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Purified chicken intestinal mucin attenuates *Campylobacter jejuni* pathogenicity *in vitro*

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Campylobacter jejuni is a major causative agent of diarrhoeal disease worldwide in the human population. In contrast, heavy colonization of poultry typically does not lead to disease and colonized chickens are a major source of *Campylobacter* infections in humans. Previously, we have shown that chicken (but not human) intestinal mucus inhibits *C. jejuni* internalization. In this study, we test the hypothesis that chicken mucin, the main component of mucus, is responsible for this inhibition of *C. jejuni* virulence. Purified chicken intestinal mucin attenuated *C. jejuni* binding and internalization into HCT-8 cells depending on the site of origin of the mucin (large intestine>small intestine>caecum). *C. jejuni* invasion of HCT-8 cells was preferentially inhibited compared to bacterial binding to cells. Exposure of the mucin to sodium metaperiodate recovered bacterial invasion levels, suggesting a glycan-mediated effect. However, fucosidase or sialidase pre-treatment of mucin failed to abrogate the inhibition of *C. jejuni* pathogenicity. In conclusion, differences in the composition of chicken and human intestinal mucin may contribute to the differential outcome of *Campylobacter* infection of these hosts.

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INTRODUCTION

Campylobacter jejuni is a major cause of bacterial gastroenteritis worldwide. In addition, *C. jejuni* infection is associated with severe postinfectious sequelae such as irritable bowel syndrome and Guillain–Barré syndrome (Janssen *et al.*, 2008). In poultry, however, despite heavy colonization, *C. jejuni* exists as a commensal (Young *et al.*, 2007). In the avian host *in vivo*, these bacteria reside in the mucus layer of the deep caecal crypts in high numbers [up to 10^{10} c.f.u. (g infected intestinal contents)$^{-1}$] but do not bind to or invade chicken intestinal epithelial cells (Larson *et al.*, 2008; Young *et al.*, 2007). The precise reasons underlying the difference in outcome following *Campylobacter* infection of humans compared to chickens remain unexplored.

Poultry is a natural source of human *Campylobacter* infections as it is widely consumed by the human population (Humphrey *et al.*, 2007). It has been shown that up to 98 % of point-of-sale chicken and chicken products are contaminated with *Campylobacter* (Hanninen *et al.*, 2000; Madden *et al.*, 1998; Pearson *et al.*, 2000). Increased understanding of the mechanisms involved in *Campylobacter* colonization of chicken mucus could potentially identify novel rational strategies to help reduce *Campylobacter* colonization of chicken in order to curb the transmission to humans of this important pathogen.

Previous work in our laboratory (Byrne *et al.*, 2007) provides provocative evidence that differences in mucus composition between humans and chickens (as opposed to host cell tropism) underlie the species-specific divergence in outcome following *Campylobacter* exposure. We used a primary cell model to show that *Campylobacter* can adhere to and invade chicken intestinal epithelial cells *in vitro* (Byrne *et al.*, 2007). This result has been mirrored in a recent study (Van Deun *et al.*, 2008). Therefore, the lack of disease in chickens following natural infection with *Campylobacter* is not due to the absence of the relevant adhesin–receptor interactions that mediate invasiveness. However, we also showed that crude chicken mucus attenuated *Campylobacter* binding and internalization into human intestinal cells *in vitro*. The magnitude of the effect depended on the site of origin of mucus, with a larger effect shown by mucus isolated from the chicken small bowel compared to mucus isolated from the caecum. In contrast, crude human mucus tended to enhance *Campylobacter* binding and internalization (Byrne *et al.*, 2007). Consequently, it is likely that components of mucus such as mucin glycoproteins may be responsible for the observed attenuation of *C. jejuni* virulence by chicken mucus or the increased virulence observed with human mucus.

Therefore, in this study, we examined the hypothesis that purified chicken intestinal mucin, the main component

of mucus, was responsible for the attenuation of *Campylobacter* virulence previously shown in our laboratory following bacterial exposure to crude chicken mucus (Byrne *et al.*, 2007). We show that purified intestinal chicken mucins attenuate *C. jejuni* binding and internalization into HCT-8 cells in a dose-dependent manner. Avian mucin preferentially impaired *C. jejuni* invasiveness (as opposed to cell adhesion) and the effect varied depending on the site of origin of the mucin. We show also that glycan structures on the purified intestinal chicken mucin may be responsible for the observed attenuation of *C. jejuni* invasion.

METHODS

Bacterial strain and routine culture. *Campylobacter jejuni* 81-176, a well-characterized and highly invasive strain that has been used widely in pathogenicity studies (Bacon *et al.*, 2002; Guerry *et al.*, 2002), was routinely cultured on Mueller–Hinton agar (Oxoid) supplemented with *Campylobacter* selective supplement (Skirrows) at 37 °C under microaerophilic conditions using Campygen gas packs (Oxoid).

Culture of *C. jejuni* for infection assays. *C. jejuni* 81-176 was cultured from frozen stocks onto Mueller–Hinton agar for 24 h. One loopful of bacteria was then transferred to biphasic medium in 25 cm^2 tissue culture flasks consisting of Mueller–Hinton agar and 6 ml RPMI 1640 tissue culture medium (Biowhittaker) for 17–21 h. RPMI used for infection assays was supplemented with 2 % fetal bovine serum (FBS). All bacterial incubations were under microaerophilic conditions generated by Campygen gas packs at 37 °C. Bacteria were harvested from biphasic medium, washed in RPMI and diluted to OD_{600} ~0.15.

Cell culture. The human ileocaecal adenocarcinoma cell line HCT-8 was purchased from the ATCC (CCL-244). HCT-8 cells were routinely maintained in RPMI 1640 supplemented with 10 % FBS. All cells were routinely grown in 75 cm^2 tissue culture flasks and incubated at 37 °C with 5 % CO_2 in a humidified atmosphere. HCT-8 cells were seeded onto 24-well PVDF plates (Corning) at a density of 5×10^4 cells per well for infection assays.

Chicken mucin isolation and purification. Avian intestinal mucins were isolated and purified in accordance with published methodology (Tierney *et al.*, 2007). Six hens were sacrificed and the intestine was dissected from each. The intestinal tract, divided into sections of small intestine, caecum and large intestine, was slit longitudinally and the mucus at the epithelial surface was scraped into 7 M GuCl in PBS. The samples were rolled at room temperature for 3–5 days to disperse the mucus.

Pooled samples (1 ml from each sample) were prepared for each tissue type (small intestine, caecum and large intestine) and a periodic acid–Schiff reaction was carried out on an aliquot of each sample to confirm the presence of carbohydrate. The samples were centrifuged at 23 000 ***g*** for 60 min at 4 °C to pellet insoluble mucin and cellular debris. The supernatant (containing soluble mucin) was carefully removed and the pellet was discarded.

Slot blots and isopycnic density-gradient centrifugation. Slot blots with PVDF membranes were used to verify carbohydrate-rich fractions throughout the mucin isolation and purification process. Carbohydrate was oxidized by 1 % periodate in 3 % acetic acid for 30 min, washed twice in 0.1 % sodium metabisulphite in 1 mM HCl and the membrane was incubated in Schiff's reagent (BDH).

Each sample was made to 1.4 g ml^{-1} by adding caesium chloride until a density of 1.395–1.405 g ml^{-1} was reached. Ultracentrifugation was carried out at 65 000 r.p.m. for 18 h at 10 °C (Beckman-Coulter Optima-LE-80K ultracentrifuge). After centrifugation, the gradient was unpacked in 1 ml aliquots from top downwards. Slot blots showed caecal and large intestinal mucin between 17 and 25 ml, and 15 and 24 ml, respectively. The density profile (from 1.35 to 1.47 g ml^{-1}) matched the carbohydrate-rich fractions obtained from the slot blots. All carbohydrate-rich fractions between 1.35 g ml^{-1} and 1.47 g ml^{-1} were pooled.

Gel filtration chromatography. Sepharose CL-2B columns (2.5×25 cm, volume 120 ml) were poured and equilibrated with 3 column volumes of buffer (50 mM Tris, 150 mM KCl, pH 7.5). A fraction collector (TYPEX) was used and 3 ml fractions were collected. The column was loaded with 3 ml pooled 1.35–1.47 g ml^{-1} fractions.

A slot blot was performed on aliquots of each fraction obtained from the column and high molecular mass carbohydrate-rich material was pooled. Dithiothreitol to a final concentration of 10 mM was added to this material and incubated at 37 °C for 3–5 h. Aliquots of the reduced material were loaded onto each Sephadex G25 (2.5×90 cm, volume 440 ml) column, pre-equilibrated with distilled water to desalt the mucin. Periodic acid–Schiff-rich fractions were collected, pooled, freeze-dried, then weighed and resuspended in sterile distilled water at a concentration of 1 mg ml^{-1}. The purified mucin was stored at −20 °C either in solid form or dissolved in distilled water.

Total association and gentamicin protection assays. The effect of chicken mucin on the viability of *C. jejuni* was determined by enumerating c.f.u. following pre-incubation of bacteria in chicken mucin compared to bacterial pre-incubation in RPMI. Different concentrations (5 %, 10 % and 20 %) of purified caecal, small and large intestinal mucin were made in RPMI 1640 (plus 2 % FBS) containing OD_{600} ~0.15 *C. jejuni* harvested from biphasic medium. Undiluted mucin was used to resuspend pelleted OD_{600} ~0.15 *C. jejuni*. Mucin and bacteria were incubated under microaerophilic conditions for 30 min to 1 h. Each mucin preparation containing bacteria was added onto HCT-8 cells in 24-well plates at an m.o.i. of 300. As a control, bacteria not pre-incubated with mucin were also used to infect HCT-8 cells. Infected 24-well plates were then centrifuged at 250 r.p.m. for 5 min, after which the infected plates were incubated at 37 °C for 3 h under microaerophilic conditions.

Total association (adherent plus internalized bacteria) and invasion assays were then performed as follows. After the incubation period, one set of wells was washed with PBS and the cells were lysed with 100 µl 0.1 % Triton X-100 in PBS for 15 min, after which serial dilutions of the cell lysates were plated on Mueller–Hinton agar to determine the amount of total associated bacteria. To quantify internalized bacteria, a second set of wells was washed in RPMI and incubated with 400 µg gentamicin sulphate ml^{-1} in RPMI for 2 h to kill all extracellular bacteria. Cells were washed in PBS, lysed with 100 µl 0.1 % Triton X-100 in PBS and serial dilutions of the cell lysates were plated on Mueller–Hinton agar plates. All washes between treatments were carried out six times with PBS. Mueller–Hinton agar plates were incubated at 37 °C under microaerophilic conditions for 48–72 h after which bacterial c.f.u. were enumerated.

Effect of sodium metaperiodate addition to mucin on *C. jejuni* invasion of HCT-8 cells. Sodium metaperiodate at a final concentration of 0.011 mM in PBS was diluted (20 %) in small and large intestinal mucin. Mucin and periodate were incubated at 4 °C for 30 min. Glycerol (0.22 %) in PBS was then added to neutralize the periodate (Stevenson *et al.*, 2004). Control samples were treated similarly to avoid a confounding effect of the mucin. The periodate-

treated mucin was used to resuspend pelleted *C. jejuni* previously diluted in RPMI to an OD_{600} ~0.15. Periodate-treated mucin and bacteria were incubated for 1 h at 37 °C and used to infect HCT-8 cells for 3 h. Wells were centrifuged at 250 r.p.m. for 5 min prior to the incubation period. As a control, 0.22 % glycerol in PBS, mucin and bacteria without periodate were coincubated and used to infect cells. In addition, bacteria that did not come in contact with either mucin or periodate were used to infect cells. The gentamicin protection assay was then carried out as described above.

Small intestinal chicken mucins were treated with an α-1,2 fucosidase (Sigma) and a broad-spectrum sialidase (Sigma) according to the manufacturer's protocol, to determine the effect of these enzymes on mucin-mediated inhibition of *C. jejuni* invasion. Mucins were treated with fucosidase or sialidase overnight at 37 °C. In order to verify enzyme activity, an aliquot of the enzyme-treated mucin was run on an SDS-PAGE gel and the mobility was compared to untreated mucin, according to the manufacturer's instructions. Pelleted *C. jejuni* was resuspended with the fucosidase- or sialidase-treated mucins and coincubated for 1 h at 37 °C under microaerophilic conditions. Enzyme-treated mucin coincubated with *C. jejuni* was used to infect HCT-8 cells for 3 h and the gentamicin protection assay was performed as described above to quantify internalized bacteria. As controls, *C. jejuni* exposed to mucin alone and *C. jejuni* not exposed to either mucin or enzyme were used to infect HCT-8 cells. Treatment of *C. jejuni* with fucosidase or sialidase did not affect bacterial invasion.

Statistics. Experiments were conducted on three separate occasions unless otherwise indicated. Results are presented as box and whisker plots with median and quartiles. The Wilcoxon two-sample test was used to estimate statistical significance. $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Chicken mucin significantly reduces *C. jejuni* binding to HCT-8 cells

To investigate the effect of purified chicken mucin on *C. jejuni* binding to cells, *C. jejuni* was pre-incubated with purified small intestinal and large intestinal mucin prior to infecting HCT-8 cells. Compared to control untreated bacteria, *C. jejuni* exposed to both small and large intestinal chicken mucin showed approximately a 6.5-fold reduction in binding to HCT-8 cells (Fig. 1a, b). Large and small intestinal chicken mucins showed a similar effect on *C. jejuni* adhesion. In addition, there was evidence of a dose-dependent effect. Purified chicken mucin did not affect the viability of the bacteria (~10^7 c.f.u. per well were recovered after mucin treatment of *C. jejuni* or from bacteria incubated in tissue culture medium alone).

Purified chicken intestinal mucin attenuates *C. jejuni* internalization into HCT-8 cells

C. jejuni was preincubated with purified small intestinal, large intestinal and caecal mucin prior to infecting HCT-8 cells, to determine the effect of purified chicken mucin on *C. jejuni* internalization. Chicken mucin significantly reduced *C. jejuni* internalization depending on the region of the intestine from which it originated (Fig. 2a).

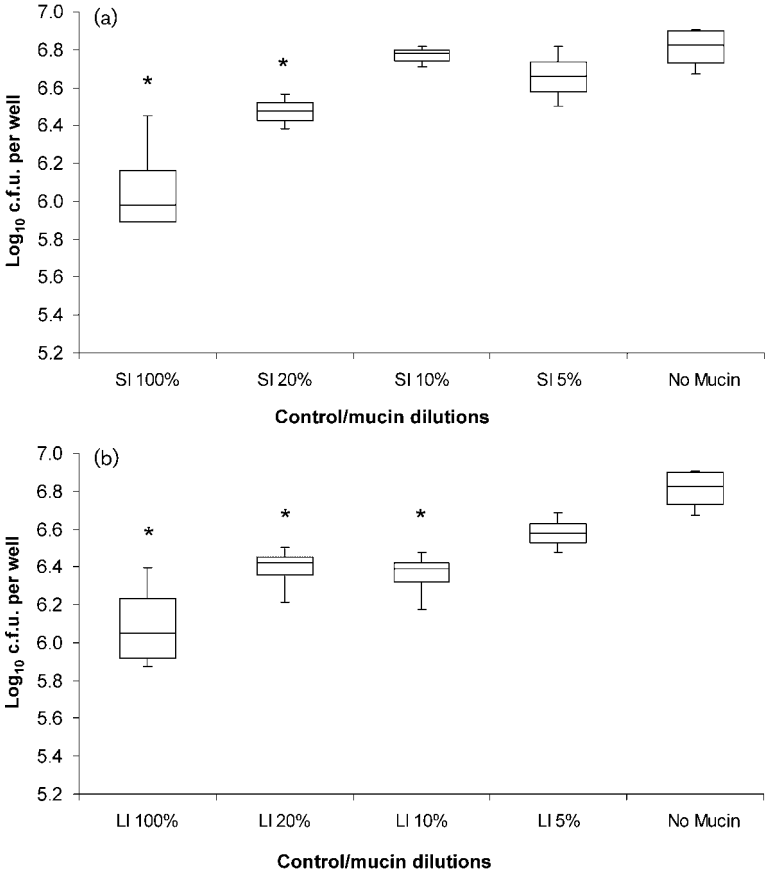

Fig. 1. Small (a) and large (b) intestinal mucins significantly attenuate *C. jejuni* binding to HCT-8 cells. *C. jejuni* was exposed to undiluted and diluted mucin prior to infecting HCT-8 cells. A total association assay was used to quantify associated bacteria. SI, Small intestine; LI, large intestine; 100 % mucin represents 1 mg ml^{-1}. *Significant difference compared to the control *C. jejuni* that was not exposed to chicken mucin. $P<0.05$ for all groups.

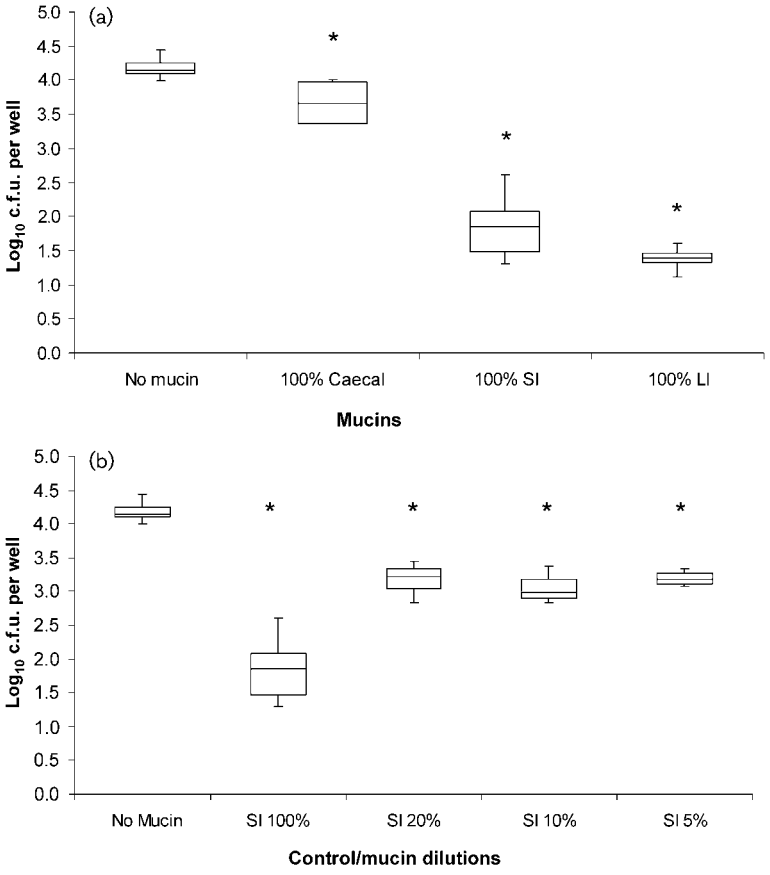

Fig. 2. Chicken mucin inhibits *C. jejuni* internalization into HCT-8 cells. (a) *C. jejuni* was treated with purified undiluted small intestinal (SI), large intestinal (LI) and caecal mucin prior to infecting HCT-8 cells. *Significant difference compared to control *C. jejuni* that was not treated with mucin. Caecal $P<0.05$, SI $P<0.005$, LI $P<0.005$. (b) Effect of dilutions of purified small intestinal chicken mucin on *C. jejuni* invasion of HCT-8 cells. *Significant difference compared to control *C. jejuni* that was not pre-treated with chicken mucin. SI $P<0.005$, 20 % SI $P<0.005$, 10 % SI $P<0.05$, 5 % SI $P<0.05$. 100 % mucin represents 1 mg ml^{-1}.

Compared to bacteria that were not preincubated with chicken mucin, large intestinal mucin had the greatest effect (more than 1000-fold reduction) followed by small intestinal mucin (~150-fold), while caecal mucin had the least effect on reducing *C. jejuni* internalization, showing approximately a 5-fold reduction. The effect elicited by chicken small intestinal mucin showed some evidence of dose-dependency (Fig. 2b).

Interestingly, there was only approximately 6.5-fold reduction in the binding of *C. jejuni* to HCT-8 cells compared with up to 1000-fold reduction in *C. jejuni* internalization. Preferential interference in bacterial internalization suggests a specific effect of avian mucin on a pathogenic mechanism that subserves invasiveness and correlates with the failure to observe host chicken intestinal cell invasion *in vivo* (Larson *et al.*, 2008; Young *et al.*, 2007). However, in light of the absence of both avian epithelial cell adhesion and invasion *in vivo* it is clear that factors other than mucin also play a role in species-specific outcomes following *Campylobacter* exposure in humans and chickens.

An intriguing finding of this study was the variation in the effect of mucin depending on its origin within the intestinal tract of the chicken. This is in keeping with our previous observations (Byrne *et al.*, 2007) regarding crude mucus. In that original study, small intestinal mucus appeared more inhibitory than large intestinal mucus. However, we had labelled caecal mucus as 'large intestinal' in those experiments, which accounts for the apparent incongruity between these two studies. It is perhaps surprising that the least invasion-inhibitory mucus originated from the avian caecum where *Campylobacter* is most abundant *in vivo*. These results suggest a complex, niche-specific interaction between the intestinal milieu and resident organisms such as *C. jejuni*. Specifically, they indicate that the effect of mucin on invasiveness, while likely helping protect the chicken from *Campylobacter* disease, is not the only factor that allows this bacterial species to flourish within the avian intestinal tract.

Sodium metaperiodate treatment of chicken mucin attenuates the inhibitory effect of chicken mucin on invasion

To investigate the possibility that the highly glycosylated structures on chicken mucin are involved in the attenuating effect of avian mucin on internalization, intestinal chicken mucins were pre-treated with sodium metaperiodate, which oxidizes the glycans on mucin glycoproteins. *C. jejuni* was then exposed to periodate-treated mucin prior to infecting HCT-8 cells. *C. jejuni* invasion levels were recovered to control (bacteria that did not come in contact with mucin or periodate) levels ($\sim 1 \times 10^5$ c.f.u. per well)

when exposed to periodate-treated mucin, compared with bacteria exposed to large intestinal mucin only (Fig. 3a). While this finding strongly supports an important role for mucin glycosylation in mediating the effect observed on *C. jejuni* pathogenicity, the nature of the interaction between the mucin-derived glycans and the identity of the specific glycan residues on the purified chicken mucins remain unexplored. Fucose/fucosylated structures previously have been identified as important in *C. jejuni* pathogenicity (Hugdahl *et al.*, 1988; Ruiz-Palacios *et al.*, 2003). However, we were unable to show a role for mucin-based fucose residues in mediating attenuation of *C. jejuni* invasion, as pre-treatment of chicken mucin with α-1,2 fucosidase did not prevent mucin-mediated inhibition of *C. jejuni* internalization (Fig. 3b). Furthermore, sialidase pre-treatment also failed to attenuate the effect of mucin on *C. jejuni* invasion (Fig. 3b). While fucose may not be a major sugar in the mucin of avian species (Fernandez *et al.*, 2000), sialylation is important (Forder *et al.*, 2007) and even may be increased following colonization with *C. jejuni* (Fernandez *et al.*, 2000). However, an alternative possibility may be that modifications on avian mucin, such as sulfation, inhibited the effectiveness of the glycosidases used in our study. Undoubtedly, avian and human mucin encoding genes differ substantially (Lang *et al.*, 2006) and there are likely marked differences in post-translational modification mediated by different species-specific glycosyltransferase activities in these two species. Further

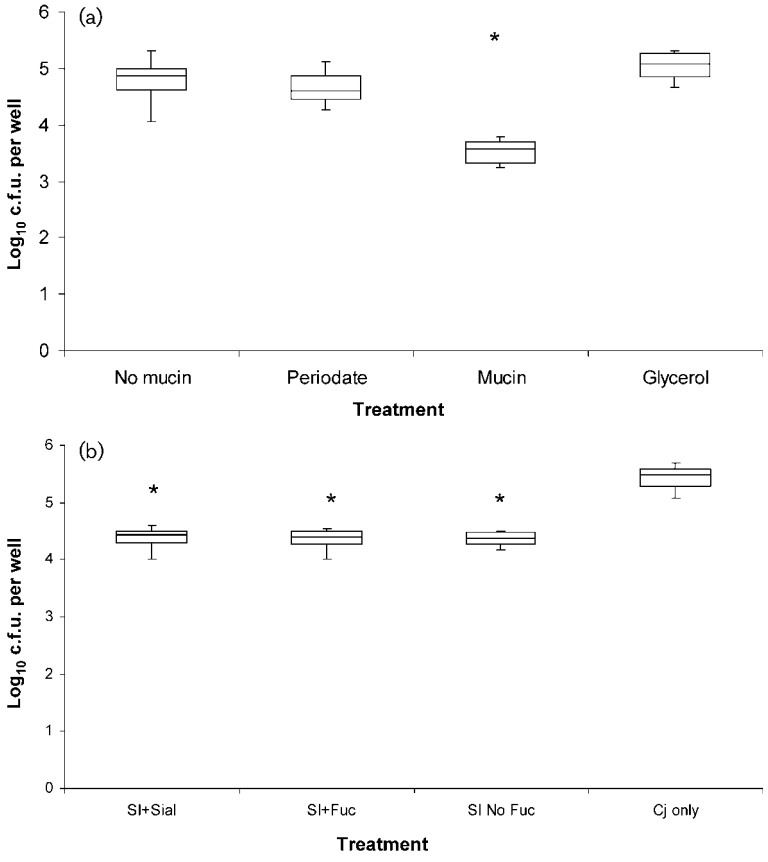

Fig. 3. Effect of mucin treatment with sodium metaperiodate, fucosidase and sialidase on mucin-mediated inhibition of *C. jejuni* invasion. (a) Mucin glycans are involved in the attenuation of *C. jejuni* internalization into HCT-8 cells following bacterial exposure to purified chicken mucin. Large intestinal chicken mucin was pre-treated with sodium metaperiodate. *C. jejuni* was exposed to periodate-treated mucin prior to infecting HCT-8 cells. Glycerol was used to stop the activity of periodate. Glycerol: *C. jejuni* pre-treated with glycerol alone. Pre-treatment of large intestinal mucin with periodate recovered bacterial invasion to control (i.e. bacteria not pre-treated with mucin or periodate) levels compared to mucin-treated bacteria. *Significant difference compared to controls (no mucin) and to periodate-treated mucin. Mucin compared to no mucin $P<0.05$; mucin compared to periodate $P<0.05$. (b) Effect of α-1,2 fucosidase and a broad-spectrum sialidase treatment of chicken intestinal mucins on *C. jejuni* invasion. *C. jejuni* was exposed to fucosidase- and sialidase-treated small intestinal chicken mucins prior to infecting HCT-8 cells. SI, Small intestinal mucin; Fuc, fucosidase; Sial, sialidase. Bacteria not pre-incubated with mucin, fucosidase or sialidase (Cj only), and *C. jejuni* exposed to mucin alone (SI No Fuc), were also used to infect HCT-8 cells as controls. *Significant difference compared to *C. jejuni* not pre-treated with either mucin, sialidase or fucosidase. $P<0.05$ for all groups compared to unexposed bacteria. Results of one of two experiments with similar results are presented.

experiments aimed at identifying major glycosylation structures on avian mucin (particularly those that differ from human mucin glycans) and subsequent modification of these glycans now are warranted in order to better understand the role of chicken mucin in the inhibition of *C. jejuni* virulence properties.

In summary, differences in human and chicken intestinal mucin likely contribute to the species-specific differences in outcomes following *Campylobacter* infection. Understanding the dynamics of *Campylobacter* chicken mucus colonization and *Campylobacter*–mucin interactions will help define why this human pathogen behaves as a commensal in chickens and might lead to intervention strategies that would curb *C. jejuni* transmission to the human population.

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