial binding was affected by temperature, strain and oxygen concentration| 7    |
|                | Lewis a2-3Neu5Ac a2-6Neu5Ac |                                                                               |      |
| Human milk     | Lewis b α1–2 fucose         | Inhibition of bacterial growth                                                | 12   |
| Monosaccharide | D-mannose O-glucose d-fucose| Decrease in association and invasion with Caco-2 human colorectal cells      | 15   |
| Monosaccharide | L-fucose                    | Chemotaxis towards L-fucose and mucins                                       | 8    |
| Bovine mucin   | O-glycans                  | Up-regulated MUC1 during infection and KO mice are more susceptible to infection | 11   |
| Murine MUC-1   | O-glycans                  | L-fucose improved bacterial growth as energy source                          | 13   |
| Monosaccharide | L-fucose                   |                                                                               |      |

Lewis a, Galβ1–3(Fucα1–4)GlcNac; Lewis b, Fucα1–2Galβ1–3(Fucα1–4)GlcNac; Lewis a, Galβ1–4(Fucα1–3)GlcNac; Lewis b, Fucα1–2Galβ1–4(Fucα1–3)GlcNac; Neu5Ac, N-acetyleneuraminic acid.
EXPERIMENTAL PROCEDURES

Chicken Mucin Isolation and Purification—Chicken intestinal sections (large intestine, small intestine, and caecum) from healthy animals were collected and epithelial mucins were purified from each. Chemicals used were of analytical grade quality and sourced from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Mucins were isolated and purified in accordance with published methodology (4, 17, 18). Six hens were sacrificed, and the intestine was dissected from each. The tract was divided into sections of caecum, small intestine, and large intestine. Each section was then slit longitudinally, and the mucus at the epithelial surface was scraped into 7 ml G6HCl in PBS using a scalpel. These samples were rolled at room temperature for up to 5 days to solubilize the mucus and allow the samples to become homogenous. Dithiothreitol was added at a final concentration of 0.01 M and incubated for 5 h at 37 °C. Iodoacetamide was added to a final concentration of 0.025 M. The samples were centrifuged at 23,000 g for 60 min at 4 °C to remove insoluble components. The supernatants were retained and the pellets discarded.

The density of dispersed mucus was then adjusted to 1.42 g/ml using cesium chloride. Samples were then centrifuged at 65,000 rpm for 18 h at 10 °C. 1 ml aliquots were removed along the gradient from top to bottom, and the density of each fraction was measured. The relative intensity of a 5 μl aliquot was assessed from each fraction when slot blotted onto a polyvinyllidene fluoride membrane and stained using periodic acid–Schiff stain (PAS staining). Based on the density and PAS analysis, the fractions containing mucin were identified and pooled. High molecular weight mucin was then separated from these pooled samples by size exclusion chromatography. The column was packed with Sepharose Cl-4B to a volume of 240 ml, and the mobile phase used was 50 mM Tris, pH 7.5. The eluent was 1.0 M NaBH4 in 0.10 M NaOH for 16 h at 50 °C. Release of carbohydrates from porcine gastric mucin (Sigma-Aldrich) on the top of a C18 Zip-tip acetic acid. Peptides were removed and samples were simultaneously desalted using a hand-packed bed of DOWEX AG-50 cation exchange resin (BioRad, Hecules, CA) on the top of a C18 Zip-tip (Millipore, Billerica, MA). Prior to the sample addition, the resin was washed with 1 ml methanol, primed with 1.0 M HCl, and equilibrated with 1 ml H2O. The sample solutions were added and spun for 5 min or until the entire sample was loaded. The resin bed was washed with three aliquots of 50 μl H2O. Samples were dried and borates were removed by a series of five washes consisting of 60 μl 1% acetic acid in MeOH followed by vacuum centrifugation between additions. Glycan samples were reconstituted in 10 μl H2O prior to LC-MS analysis.

LC-MS—Mass spectrometry analysis was performed as described with minor adaptations (20). Briefly, analysis of glycan adducts was performed using a HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), Agilent 1100 HPLC binary pump (Agilent Technologies, Palo Alto, CA), and Thermo LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA). HPLC analytical columns, consisting of 5 μm porous graphitized carbon particles (Hypercarb, Thermo Hypersil-Keystone, Bellefonte, PA), were packed in a fused silica capillary (20 cm × 180 μm inner diameter; outer diameter 375 μm; Polymicro Technologies, Phoenix, AZ). A linear gradient from 0–40% acetonitrile was used consisting of 100% 8 mM NH4HCO3 (solvent A) and 8 mM NH4HCO3 in 80% acetonitrile/20% H2O (solvent B). The flow rate was between 8–12 μl/min generated by a preinjection flow splitter. MS data were acquired in negative mode, and MS2 spectra were obtained automatically using the Xcalibur 2.1 MS software. Glycan ions were detected as [M–H]− and [M–2H]2− ions and elution time ranged between 6–27 min. The collision-induced dissociation energy was set to 30%, and the spray voltage was 3.2 kV. FMS and MS2 data were annotated manually with the assistance of the Unicarb-DB glycan database for spectral matching (http://www.unicarb-db.com) (21).

Reverse Phase HPLC: Sialic Acid Speciation—The LuderTag™ DMB sialic acid release and labeling kit was purchased from Luder Ltd (Oxfordshire, UK). 100 μg aliquot of purified large intestinal mucin was dried and 25 μl 2 M acetic acid was added. It was then incubated at 80 °C for 2 h. Samples were cooled, and 5 μl was removed, transferred to a 0.5 ml centrifuge tube, and 20 μl 1,2-diamo-4,5-methylenedioxybenzene (DMB) labeling mix was added. The labeling mix was prepared by adding 440 μl mercaptoethanol solution to the sodium dithionite vial and mixed until completely dissolved. This mixture was added to the DMB vial and mixed. The DMB labeling reaction was carried out for 3 h at 50 °C. The reaction was quenched with the addition of 475 μl water. The sialic acid quantitative standards were prepared as outlined in the kit protocol. Analysis was performed using a Waters XBridge™ C18 3.5 μm particle size 2.1 × 150 mm column, Waters Alliance 2695 HPLC with a 2475 Multi-Wavelength Fluorescence Detector (Waters Corp., Milford, MA). Solvent A was 10 mM ammonium formate adjusted to pH 4.4, and solvent B was acetonitrile. The flow rate was 0.2 ml/min and separation was performed with a 40 min linear gradient running from 0% to 50% solvent B. The λex and λem were 373 nm and 448 nm, respectively.

RESULTS

Here, we present a detailed analysis of O-glycans released from purified chicken caecum, small intestinal, and large intestinal mucins by LC-MS+ analysis using porous graphitized carbon, which is a well-established technique (20) for assigning glycan structures. Annotation of fragmentation spectra was performed de novo where glycan fragments were assigned manually and checked with the aid of UniCarb-DB, an on-line database of LC-MS mucin O-glycan fragmentation data (21). The online Mucin Glycan Database from the Mucin Biology Group at the University of Gothenburg was also a resource for MS2 spectral matching (22); however, not all glycan structures assigned in this study were represented in these databases. GlycoworkBench (23) was also used to verify glycan assignments as follows; MS2 peak lists (fragment ions with a relative intensity >1%) were imported, and fragment ions were annotated from candidate structures. These data were uploaded to Unicarb-DB and are freely available. Branching and topology were established from MS2 spectra, and the assignment of specific structural features (i.e., Lewis and blood group epitopes) was based on structural assumptions of the individual monosaccharide identification, namely...
Hex and HexNAc residues as galactose and N-acetylgluco-
samine, respectively.

Glycans were not modified prior to analysis and were ob-
served as deprotonated species, primarily $[\text{M-}H^{-}]$ with minor
$[\text{M}-2\text{H}]^{2+}$ ions seen for larger structures. A control sample of
porcine gastric mucin (PGM) from Sigma Aldrich was run prior
to analysis to ensure instrument settings were optimized to
limit in-source fragmentation. Observed and theoretical mass-
to-charge values with corresponding glycan structures are
presented in Table II. In the absence of diagnostic fragment
ions, as seen in the higher mass O-glycans where detailed
assignments were not possible, only a compositional descrip-
tion was assigned and presented. Additionally, the relative
abundance of each precursor ion from the combined spectra
(spanning 6–27 min) was determined. These values were
calculated as a ratio of the measured intensity of each ion to
the sum of all ion intensities. The distribution within each type
(neutral, sialylated, sulfated, and sulfo-sialylated) was also
determined under the same principle but with the ratio of each
O-glycan structure as a ratio to the sum of peaks within each
group. Glycan fragmentation vocabulary used was defined by
Domon and Costello (24), and O-glycan cartoon representa-
tion was the CFG glycan nomenclature (Supplemental Fig. 1)
(25). Structures were assigned (Table II) from collision-in-
duced dissociation spectra and were found to be primarily
extended core III (GlcNAc$\beta$1–3GalNAc) and core IV
(GlcNAc$\beta$1–3[GlcNAc$\beta$1–6]GalNAc) O-glycans. Core I and
core II O-glycans were also detected in the caecum.

**Mucin O-glycan Diversity Is Correlated to C. jejuni Colonization
Density**—The overall glycan compositions in each sec-
tion of the intestinal tract examined, determined from the
combined MS profiles (Fig. 1), showed the diversity and range
in the number of O-glycans in the chicken intestinal tract.
Mucin glycosylation heterogeneity increased from the cae-
cum toward the large intestine, with 23 structures found in the
caecum, 40 in the small intestine, and 60 in the large intestine.
The increase in glycan microheterogeneity also corresponds
with the greatest inhibition of *C. jejuni* binding to HCT-8 cells
(i.e. large intestines glycans inhibited the most). There were 16
structures unique to the chicken large intestine compared
with other sections. The intensity (signal strength) of the com-
bined large and small intestine MS$^1$ spectra (6–27 min) were
comparable (NL: 1.12E1 and NL: 2.01E1), whereas the cae-
cum O-glycan sample intensity was an order of magnitude
lower (NL: 4.31), indicating a lower degree of glycosylation of
mucins in caecum.

The distribution of glycans by type in the large intestine was
26% neutral, 29% sialylated, 34% sulfated, and 11% sulfo-
sialylated. O-glycan distribution in the small intestine was
similar to the large intestines with 40% neutral, 23% sialy-
lated, 33% sulfated, and 4% sulfated-sialylated glycans, while
the caecum contained more sulfated (57%) and less sialylated
(14%) with a similar amount of neutral (22%) and sulfat-
sialylated (7%) glycans as the other two sections. Neutral

glycans only represent a small number of structures identified
in the chicken intestines, especially in the caecum. There were
15 neutral structures in the large intestines, 13 in the small
intestines, and five in the caecum. Most noticeable between
samples was the presence of highly anionic structures that
contained sulfate and/or sialic acid residues.

**O-glycans Were Predominantly Extended Core III and Core
IV Extended with Both Type I and II LacNAc—O-glycan cores
I, II, III, and IV were identified in all mucin sections (Fig. 2) but
core III and core IV predominated based on ion intensities in
the MS$^1$ spectra. Core III structural isomers were identified
from the collision-induced dissociation fragmentation of the
$m/z$ 587 ion (HexHexNAcHexNAc), confirming the presence of
an extended core III structure with both type I Gal$\beta$1–3GlcNAc
and type II Gal$\beta$1–4GlcNAc residues. Core II (Gal$\beta$1–3
GlcNAc$\beta$1–6GalNAc) structures were detected in the large
and small intestines and comprised a small percentage (0.3%
and 0.4%, respectively). The most intense fragment in the
core II MS$^2$ spectrum was a 407 ion, resulting from the elim-
ination of the galactose linked at the C-3 position of the
reducing end GalNAcol. This characteristic C-3 fragmentation
was used to identify the branching of neutral and sialylated
core II and core IV structures from the MS$^2$ spectra through-
out the analysis. The B type fragment ion of m/z 364 repres-
ents the loss (-223) of the reducing end GalNAcol residue,
and this type of neutral loss was informative for assigning
linear core types (i.e. core I and III) together with the 108 Da
loss (C$\alpha$H$_2$O$_2$).

Evidence for both type I and II LacNAc extensions was based
on diagnostic ions in MS$^2$ spectra and description of
fragmentation mechanisms (20, 26). Evidence for the Gal$\beta$1–
4GlcNAc linkage (type II) of a core III structure was supported
by the presence of the $m/z$ 263 ion ($^{2-2}A$H$_2$O) and $m/z$ 221
($^{2-4}A$) fragment (Fig. 2). However, the $m/z$ 263 ion was also
observed in the core III type I spectrum and is likely from
coelection of the precursor ion during analysis. It is important
to note that the 263 ion was detected in the MS$^2$ spectrum
from the proposed core III type II structure in the small intes-
stenes but not in the type I spectrum (raw data are found in
UniCarb-DB). When detected, the m/z 263 ion was used for
differentiating type I or type II as was the case for the $m/z$ 790
ion (core III, type II with an additional HexNAc). For the type I
glycan, it was found that ions resulting from a neutral loss of
108 Da on the reducing end as described above could be
further fragmented by a galactose neutral loss (−162) giving
the type I diagnostic ion fragment of m/z 317. Furthermore,
the m/z 202 ion ($\gamma_2/B_2$ cleavage) was detected in the type I
structure and not the type II. However, in the absence of
diagnostic ions to specify either type, O-glycan structures
were designated with a “Hex-3/4” linkage as seen in Table II.

Core IV structures were indicated by presence of a trisac-
charide with the composition HexNAc$\beta$HexNAcol ([M - H]$^-$
628 ion). Further evidence was provided from the MS$^2$ where
a predominant Z type m/z 407 fragment showed that one
### TABLE II

**Mucin O-glycans identified from chicken caecum, small intestine, and large intestine**

| Neutral O-glycans | Relative Abundance | Caecum | CMSI | CMLI | Human |
|-------------------|--------------------|--------|------|------|--------|
| Obs m/z            | Theor m/z          | Proposed Structure | → Increased C. jejuni Inhibition → |        |        |
| 384.08            | 384.15             | Hex-3HexNAcOol     | 9.2 (41.1) | 7.6 (19.0) | 5.2 (20.0) |
| 425.08            | 425.18             | HexNAcO-3HexNAcOol | 8.8 (39.5) | 9.8 (24.5) | 5.7 (22.3) |
| 587.17            | 587.23             | Hex-4HexNAc-3HexNAcOol | 0.9 (3.8) | 1.3 (3.2) | 0.4 (1.6) |
|                   |                    | Hex-3HexNAc-3HexNAcOol | 0.3 (1.4) | 1.8 (4.4) | 1.2 (4.7) |
|                   |                    | Hex-3HexNAc-3HexNAcOol | 0.3 (0.7) | 0.4 (1.6) |
| 628.25            | 628.26             | HexNAcO-3HexNAcOol | 3.2 (14.2) | 3.9 (6.8) | 1.5 (5.7) |
| 733.17            | 733.29             | Hex-43Fuc-34HexNAcOol | 2.5 (8.3) | 2.3 (8.8) |
| 749.17            | 749.28             | Hex-3HexNAc-3HexNAcOol | 1.1 (4.4) |
| 790.17            | 790.25             | Hex-4HexNAcOol     | 2.7 (6.7) | 0.9 (2.5) |
| 895.17            | 895.34             | Fuc-2Hex-3HexNAcOol | 1.3 (3.2) | 1.2 (4.5) |
| 936.25            | 936.37             | Hex-43Fuc-34HexNAcOol | 3.8 (9.4) | 1.0 (3.9) |
|                   |                    | HexNAcO-3HexNAcOol | 1.4 (3.5) | 1.3 (4.9) |
| 1041.17           | 1041.40            | Fuc-2Hex-3HexNAcOol | 1.3 (3.5) |
| 1086.25           | 1086.42            | Fuc-2Hex-3HexNAcOol | 1.7 (4.3) | 0.9 (2.7) |
| 1244.25           | 1244.48            | Fuc-2Hex-3HexNAcOol | 2.1 (5.2) | 1.4 (5.5) |
|                   |                    |                   | 22.4     | 40.1   | 25.8   |

| Sialylated O-glycans | Relative Abundance | Caecum | CMSI | CMLI | Human |
|----------------------|--------------------|--------|------|------|--------|
| Obs m/z              | Theor m/z          | Proposed Structure | → Increased C. jejuni Inhibition → |        |        |
| 716.25              | 716.27             | HexNAcO-3Neu5Ac-3HexNAcOol | 3.8 (29.1) | 2.6 (10.3) | 1.9 (6.6) |
| 869.26              | 868.31             | Neu5AcHex-3HexNAcOol | 1.2 (5.3) | 1.4 (4.6) |
| 884.26              | 884.26             | Neu5GcFucHex-3HexNAcOol | 1.8 (6.0) |
| 1024.33             | 1024.38            | Hex-343Fuc-3HexNAcOol | 0.6 (2.4) | 0.6 (1.9) |
|                     |                    | Neu5AcHex-343Fuc-3HexNAcOol | 1.4 (5.7) | 2.5 (8.4) |
| 1040.25             | 1040.38            | Neu5GcHex-343Fuc-3HexNAcOol | 1.1 (3.8) |
| 1081.33             | 1081.41            | HexNAcO-3Neu5Ac-3HexNAcOol | 2.6 (11.1) | 1.3 (4.6) |
| 1169.25             | 1169.42            | Neu5AcHex-3HexNAcOol | 1.5 (5.2) |
| 1186.33             | 1185.44            | Fuc-2Hex-3Neu5Ac-3HexNAcOol | 1.8 (6.8) | 1.0 (3.5) |
| 1227.46             | 1227.46            | Neu5AcHex-343Fuc-3HexNAcOol | 1.6 (6.8) | 1.0 (3.5) |
| 1243.43             | 1243.46            | Hex-343HexNAcOol | 5.8 (36.8) | 6.1 (25.7) | 1.7 (5.7) |
| 1259.45             | 1259.45            | Hex-3HexNAcOol | 5.0 (36.8) | 6.1 (25.7) | 1.7 (5.7) |
| 1315.42             | 1315.48            | Hex-43Fuc-3HexNAcOol | 4.8 (36.0) | 4.6 (36.0) |
| 1389.42             | 1389.52            | Fuc-2Hex-3HexNAcOol | 2.7 (11.4) | 2.0 (8.8) |
| 1405.51             | 1405.51            | HexNAcO-343Fuc-3HexNAcOol | 2.1 (9.0) | 1.9 (6.4) |
| 1535.53             | 1535.57            | Neu5AcFucHex-3HexNAcOol | 1.9 (6.8) | 1.7 (4.9) |
| 1551.57             | 1551.57            | Hex-343Fuc-2Hex-3HexNAcOol | 1.6 (14.4) |
| 1587.56             | 1587.56            | HexNAcO-343Fuc-3HexNAcOol | 1.7 (7.7) |
| 1680.42             | 1680.61            | Neu5AcFucHex-3HexNAcOol | 1.9 (5.5) |
| 1826.33             | 1826.67            | Neu5AcFucHex-3HexNAcOol | 0.9 (4.0) | 0.9 (3.2) |
|                   |                    |                   | 13.6     | 23.6   | 29.4   |

| Sulphated O-glycans | Relative Abundance | Caecum | CMSI | CMLI | Human |
|---------------------|--------------------|--------|------|------|--------|
| Obs m/z             | Theor m/z          | Proposed Structure | → Increased C. jejuni Inhibition → |        |        |
| 464.08              | 464.11             | (SO)3Hex-3HexNAcOol | 4.2 (12.4) |
| 505.08              | 505.13             | (SO)3HexNAcOol | 3.9 (11.6) | 3.2 (9.3) |
| 610.16              | 610.16             | Fuc-2(SO)3HexNAcOol | 3.2 (5.6) | 1.5 (4.5) |
| 628.75              | 628.86             | (SO)3Hex-4HexNAcOol | 3.2 (5.6) | 3.8 (11.9) | 1.5 (4.3) |
| 667.25              | 667.19             | (SO)3Hex-3HexNAcOol | 11.0 (19.3) | 8.0 (24.7) | 1.7 (4.9) |
|                     |                    | (SO)3Hex-3HexNAcOol | 1.9 (3.3) | 0.5 (1.6) | 0.7 (2.0) |
|                     |                    | (SO)3Hex-3HexNAcOol | 0.7 (2.1) | 1.0 (2.8) |
| 701.67              | 701.69             | (SO)3Hex-43Fuc-3HexNAcOol | 6.0 (10.6) |
| 813.17              | 813.25             | Hex-43Fuc-3HexNAcOol | 3.1 (5.5) | 0.9 (2.5) | 3.7 (11.0) |
| 828.25              | 829.34             | (SO)3HexNAcOol | 3.1 (5.5) | 0.9 (2.5) | 3.7 (11.0) |
| 870.25              | 870.27             | (SO)3HexNAcOol | 5.1 (8.9) | 5.6 (17.1) | 1.7 (5.1) |
| 975.25              | 975.30             | Fuc-2Hex-3(SO)3HexNAcOol | 3.8 (6.6) | 1.3 (4.0) | 1.7 (4.9) |

**Notes:**
- As described in the text, the implications of increased C. jejuni inhibition are crucial for understanding the role of these O-glycans in gut health and disease.
Temporal & Sulphated O-glycans

| m/z     | Sialylated & Sulphated O-glycans | — Increased C. jejuni Inhibition — |
|---------|----------------------------------|----------------------------------|
| 847.17* | 847.24*                          | 2.2 (20.6)                       |
| 958.17  | 958.28                           | 1.8 (14.8)                       |
| 1104.17 | 1104.34                          | 1.1 (31.2)                       |
| 1120.25 | 1120.34                          | 1.1 (10.4)                       |
| 1469.25*| 1469.47                          | 1.4 (39.7)                       |
| 1615.33*| 1615.53                          | 1.1 (29.1)                       |

GlcNAc was linked to the C-3 of GalNAcO, leaving the additional GlcNAc attached to the C-6 position. The characteristic 262.2* cleavage from the reduced GalNAc indicative of a GlcNAcβ1-6GalNAc was also identified. Overall, the most common core O-glycans were type III and IV and subsequent structures throughout this study were primarily extensions of these structures.

The C. jejuni Chemo-Attractant α1–2 Fucose Is Present on Large and Small Intestine Mucins—Fucose-containing glycans were present throughout the intestinal tract and were identified with blood group B (Galβ1–3(Fucβ1–2Gal)-) like and blood group B (Galβ1–3(Fucβ1–2Gal)-) like epitopes as well as α1–3/4 linkages present as Lewisα/β (Galβ1–3(Fucβ1–3/4)Gal-NAc-) epipitopes. The sequence of blood group H-like structures in the large and small intestine is illustrated by the fragmentation of the m/z 895 ion where the fucose residue was linked to the penultimate hexose residue, presumably galactose (Fig. 3). Ions 731 and 749 (Z- and Y-type fragments) were from the neutral loss of a fucose, and ions 715 (Z-type) and 733 (Y-type) resulted from a hexose loss, but these ions alone were not diagnostic of a blood group epitope. The m/z 569 (Z-type) and m/z 731/749 (Z- and Y-type) fragment ions showed the fucose-hexose disaccharide is in a linear conformation. The 569 ion, which corresponds to the neutral loss of a hexose and fucose, contains the reducing end HexNAcO with a hexose and HexNAc residue. This ion could arise from a structure with a terminal hexose-fucose disaccharide, and together with Z- ion of m/z 407 and the m/z 389 ion (407 - H2O) support the presence of a blood group H-like epitope on the C-3 branch of the GalNAcO. Spectral and retention time matching to the equivalent m/z 895 ion isolated from the well-characterized blood group H expressing PGM standard provided further evidence for the presence of blood group H epitopes in chicken intestinal mucins (Fig. 3, inset). An additional blood group H containing structure was found in the large intestines (m/z 1041) as an extension of the m/z 895 structure with an additional fucose, and comparison to the PGM equivalent also supported this structural assignment. These two structures represent the only neutral blood group H-like glycans, but several blood group H-like sulfated and sialylated structures were also found (Table II). A blood group B-like epitope was detected in the MS2 spectra of Table II.

Other neutral-fucosylated structures with α1–3/4 linked fucose were identified (m/z 733, 936, 1098, and 1244) in the small and large intestines only. The fragmentation spectra of the m/z 733 glycan ion from the large and small intestines were consistent with a Lewisα/β type epitope (Fig. 3). The predominant fragment ion at m/z 359 was produced from the loss of the hexose and fucose residues (a double Z-type fragment ion; m/z 389) together with a loss of the CH2O side group (~30 Da). Spectral matching of the 733 MS2 to a previous report characterizing Lewis and blood group MS2 fragments of five structures with identical compositions (20).
further supported the structural assignment in this study. The 936 and 1098 structures were extended core IV structures with Lewisα/β epitopes present on either the C-3 or C-6 branch (Supplemental Fig. 2). Collision-induced dissociation of m/z 936 resulted in a HexNAc-HexNAcol 425 ion from a Y-type cleavage at the C-3 position of the HexNAcol identifying the presence of Hex-Fuc1-HexNAc residues at that site. The relative lower intensity of the m/z 407 ion versus the 715 ion suggests the predominant isomer contains the Hex-Fuc-HexNAc substituents on the 6-arm as shown in Supplemental Fig. 2. This is also the case for the m/z 1098 structure (m/z 527 versus the m/z 715 ion), also suggesting the major isomer having the Hex-Fuc-HexNAc on the 6-arm. Similarly, the m/z 733 fragment ion (Y-type at the 3-arm HexNAcol) identifies the Lewisα/β epitope linked at the 6-position. Comparing the ion intensities may suggest the Lewis epitope is present more at the 6-position. The overall identification of the Lewisα/β epitope for both structures was further supported by the neutral loss of hexose (m/z 774) and fucose (m/z 790). Correspondingly, the m/z 587/569 and m/z 733/715 fragment ions in the m/z 1098 spectrum also identify the Lewis epitopes primarily on the 6- arm, respectively.

Mucin Sialylation and the Presence of N-glycolylneuraminic in the Large Intestines—The presence of sialylated glycans was highest in the large intestine (n = 22), followed by the small intestines with 13 structures. Only three sialylated O-glycans were detected in the caecum, all of which were extended core III structures. In most cases, the sialic acid monosaccharide was N-acetyllneuraminic acid, but interestingly,
N-glycolylneuraminic acid (NGNA)-containing O-glycans were found in the large intestine (m/z 1405, 1567, and 884 [M-2H]2-). Chickens lack the enzyme to produce NGNA residues but trace amounts have previously been reported (27), and the presence of NGNA identified by MS in this study was further confirmed by sialic acid speciation HPLC (Fig. 4A).

Several O-glycans were found in the chicken large intestines that were not in the small intestines or caecum. These ions were m/z 1040, 1169, 1259, 1405, 1551, 1567, 1680, and 884 (M-2H)2-. Four of these contained N-glycolyneuraminic acid (m/z 1040, 1259, 1405, and 1567). Structures containing two sialic acid residues were present; the most prominent was the Neu5NAc2Fuc2Hex2HexNAc2HexNAcol composition (detected as m/z 1680 [M-H]- and 840 [M-H]2- ions). This glycan represented the largest molecular weight glycan and constitutes ~8% of glycans in the large intestine based on MS intensities. The m/z 1040 NGNA-containing glycan had a single structure compared with the NANA containing equivalent m/z 1024 ion. The m/z 1024 ion contained two isomers, one with NANA on the nonreducing end hexose (Neu5Ac3Hex3HexNAc3HexNAcol) and the second isomer containing NANA linked to the reducing end (Hex3Hex3/Fuc3Hex3/HexNAc3(Neu5Ac6HexNAcol) (Fig. 4B). These isomers had different retention times and MS2 fragmentation spectra, confirming the structural assignments (Supplemental Fig. 3).

The m/z 1551 ion and the corresponding N-glycolyneuraminic acid containing structure (m/z 1567) had matching fragmentation MS2 spectra suggesting the structures were identical except for the sialic acid residue (Fig. 5). There is
evidence for both blood group B-like epitopes and Sdα-like antigens that were determined from several important diagnostic ions. The presence of the 772 ion suggested substitution at the C-6 and was consistent with HexNAc-Hex-HexNAc linked to the reducing end HexNAcol (following the loss of sialic acid). Furthermore, the 466 ion results from the loss of sialic acid (Z-type) and a 1,2A cross-ring cleavage of HexNAc residue linked to the reducing end HexNAcol. These ions in addition to the 569 fragment ion that comes from a HexNAc loss of the 772 fragment, verifies a HexNAc-Hex-HexNAc sequence. Additional evidence for Sdα-like-containing structures was also detected for the 1243/1259 and 1389/1405 ions. Sdα-like antigens appear to be a common feature for chicken large intestines, and Sdα antigens have been found in other species such as human, mice, and rat. For the blood group B-like (Galβ1–3(Fucβ1–2)Gal-) assignment, the determining fragments were from the 895 (hexose loss on the C-3 arm) and 730 (additional fucose loss) fragment ions. Together, these show the presence of a terminally linked hexose and fucose residues linked to the hexose at the C-3 position indicative of a blood group B epitope.

**Chicken Intestinal Mucin Glycans Are Highly Sulfated**—The most recurrent structural motif in all chicken intestinal sections was sulfation. There were 19 sulfated structures in the large intestines and 13 sulfated structures in the caecum and small intestine. Despite the relatively reduced diversity of nonsulfated structures in the caecum, surprisingly, the number of sulfated glycans was nearly equal in number to structures in the small and large intestines. MS² spectra of sulfated structures confirmed that sulfates were present on hexose and N-acetylgalactosamine residues as shown from fragmentation of the most commonly observed sulfated ions (Fig. 6) and additional sulfated species (Supplemental Fig. 4). Sulfated residues were identified on hexose residues typically as B-type ions m/z 241 and on N-acetylgalactosamine residues (B-type ions m/z 282). Sulfated O-glycans were found in most cases to be extensions of the neutral or sialylated glycans reported in Table II with the sulfate moiety located primarily on terminal residues presumably linked to the 3 or 6 position on galactose residues. We did not identify these linkages specifically, and thus, they were not reported.

**Large Intestine Mucins Contain Keratan Sulfate-Like LacNAc Repeats**—Four structures containing sulfated galactose β1–4N-acetylgalactosamine repeats, characteristic of keratan sulfate, were identified. These structures (m/z 1178, 1324, 628 [M-2H]², and 701 [M-2H]²) also contained one or two fucose residues linked to HexNAc residues, presumably forming Lewisα decoration of the sulfated galactose β1–4N-acetylgalactosamine repeats. The assigned structures corresponded to extended core III glycans with sulfates on the hexose and/or HexNAc residues. The doubly charged ions 628 and 701 [M-2H]² structures contained two sulfates linked to hexose/HexNAc residues and only differed by one fucose residue (Supplemental Fig. 5). Similar structures were identified with one sulfate (m/z 1324, 1178, and 1032) and two, one, and zero fucose residues. These structures were identified in all three intestinal sections.

**Sialylated-Sulfated O-glycans**—O-glycans with both sulfate and sialic acid residues were identified in the chicken mucin. There were seven structures in the large intestines, three in the small intestines, and two in the caecum. Five of the seven...
structures in the large intestines contained fucose residues, and all the structures in the small intestines and caecum were fucosylated. The degree of sulfation in chickens increases from the ileum toward the rectum. The masses of sialylated-sulfated O-glycans ranged from 958 \([\text{M-H}]^-\) to 847 \([\text{M-2H}]^{2-}\) (1695 \([\text{M-H}]^-\)). Interestingly, the location of sulfate was primarily on hexose residues in this class of structure.

Differences to Human Intestinal Mucin Structures—A previous report of human O-glycan structures isolated from different gut sections obtained from two male adults of A-Lewis\(^b\) blood groups provides useful information for comparison of structures identified in this study. Evaluation of chicken mucin O-glycans to the equivalent sections in humans (caecum, ileum, and transverse colon) shows considerable differences in the type of glycans present in the gut (Fig. 7). The number of O-glycans was the highest in the ileum (n = 56 and 40) and caecum (n = 42 and 46), followed by the transverse colon (n = 41 and 34). These values contrast to our findings specifically in the caecum section where we identified only 23 O-glycans, which corresponds to the lowest level of \(C.\ jejuni\) inhibition in vitro. Several structures were identified in chicken mucins that are not present in any human intestinal mucins. Overall, there were 33 structures unique to the chicken not reported in any section of the human gut. Of these, nine were unique to the large intestines where \(C.\ jejuni\) inhibition was the greatest.

When comparing the degree of sulfation to the human gut, it becomes evident that mucin sulfation is significantly more predominant in chicken (Fig. 7). We identified 19 sulfated structures in the large intestine (LI) and 13 in the small intestine (SI) and caecum. This contrasts to the amount of sulfation found in the human transverse colon (n = 6 and 2), ileum (n = 2 and 0), and caecum (n = 2 and 6). Sulfated glycans in
LC-MS Analysis of Chicken Mucin O-Glycans

Humans were most predominant in the sigmoidal colon (n = 5 and 7) and rectum (n = 7 and 8). The location of sulfate was found on both hexose (Gal) and HexNAc (GlcNAc) residues. Many sialylated structures were similar between species (i.e. extended core II and III) with the exception of extended core IV structures (m/z 870, 1016, and 1032) found in all three chicken sections. There were not any sulfated core IV glycans in the human gut.

Sialic-acid-containing glycans reported in the human intestinal tract were primarily extended core III, with the sialic acid residue linked at the 6 position on the reducing end GalNAc-ol. There were three structural isomers for the 1024 ion, two of which were present in all human sections. These two structures were a core III with the Neu5Ac linked to the reducing end GlcNAc and a Lewis^a^-epitope (Hex→3/4(Fuc→3/4)-HexNAc→3(Neu5Ac→6)HexNAc). This structure was found in the chicken small and large intestines (Fig. 4), but an additional isomer with a terminal sialic acid was more predominant (Neu5Ac→3Hex→3/4(Fuc→3/4)HexNAc→3HexNAc). This structure was reported in the sigmoidal colon of one patient. In general, we observed sialylation both at the reducing end GalNAc-ol but more commonly linked at terminal positions.

DISCUSSION

This analysis represents a follow-up study from previous work demonstrating that chicken mucin O-glycans inhibit binding and internalization of C. jejuni on the human colorectal HCT-8 cell line. Here, we identified the mucin O-glycan structures and identified unique characteristic structural features that offer insights that may enhance understanding of how chicken mucins inhibit C. jejuni infection. Most notable was the consistent expression of sulfates in all sections of the intestinal tract. Terminal epitopes that were identified as well as the degree of sulfation present on the entire O-glycan is highlighted in Table III. The most common epitope were Lewis^a^-structures with and without sulfate followed by blood group H-like structures. Secondly, the overall number of glycans correlated inversely with the density of C. jejuni in vivo, with the caecum having the highest number of colony-forming units (10^{10} cfu/gram), the lowest glycan diversity, and least inhibitory effects in in vitro studies. Above all, C. jejuni are unable to cross the mucosal barrier in any section of the chicken intestines, and therefore, bacterial resistance may be attributable to the presence of a common structural feature throughout the tract. The extent of chicken mucin sulfation is further emphasized when compared with results from intestinal sections isolated from two healthy human donors. As highlighted in Fig. 7, the number of sulfated structures in chicken caecum was 13 and represents roughly 57% of all O-glycans. By comparison, sulfation in the caecum from two healthy human samples was 13 and 5% (28). These numbers were comparable in the other sections: small intestines 33% (chicken) and 0 and 4% (human); large intestines 30% (chicken) and 6 and 15% (human). However, another report investigating the glycosylation of MUC2 isolated from 25 human colon biopsies classified 42 sulfated O-glycans (29), but it is important to note that these values represent all structures identified and not necessarily the number present in each individual.

Sulfation is a common modification of gut mucin O-glycans and can protect the host from mucin degradation by glycosidases (30, 31). The link between sulfated mucins and C. jejuni remains uncharacterized, but inferences can be made based on previous studies where sulfation has been shown to confer protective properties. The NaS1 sulfate transporter knockout mouse that exhibits a low level of circulating serum SO_{4}^{2-} showed a reduction in sulfomucins in goblet cells in the jejunum, ileum, and colon (32). The NaS1^-/- mouse had equal amounts of C. jejuni in the small and large intestines compared with NaS1^+/+ mice, but the bacteria were detected in the liver of the null mice, suggesting that a decrease in available sulfate or potentially an overall reduction in mucin content promotes transepithelial transit of the organism. Additionally, gene expression studies in two healthy chicken lines that differ in C. jejuni resistance revealed a 3.346-fold increase in sulfotransferase activity in the more resistant animals as well as an increase in galactose 3-O-sulfotransferase-2 (0.104-fold) and heparinsulfate 6-O-sulfotransferase-3 (0.412-fold).
from RNA isolated from the caeca (33). Furthermore, treatment with human MUC2, which contain sulfated O-glycans (29), inhibits C. jejuni growth in vitro and significant up-regulation of mucin-degrading enzymes as well down-regulation of a sialic acid synthase (34). A thorough glycan array study using an attenuated C. jejuni strain adapted to laboratory conditions through multiple passages (11168-GS) and the original more invasive strain (11168-O) revealed the importance of sulfate in binding (7). Both strains bound fucosylated glycans, especially Galβ1-3(Fucα1–4)GlcNAC (Lewisx), but binding of the 11168-O strain to SO3−Galβ1–3(Fucα1–4)GlcNAC (sulfo-Lewisx) was significantly reduced. From these previous reports and from the prevalence of this epitope with and without sulfate (Table III), it appears that mucin sulfation may play a significant role in protecting the intestinal wall against C. jejuni adhesion and invasion. Continued studies should target the binding properties of C. jejuni against glycans with and without sulfate moieties to determine changes in affinity or identify epitopes with greater inhibitory effects.

The degree of charged structures (sulfated, sialylated and sulfated/sialylated) increased from the caecum to the large intestines, with the number of sulfated and sialylated glycans most prevalent in the large intestines. One interesting result was the identification of N-glycoly neuraminic acid (Neu5Gc) residues in the large intestines despite the observation that chickens lack the gene required to synthesize Neu5Gc ((cytidine monophosphate (CMP)-N-acetylneuraminic acid hydroxylase (CMAH)), although trace amounts have been previously reported (27). Interestingly, chickens are the only other animals aside from humans that generate antibodies against injected Neu5Gc monosaccharides (35). Uptake and incorporation of dietary Neu5Gc into salivary mucin O-glycans has been observed in humans (36), and the adsorption of Neu5Gc from the diet may be the source of the O-glycans containing N-glycoly neuraminic acid in this study. Previous reports have shown that alterations in chicken diet affect mucin glycosylation in goblet cells and also C. jejuni colonization in broiler chicks (37). A diet supplemented with xylanase resulted in a reduction in C. jejuni in the intestines and an increase in sialic-acid-containing residues in goblet cells. Additionally, an increase in sulfation on the surface and upper crypt cells was observed with the xylanase supplemented diet. These results further support a possible correlation between an increase in anionic glycans and a decrease in C. jejuni colonization in broiler chicks, but the specific role of N-glycoly neuraminic acid residues in bacterial defense remains unclear. The biological significance of NGNA residues linked terminally as opposed to being linked to the GalNAC core (as seen with NANA residues) is not ostensibly obvious. One postulation is that substrate recognition of the enzyme responsible for terminal additions has a broad affinity for NGNA as well as NANA. We cannot infer the biological importance for the presence or absence of NANA/NGNA as it pertains to C. jejuni binding, but this result underscores the need for additional binding assays such as glycan arrays. Importantly, it may be possible to limit C. jejuni infection by shifting the mucin bilayer toward a more anionic O-glycan composition through altering broiler chick diets.

It is well known that a strong affinity between C. jejuni and fucose monosaccharides exists, where previous work has shown that C. jejuni bind milk oligosaccharides, particularly neutral α1,2-fucosylated structures (12). L-fucose, and hog gastric mucins have also been show to chem o-attract C. jejuni (8). Additionally, Chinese hamster ovary (CHO) cells overexpressing α1,2-fucosyltransferase (FUT1) inhibited bacterial binding; whereas wild-type CHO cells did not (12). FUT1 transfected CHO cells that express the H(O) Fucα1,2Galβ1,4GlcNAc-R epitope showed greater C. jejuni binding compared with CHO cells transfected with FUT3 (α1,3/4-fucosyltransferase) or FUT4 (α1,3-fucosyltransferase). Our analysis identified fucose-containing structures with α1,2-fucose and/or α1,3-fucose and α1,4-fucose linkages. Interestingly, the site with the most neutral O-glycans with α1,2-fucose was in the large intestines where colonization population is the lowest. The expression of this linkage in mucins may therefore act as an inhibitory binding decay in the chicken large intestine. However, this effect may be modulated where glycans carry charged residues. Interestingly, the greatest colonization by C. jejuni in the chicken gut occurs in the caecum where we did not detect neutral fucosylated structures. Ruiz-Palacio et al. state that α1,2-fucose-containing glycans may serve as a potential ligand in the initializing step toward infection. C. jejuni also express enzymes for the metabolic utilization of L-fucose (13). Currently, it remains unclear whether α1,2-fucose is the preferred substrate and first recognition step or acts as a decay ligand to prevent infection.

The analysis of chicken intestinal mucin glycans provides a platform for greater understanding on how thematic structural differences in mucin glycosylation could explain how C. jejuni exists as a commensal within the mucus of the chicken gut but as a pathogen in the human gut. Based on this work, the following observations differentiate different sites with different levels of colonization in chickens: The site with the greatest inhibition in vitro and the least colonization in vivo (large intestines) had the greatest number of O-glycans as well as the most diversity of structures. Where colonization is highest and inhibition was weakest in vitro (caecum), there were far fewer O-glycan structures detected. There are two plausible postulations for the increased inhibition in the large intestines. The total increase in glycan diversity creates a barrier that the bacteria cannot bypass in order to invade the gut epithelium or that there are specific glycan structures/epitopes that inhibit the bacteria and eliminate their ability to travel through the outer mucosal barrier. However, considering that chicken mucins protect against C. jejuni in vivo, further observations can be made differentiating the characteristics of the mucus barrier in humans and chickens. First, the level of sulfation is
much greater in chickens, and second, the overall charge density of anionic glycans is increased. The increase in anionic glycans may provide a shielding or charge-repulsion effect as well as maintaining the relatively low pH in the intestines (5.7–6.4) (38) that could also reduce the pathogenicity of the bacterium. Overall, the increase in anionic glycans through sialylation, sulfation, and sialylation/sulfation has already been shown to have protective effects. However, the mode in which the bacterium interacts with, or might be deterred by, these factors is not clear. Together, these observations provide rational avenues and a library of structures for further investigation of the chicken mucin glycome for glycan structures with therapeutic or prophylactic potential against C. jejuni.

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