The Human Milk Oligosaccharide 2′-Fucosyllactose Quenches Campylobacter jejuni–Induced Inflammation in Human Epithelial Cells HEp-2 and HT-29 and in Mouse Intestinal Mucosa1–3

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Abstract

Background: Campylobacter jejuni causes diarrhea worldwide; young children are most susceptible. Binding of virulent C. jejuni to the intestinal mucosa is inhibited ex vivo by α1,2-fucosylated carbohydrate moieties, including human milk oligosaccharides (HMOSs).

Objective: The simplest α1,2-fucosylated HMOS structure, 2′-fucosyllactose (2′-FL), can be predominant at ≤5 g/L milk. Although 2′-FL inhibits C. jejuni binding ex vivo and in vivo, the effects of 2′-FL on the cell invasion central to C. jejuni pathogenesis have not been tested. Clinical isolates of C. jejuni infect humans, birds, and ferrets, limiting studies on its mammalian pathobiology.

Methods: Human epithelial cells HEp-2 and HT-29 infected with the virulent C. jejuni strain 81-176 human isolate were treated with 5 g 2′-FL/L, and the degree of infection and inflammatory response was measured. Four-week-old male wild-type C57BL/6 mice were fed antibiotics to reduce their intestinal microbiota and were inoculated with C. jejuni strain 81-176. The sensitivity of the resulting acute transient enteric infection and immune response to inhibition by 2′-FL ingestion was tested.

Results: In HEp-2 and HT-29 cells, 2′-FL attenuated 80% of C. jejuni invasion (P < 0.05) and suppressed the release of mucosal proinflammatory signals of interleukin (IL) 8 by 60–70%, IL-1β by 80–90%, and the neutrophil chemoattractant macrophage inflammatory protein 2 (MIP-2) by 50% (P < 0.05). Ingestion of 2′-FL by mice reduced C. jejuni colonization by 80%, weight loss by 5%, histologic features of intestinal inflammation by 50–70%, and induction of inflammatory signaling molecules of the acute-phase mucosal immune response by 50–60% (P < 0.05). This acute model did not induce IL-17 (adaptive T cell response), a chronic response.

Conclusions: In human cells in vitro (HEp-2, HT-29) and in a mouse infection model that recapitulated key pathologic features of C. jejuni clinical disease, 2′-FL inhibited pathogenesis and its sequelae. These data strongly support the hypothesis that 2′-FL represents a new class of oral agent for prevention, and potentially for treatment, of specific enteric infectious diseases.

Keywords: human milk oligosaccharides, enteric infection, mucosal immune response, cytokines, inflammation

Introduction

Campylobacter jejuni is the leading cause of enteric bacterial infection worldwide and poses a substantial challenge to public health (1), especially for young children. Domestic fowl serve as a reservoir for human infection, with further transmission through contaminated food, water, and direct fecal-oral contact. C. jejuni infection causes local acute inflammatory changes in both the small and large bowel (2). After an incubation period of ~24–72 h, C. jejuni infection typically causes cramping and abdominal pain and is often associated with fever, vomiting, and headache (3). The subsequent diarrhea can vary from a mild, noninflammatory, watery presentation to one that is severe and bloody (4) and generally lasts for ~7 d in otherwise healthy, immunocompetent individuals. After a C. jejuni infection resolves, there remains a residual elevated risk of severe complications, including Guillain-Barré syndrome and irritable bowel syndrome (5).
Virulence factors and mechanisms of *C. jejuni* infection are being actively investigated. A wide array of surface glycans of *C. jejuni* allow evasion of host defenses and resistance to proteolytic degradation (6). *C. jejuni*-infected ferrets (7) reflect human infection well, but this expensive model lacks a full complement of reagents to define the host response to infection. In *C. jejuni*-inoculated mice, the host response of the intestinal mucosa is inconsistent (8–11), suggesting that variation in murine microbiobial colonization could influence the transition from *C. jejuni* colonization to *C. jejuni* infection. Taking this into account, mice were treated with a cocktail of antibiotics for 6–8 wk before inoculation with *C. jejuni*. Antibiotic treatment allows murine *C. jejuni* infection, but the high counts of *C. jejuni* in the stomach and proximal intestine are not typical of human infection (12). Another study compared wild-type and *Sigirr*−/− knockout mice that had been inoculated with *C. jejuni* 4 h after vancomycin gavage; wild-type mice showed minimal pathology, indicating that this treatment does not closely model human infection (13). To allow testing of potential therapeutic agents in the context of an intact appropriate host mucosal immune response, we developed a model of antibiotic-treated wild-type mice that become susceptible to infection by a clinical isolate of *C. jejuni*. In this model, the antibiotics per se did not elicit an inflammatory response, and the shift in microflora allowed *C. jejuni* infection that closely recapitulated the pathobiology of human disease with regard to time course, inflammatory response, and spontaneous clearance of mucosal and systemic infection.

Human milk oligosaccharides (HMOs) are putative protective agents against enteric infection (14). Oligosaccharides, the third-largest fraction of human milk, consist of thousands of components and, in most mothers, are predominantly fucosylated. *C. jejuni* binds diverse host glycans, including fucosylated glycans, that are crucial for the initial attachment to and continued colonization of the host (14, 15). Fucose binding is a critical determinant of *C. jejuni* pathogenesis and is strongly inhibited by HMOs and by 2'-fucosyllactose (2'-FL), a major constituent of the HMOs (10). The ability of 2'-FL to inhibit *C. jejuni* binding is consistent with the inverse relation between concentrations of 2-linked fucosyl oligosaccharides in milk consumed by infants and the risk of *C. jejuni*-caused moderate-to-severe diarrhea in a prospective study in breastfeeding mother-infant dyads (16, 17). Prebiotic activity of oligosaccharides also may be protective (18, 19); these indigestible glycans provide a carbon source for mutualistic bacteria that promote the colonization of the gut by a protective community of microbes.

Elucidating the molecular mechanisms whereby *C. jejuni* pathobiology could be inhibited by HMOs (20) requires an appropriate model. In mice, reducing the gut microbial diversity by using a specific cocktail of antibiotics before inoculation allows *Salmonella typhimurium*, *Clostridium difficile*, and *Shigella dysenteriae* to infect with pathology that recapitulates human disease: inflammation is followed by spontaneous clearance of the infection after several weeks (21, 22). This strategy was tested for its ability to allow infection by *C. jejuni* and was modified to accommodate specifics of *C. jejuni* pathobiology.

A study was designed to test whether HMOs inhibit infection and its sequelae by a clinical isolate of *C. jejuni* and whether pure 2'-FL can account for this activity. First, cell culture models of human epithelium, HEp-2, and of human intestinal epithelium, HT-29, were used to test the ability of synthetic 2'-FL to inhibit *C. jejuni* infection. When 2'-FL proved to be effective, a novel murine *C. jejuni* model that exhibited typical acute transient enteric infection, immune response, and spontaneous clearing was developed and used to test the hypothesis that a single HMO, 2'-FL, could inhibit *C. jejuni* infection of the intestinal epithelium and *C. jejuni*-associated mucosal inflammation.

**Methods**

HMOs. HMOs were prepared from pooled human milk as described previously (16). Pure 2'-FL was produced by microbial fermentation and brought to >99% purity by Glycosyn LLC.

**Bacterial strains.** *C. jejuni* strain 81-176 (a kind gift by Patricia Guerry, Naval Medical Research Center, Maryland) was cultured through one passage on trypticase soy agar plates containing 5% sheep’s blood (BD) for 2 d under microaerophilic conditions at 37°C.

**Intestinal epithelial cells.** The effects of *C. jejuni* infection are not robust in many common cell lines (23). Thus, the human epithelial cells (HECs) HEp-2 (cervical origin) and HT-29 (intestinal origin) were cultured as polarized epithelial cell monolayers to model *C. jejuni* infection and its inhibition. HEp-2 cells were grown in MEM and HT-29 were grown in McCoy’s medium, each supplemented with 10% FBS (Gibco), without antibiotics, in 75-cm² flasks (Falcon) at 37°C in a 5% CO₂ humidified atmosphere. Trypsinized HEp-2 or HT-29 cells were seeded (5.0 × 10⁴ in 1 mL) in 6-well tissue culture plates. After 18 h of incubation, the semiconfluent monolayers were washed and incubated with MEM containing 1% FBS; confluent monolayers were used for the invasion study. Invasion was measured by staining with fluorescein isothiocyanate–labeled *C. jejuni*, with nuclei stained with 4',6-diamidino-2-phenylindole dihydrochloride. Inflammatory response to infection was determined by measuring IL-8, IL-1β, macrophage-inflammatory protein 2 (MIP-2), and TNF-α, as well as levels of their corresponding mRNA.

**Invasion assay.** In Expt. 1, binding by *C. jejuni* or heat-killed *C. jejuni* to HEp-2 or HT-29 HECs was assessed as described previously (10, 24). *C. jejuni* (1 × 10⁸ CFUs/mL) alone or previously treated for 1 h with 2 g 2'-FL/L, *Ulex europaeus* agglutinin I (UEA-I) lectin (positive control), *Lotus tetragonolobus* lectin, α-fucose, or Dolichos biflorus agglutinin (negative control; EY Laboratories) were inoculated into wells containing HEp-2 or HT-29 cells. The cells were incubated for 2 h followed by the removal of extracellular *C. jejuni* by using 100 mg gentamicin/L (Sigma) as described previously (10, 24). *C. jejuni* colonies formed by plated epithelial cell lysates, with 4 replicate wells/Expt. in 3 independent Expts. were analyzed as 3 independent measurements.

In Expt. 2, the dose response of 2'-FL inhibition of invasion was likewise assessed over the range that can be found in human milk, from a high of 5 g/L, through the median value of 2 g/L, through low values of 1 and 0.5 g/L, and to 0 g/L. The lowest values represent milk of nonsecretors who are homozygous recessive for the FUT2 gene and do not secrete α1,2-linked fucosylated epitopes in their exocrine secretions, including milk.

**Bacterial infection of mice.** A C57BL/6 murine model was developed to minimize variable microbiota effects on *C. jejuni* infection (Boston College Institutional Animal Care and Use Committee protocol 2011010). Four-week-old C57BL/6 male mice (Jackson Laboratories) were housed with a 12-h light-dark cycle and had access to a standard unpurified mouse diet (Prolab RMH 3000, 50/50, LabDiet) and water ad libitum. LabDiet 5000 contains ~22% protein, ~5% fat, and ~5% crude fiber.
Expt. 3 tested conditions that promoted *C. jejuni* infection of mice. Groups of 4 mice were treated for 1, 3, or 7 d with an antibiotic cocktail in drinking water to disrupt the microbiota (25) followed by an inoculum of 10^8 CFUs *C. jejuni*/mouse for 3 consecutive days; 7 d of antibiotic pretreatment provided the highest level of infection. Next, 4 groups of 4 mice pretreated with 7 d of antibiotics were inoculated with 1 × 10^3, 10^3, or 10^4 CFUs/mouse in a 100-μL intraocular challenge or with vehicle alone as a negative control; 10^3 CFUs/mouse provided the highest level of infection. With the use of the inoculum of 10^3 CFUs *C. jejuni* mouse, mice pretreated with 7 d of antibiotic cocktail in their drinking water were inoculated by daily gavage with *C. jejuni* (10^5 CFUs/mouse) for 1, 3, or 7 d. Mice were weighed and feces collected daily. Mice were killed 1 d after the final *C. jejuni* gavage, and tissues were collected. Body-weight data were normalized to the body weight of each mouse at the outset of the experiment (the day of initiation of antibiotic treatment) to isolate the variance in changes due to the experimental treatment from the initial variance in body weights. A 3 d inoculum was sufficient for full infection. The experimental murine model for *C. jejuni* infection was thus 7 d of antibiotic cocktail followed by 3 daily inocula of 10^8 CFUs/mouse in 100 μL saline gavage.

Expt. 4 tested whether 2'-FL could inhibit *C. jejuni* infection and inflammatory response. The 5 arms each consisted of groups of 4 4-wk-old C57BL/6 mice pretreated with the antibiotic cocktail, as follows: 1) uninfected negative controls, 2) uninfected mice administered 5 g 2'-FL/L in their water for 3 d, 3) mice infected with C. jejuni with no further intervention (positive controls), 4) mice infected with an inoculum of *C. jejuni* that also contained 2'-FL, and 5) mice administered 5 g 2'-FL/L in their drinking water for 3 d before and concurrent with the inoculum, which, as in group 4, contained both *C. jejuni* and 2'-FL.

This combined inoculum does not affect *C. jejuni* viability per se (10), and 5 g 2'-FL/L in drinking water recapitulates the upper range of its concentrations in human milk. Mice were examined daily and weighed, and the presence and severity of rectal bleeding were recorded. On alternate days, fresh fecal samples were collected and stored at −20°C. The disease activity index (DAI) is a composite of weight loss, bleeding, and diarrhea symptoms (26). Scoring was performed by researchers blinded to the experimental status (e.g., 2'-FL, *C. jejuni* treatment) of the mice.

### C. jejuni infection.

*C. jejuni* was quantified in fecal samples obtained from the colon after mice were killed. Log dilutions of 100 μL feces in 9 volumes of PBS were plated in duplicate on *C. jejuni*–selective agar (Oxoid; 48 h, 37°C microaerophilic). *C. jejuni* was also quantified in feces and tissues by real-time PCR with the use of *C. jejuni*–specific primers VS15 and VS16 from VS1 sequence (X71603), as described previously (27).

### Tissue collection.

Isoflurane-anesthetized mice were killed by cervical dislocation. Proximal and distal segments of colons were cleaned with ice-cold PBS, spleen, and mesenteric lymph nodes (MLNs) were frozen immediately in liquid nitrogen for isolation of total RNA. Other colonic tissues fixed in 4% buffered paraformaldehyde for 12 h at 4°C were imbedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. Colonic tissue sections washed in PBS containing penicillin and streptomycin were cut into 5 × 5 mm^2 explants for culture.

### Histology, pathologic scoring.

Bowel tissues fixed in 10% formalin, paraffin embedded, cut for histologic analysis, and stained with hematoxylin and eosin were subjected to pathologic scoring by 3 blinded observers (13, 28).

### Immunohistochemistry.

In situ immunohistochemical analysis was performed on the colonic paraffin sections by using primary antibodies against CD3 (dilution of 1:100; Abcam) and myeloperoxidase-7 (dilution of 1:100; Abcam). For each mouse, the mean number of positively stained cells within ≥6 high-power fields (400× magnification) was determined microscopically by 3 independent investigators.

### Cytokines and chemokines.

The mucosal immune response to infection was determined by measuring selected NF-kB–dependent inflammatory mediators in the colon, the primary site of *C. jejuni* infection. IL-6 and TNF-α induction represented overall acute inflammatory response, MIP-2 induction exemplified the degree of expression of a neutrophil chemoattractant, IL-1β induction represented the degree of activation of inflammation, and IL-17 induction reflected the degree of polarization of the Th-helper cells in response to infection as a measure of adaptive immune response.

Proximal and distal colon segments were cultured in 24-well flat-bottom culture plates (Falcon) containing serum-free DMEM ( Gibco) with penicillin (100 U/L) and streptomycin (100 U/mL) (29). The media were collected after 20 h, centrifuged, frozen, and stored at −20°C, as described (20). IL-1β, IL-6, TNF-α, MIP-2, and IL-17 concentrations were measured by ELISA (R&D Systems).

The relative inhibition of the activation of phosphorylated protein 38 (p-p38) by 2'-FL and by SB203580 (10 μM; Cell Signaling Technology), an inhibitor of protein 38 (p38), MAPK, was compared in *C. jejuni*-infected HT-29 cells and C57BL/6 mouse colon. Values were normalized to total mucosal protein, as assessed by the bicinchoninic acid assay (BCA kit, Pierce-ThermoFisher).

### Cytokine and chemokine mRNA.

RNA from tissue homogenates (RNeasy Mini Kit; Qiagen) generated cDNA by using random hexamers (GeneAmp RNA PCR Kit; Applied Biosystems). Inflammatory cytokine and chemokine mRNAs were quantified by RT-PCR. Primer sequences (30–32) are shown in Supplemental Table 1.

### Statistical analysis.

Results from each in vitro experiment are from 3 independent Expts. (each the mean of 4 wells) and are expressed as means ± SDs. Treatment effects were analyzed in Supplemental Figure 1 by single-tailed Student’s *t* test. Experimental results in Figure 2 and Supplemental Figure 2, which were normally distributed, homogeneous, and composed of independent data with only 1 independent variable, were analyzed by Bonferroni-corrected 1-factor ANOVA; if the ANOVA produced *P* < 0.05, a post hoc Tukey’s honestly significant difference test determined the significance of individual group comparisons. Data from experiments with 2 independent variables, *C. jejuni* infection and 2'-FL treatment, after testing for homoscedasticity by Levene’s median test, were analyzed by 2-factor ANOVA (*C. jejuni* infection × 2'-FL treatment) followed by mean comparisons with Bonferroni adjustment. Results are expressed as means ± SDs. *P* ≤ 0.05 was considered significant. In Figure 1B, decreases in infection as a function of 2'-FL concentration were analyzed by generating best-fit regression curves, from which R and *P* for the closeness of fit were calculated (Microsoft Excel for MAC, version 14.5.4).

### Results

*C. jejuni* infection in vitro. Infection of HEp-2 or HT-29 cells with fluorescein isothiocyanate–labeled *C. jejuni* was measured as number of *C. jejuni* per cell. Expt. 1 indicated that the infection was similar for both HEp-2 and HT-29 epithelial cells (Supplemental Figure 1). Expt. 1 also indicated that *C. jejuni* infection was inhibited by 2 g HMOs/L, 2'-FL or UEA-I, in both HEp-2 and HT-29 cells (*P* < 0.05) but was not affected by *Dolichos biflorus* agglutinin, 1-fucose, or *Lotus tetragonolobus* lectin treatment (Figure 1A).

Expt. 2 determined that the inhibition of *C. jejuni* infection was dose dependent in both HEp-2 (*P* = 0.01 for closeness of fit to curve) and HT-29 (*P* = 0.02) cells (Figure 1B). The half-maximum inhibitory concentration of 2'-FL is ~1 g/L for both cell types; typical secretor human milk contains 2–3 g/L. Thus, the inhibition by 2'-FL alone can account for the complete inhibition of *C. jejuni* infection by concentrations of HMOs that represent human milk. Inhibition by 2'-FL is mediated by attenuating pathogen-induced p38 activation in the epithelial cells (Supplemental Figure 3A).

In HEp-2 cells, *C. jejuni* infection induced the expression of IL-8 (*P* < 0.0005), IL-1β (*P* < 0.0005), MIP-2 (*P* = 0.001), and TNF-α (*P* < 0.0005) (Figure 1C) and the corresponding expression of chemokine (C-X-C motif) ligand 8 (gene for IL-8)
C. jejuni infection of HT-29 cells for the induction of protein mRNA (indicated a significant interaction between CXCL8 mRNA, TNFA mRNA by 19% (Figure 1C). Treatment with 2'-FL reduced IL-8 mRNA expression by 61%, and TNFA mRNA expression by 64% (P < 0.0005) (Figure 1D). The inability of heat-killed C. jejuni infection per se rather than by an epithelial response to bacterial binding or activation of innate immune receptors by LPS or other microbial pattern molecules.

**FIGURE 1** Invasion of HEp-2 and HT-29 cells by live Campylobacter jejuni was inhibited by 2'-FL. Treatment with 2'-FL inhibited infection (Expt. 1; A) in a dose-dependent manner (Expt. 2; B) and in C. jejuni-infected cells, 2'-FL attenuated protein (Expt. 1; C) and its corresponding mRNA (D) levels of inflammatory markers. Bars represent means ± SDs, n = 3 independent Expts. (4 wells/Expt.). The statistical evaluation of HEp-2 data is independent of the evaluation of HT-29 data, and they are shown together only to illustrate visual concordance of changes induced by the experimental variables. Within panels A and B, means without a common letter differ (P < 0.05); within panels C and D, asterisks (*) indicate differences between infected cells that were or were not treated with 2'-FL (P < 0.05). CXCL2, chemokine (C-X-C motif) ligand 2 (gene for MIP-2); CXCL8, chemokine (C-X-C motif) ligand 8 (gene for IL-8); DBA, Dolichos biflorus agglutinin; HK, heat-killed; HMOS, human milk oligosaccharides; LTL, Lotus tetragonolobus lectin; MIP-2, macrophage inflammatory protein-2; TNFA, tumor necrosis factor α; UEA-I, Ulex europaeus agglutinin I; 2'-FL, 2'-fucosyllactose.

C. jejuni infection in vitro. Oral inoculation of conventional specific pathogen–free mice with clinical isolates of human pathogens often does not result in the infection and sequelae of human disease, but disruption of the murine microbiota by antibiotic treatment can render mice susceptible. A minimum antibiotic treatment appropriate for infecting mice with a clinical human isolate of pathogenic C. jejuni was determined empirically (Supplemental Figure 2). Expt. 3 tested various permutations of timing and duration of an antibiotic cocktail in drinking water before C. jejuni inoculation that would allow the most appropriate infection. Seven days of antibiotic treatment produced greater C. jejuni colonization than treatment for 1 or 3 d, with sequelae resembling human infection. One-factor ANOVA of decrements in body weight (time 0 = 100% for each individual mouse) after inoculation that followed variable antibiotic treatments indicated a significant effect of duration (P < 0.0005). Subsequent Tukey's honestly significant difference test indicated that the 3- and 7-d antibiotic treatment group body weights differed from one another and from their initial weights on 0 and 1 d (P < 0.05). Colonization, measured as C. jejuni plate count in fresh feces, also differed by variable antibiotic treatments (P < 0.0005). Subsequent post hoc Tukey's test indicated that fecal colonization after 3 and 7 d of antibiotic treatment differed from one another and from days 0 and 1 (P < 0.05) (Figure 2). When 7 d antibiotic-treated mice were inoculated with 10³, 10⁴, or 10⁸ C. jejuni CFUs/mouse, 10⁸ supported the greatest colonization (Supplemental Figure 2). When the 10⁸ daily inoculation was for a 1, 3, or 7 d duration, 3 d was sufficient for full colonization (P < 0.05). Plate count in fresh feces, also differed by variable antibiotic treatments (P < 0.0005). Subsequent post hoc Tukey’s test indicated that fecal colonization after 3 and 7 d of antibiotic treatment differed from one another and from days 0 and 1 (P < 0.05) (Figure 2). When 7 d antibiotic-treated mice were inoculated with 10³, 10⁴, or 10⁸ C. jejuni CFUs/mouse, 10⁸ supported the greatest colonization (Supplemental Figure 2). When the 10⁸ daily inoculation was for a 1, 3, or 7 d duration, 3 d was sufficient for full colonization. Thus, 7 d of pretreatment by antibiotics followed by 3 d of inoculation by daily gavage provided a murine model of human C. jejuni enteritis. This validated murine protocol was used to investigate mechanisms of C. jejuni infection and its proinflammatory host response, and of the efficacy of 2'-FL to prevent C. jejuni infection and infection-mediated inflammation.

2'-FL inhibition in vivo. Expt. 4 consisted of 5 groups of four 4-wk-old C57BL/6 mice pretreated with the antibiotic cocktail.
Group 1 consisted of uninfected negative controls. Group 2 consisted of uninfected mice that received 5 g 2'-FL/L for 3 d, which were essentially similar to untreated controls in all variables measured. Group 3 consisted of positive controls infected by *C. jejuni* with no further intervention, which lost 9.8% of their initial (before antibiotic treatment) body weight after 3 d (*P* < 0.014; Figure 3A), with a high DAI (*P* < 0.001; Figure 3B), fecal shedding of *C. jejuni* (*P* < 0.001; Figure 3C), and infection of the intestines, spleen, and MLNs (*P* < 0.0005; Figure 3C) relative to groups 1 and 2 (uninfected negative controls). Group 4 was infected with a *C. jejuni* inoculum containing 2'-FL. Two-factor ANOVA indicated a significant interaction between 2'-FL treatment and *C. jejuni* infection for the following: body weight (*P* = 0.001) (Figure 3A), DAI (*P* < 0.0005) (Figure 3B), shedding of fecal *C. jejuni* (*P* < 0.0005), infection of intestines (*P* < 0.0005), infection of spleen (*P* < 0.0005), and infection of MLNs (*P* < 0.0005) (Figure 3C). Mean comparisons with Bonferroni adjustment indicated that 2'-FL attenuated the weight loss associated with *C. jejuni* infection (*P* = 0.02) with infected, but treated, mice weighing within 1% of uninfected controls. The reduction of infection by 2'-FL is associated with attenuation of pathogen-induced p-38 activation in the colonic mucosa (Supplemental Figure 3B). DAI's were reduced by 57% (Figure 3A, B; *P* < 0.0005), fecal shedding was reduced by ~90% (*P* < 0.005), infection of intestine reduced by 80% (*P* < 0.0005), infection of spleen was reduced by 96% (*P* < 0.0005), and infection of MLNs was reduced by 93% (*P* < 0.0005; Figure 3C).

Group 5 received 2'-FL for 3 d before and concurrent with the 3 d of inoculation; the inoculum, as in group 4, contained both *C. jejuni* and 2'-FL. Two-factor ANOVA indicated a significant interaction between 2'-FL treatment and *C. jejuni* infection for the following: body weight (*P* < 0.005; Figure 3A), DAI's (*P* < 0.0005; Figure 3B), shedding of fecal *C. jejuni* (*P* < 0.0005), infection of intestines (*P* < 0.0005), infection of spleen (*P* < 0.0005), and infection of MLNs (*P* < 0.0005; Figure 3C). Mean comparisons with Bonferroni adjustment indicated that 2'-FL attenuated the weight loss associated with *C. jejuni* infection (*P* = 0.02); infected but treated mouse weights were within 1% of uninfected controls. Relative to infected positive controls, treatment with 2'-FL reduced the DAI's by 77% (Figure 3A, B; *P* < 0.005), fecal shedding by 99% (*P* < 0.0005), infection of intestine by 97% (*P* < 0.0005), infection of spleen by 97% (*P* < 0.0005), and infection of MLNs by 98% (*P* < 0.0005; Figure 3C).

Note that these biomarkers of *C. jejuni*-induced disease and inflammation were reduced by the 2'-FL treatment by such an extent that they were not significantly different from those of the negative controls. Thus, prophylactic treatment with 2'-FL added to the protection afforded by simultaneous treatment, offering almost complete protection.

**Histology of mucosal injury.** Histopathology of *C. jejuni*-infected mucosal sections of colonic mucosa showed extensive infiltration by immune cells, including neutrophils (*P* < 0.0005), epithelial ulceration (*P* < 0.0005), goblet cell depletion (*P* = 0.007), submucosal edema (*P* < 0.0005), and a histopathology score reflecting crypt hyperplasia with abscesses (*P* < 0.0005), reminiscent of the histopathology of human *C. jejuni*-infected mucosal colitis (Figure 4). Mice whose inoculation was accompanied by 2'-FL exhibited attenuated mucosal colitis with some submucosal edema (Figure 4C), whereas pretreatment with 2'-FL before and during inoculation protected mucosa from *C. jejuni*-associated injury even more strongly than concurrent treatment alone, exhibiting no apparent mucosal colitis or injury relative to uninfected negative controls (Figure 4D).

Two-factor ANOVA indicated a significant interaction between 2'-FL treatment and *C. jejuni* infection for the following: goblet cell depletion (*P*-interaction < 0.0005; Figure 4E), histopathology score (*P*-interaction < 0.0005; Figure 4F), myeloperoxidase 7 positive (MPO7)+ neutrophil infiltration (*P*-interaction < 0.0005; Figure 4G), and CD3+ lymphocyte infiltration (*P*-interaction < 0.0005; Figure 4H). Mean comparisons with Bonferroni adjustment indicated that 2'-FL attenuated *C. jejuni* infection-mediated goblet cell depletion by 53% (*P* = 0.007), histopathology score by 72% (*P* < 0.0005), MPO7+ neutrophil infiltration by 47% (*P* < 0.0005), and CD3+ lymphocyte infiltration by 54% (*P* < 0.0005).

**Mucosal inflammatory signaling.** *C. jejuni* infection strongly induced mucosal secretion in mouse colons of NF-kB–dependent inflammatory mediators IL-1β (*P* < 0.0005), IL-6 (*P* < 0.0005), TNF-α (*P* = 0.006, and MIP-2 (*P* = 0.002) and their corresponding IL1β (*P* = 0.001), Il6 (*P* < 0.0005), Tnfa (*P* < 0.0005), and Cxcl2 (*P* < 0.0005) mRNAs (Figure 5). Two-factor ANOVA indicated a significant interaction between 2'-FL treatment and *C. jejuni* infection for pathogen-induced expression in colonic mucosa of the following: IL-1β (*P*-interaction < 0.0005; Figure 5A), IL-6 (*P*-interaction < 0.0005; Figure 5B), TNF-α
FIGURE 3  Sequelae of Campylobacter jejuni infection in C57BL/6 mice treated with 2'-fucosyllactose (Expt. 4). Treatment with 2'-fucosyllactose prevented weight loss (A), reduced the DAI (B), and reduced C. jejuni in feces and C. jejuni invasion of colonic tissue, MLNs, and spleen (C). Values are means ± SDs or single points with bars for means, n = 4 mice/group. Means without a common letter differ, P < 0.05. Cj, pretreated with antibiotics and inoculated with C. jejuni; Cj+2'-FL, inoculated with C. jejuni + 2'-fucosyllactose; Cont, control mice pretreated with antibiotics only; DAI, disease activity index; MLN, mesenteric lymph nodes; 2'-FL, pretreated with antibiotics and then with 2'-fucosyllactose; 3d2'-FL-Cj+2'-FL, pretreated with 2'-fucosyllactose for 3 d before inoculating with C. jejuni + 2'-fucosyllactose.

Discussion
The reduced risk of infectious disease by breastfeeding had been attributed primarily to secretory antibodies in human milk.

2'-Fucosyllactose averts inflammation by C. jejuni
HMOs are now recognized as part of an innate immune system whereby the mother protects her infant (33) by 3 distinct activities: antiadhesive, prebiotic, and anti-inflammatory.

Although many HMOs are fucosylated, the expression of specific fucosylated oligosaccharides in milk varies by maternal genotype. In breastfed infants, differences in milk content are strongly associated with the risk of infectious disease in the neonate (34). A high content of 2'-fucosyllactose before inoculation with C. jejuni strain NCTC 11168 binds glycans terminating in fucose and that 2'-FL inhibits C. jejuni binding (35).

The binding specificity of C. jejuni varies by strain, and as with the related species Helicobacter pylori (36, 37), C. jejuni has broad binding specificity for mucins and other complex glycans (38, 39). In glycan arrays, C. jejuni strain NCTC 11168 binding specificity varies according to growth conditions; under growth conditions mimicking mammalian and avian hosts, NCTC 11168 binds glycans terminating in α- and β-linked galactose and to fucosylated glycans. Mannose and sialic acid are required for initial binding to host tissues, but long-term infection requires fucose and terminal galactose moieties (15). Lewis B and H-2 are the primary glycan moieties recognized (14). Accordingly, high concentrations of fucose (39), the fucose binding lectin UEA-I, and the galactose recognizing lectin Ricinus communis agglutinin I (RCA-I) inhibit adherence of C. jejuni strain NCTC 11168 to epithelial cells (15).

Other strains of C. jejuni also depend on fucose for binding. Binding of a virulent clinical isolate, C. jejuni strain 287ip, to HEp-2 cells is inhibited by carbohydrate moieties containing fucosylated H-2 moieties (10). Strain 287ip binds to Chinese hamster ovary cells only when the cells are transfected with a human α1,2-fucosyltransferase gene that causes overexpression of H-2 antigen. This binding is inhibited by anti-H-2 monoclonal antibodies (block H-2) and by competitive inhibitors of H-2 binding, including 2'-FL. Pathogenic (invasive) strains of C. jejuni bind strongly and specifically to H-2 glycoconjugates, whereas adherent (nonpathogenic) strains do not (10).

Binding of strain 287ip to human intestinal mucosa ex vivo is inhibited by HMOs or 2'-FL (10). Here, infection by C. jejuni strain 81-176 consistently and robustly inhibited by HMOSs or 2'-FL (10). Histopathology of Campylobacter jejuni infection in C57BL/6 mice treated with 2'-fucosyllactose (Expt. 4). Treatment with 2'-fucosyllactose ameliorated C. jejuni-induced histopathology of mouse colon tissues (A–D), preserved or restored mouse goblet cell numbers (E) and histopathology scores (E), and prevented or reduced mouse neutrophil granulocyte infiltration (measured as MPO7) (G) and CD3-positive T lymphocyte mucosal infiltration (H). Values are means ± SDs, n = 4 mice/group. Means without a common letter differ, P < 0.05. Cj, pretreated with antibiotics and inoculated with C. jejuni; Cj+2'-FL, inoculated with C. jejuni + simultaneous 2'-fucosyllactose; Cont, control mice pretreated with antibiotics only; HPF, high power field; MPO7, myeloperoxidase 7; 2'-FL, pretreated with antibiotics and 2'-fucosyllactose; 3d2'-FL-Cj+2'-FL, pretreated with 2'-fucosyllactose before inoculating with C. jejuni + 2'-fucosyllactose.

The use of modified murine models of C. jejuni colonization (8–12) is based on the importance of microbiota in the natural resistance of mice to human strains of C. jejuni. Previous murine models of C. jejuni infection used pretreatment with antibiotics. A single gavage of vancomycin 4 h before C. jejuni inoculation did not induce true infection in mice (12). Conversely, treatment of mice with a cocktail of antibiotics for 6–8 wk before inoculation resulted in massive C. jejuni infection equally from the stomach to the colon, a degree of infection not seen in human patients (11). To develop our model, varying times of treatment with a particular cocktail of antibiotics were tested for their ability to produce features that most resembled those of clinical C. jejuni infection; treatment for 7 d optimized infection and signaling. C. jejuni strain 81-176 consistently and robustly
FIGURE 5  (A–E) Colonic inflammatory markers in Campylobacter jejuni–infected C57BL/6 mice treated with 2’-fucosyllactose (Expt. 4). The induction of inflammatory mediator proteins and mRNAs by C. jejuni infection in the mouse colon, measured in media from organ culture, was suppressed by 2’-fucosyllactose. Values are means ± SDs, n = 4 mice/group. Within the protein or mRNA data sets, means without a common letter differ (P < 0.05). Cj, pretreated with antibiotics and inoculated with C. jejuni; Cj+2’-FL, inoculated with C. jejuni with simultaneous 2’-fucosyllactose; Cont, control mice pretreated with antibiotics only; Cxcl2, chemokine (C-X-C motif) ligand 2 (gene for MIP-2); MIP-2, macrophage inflammatory protein 2; Tnfa, tumor necrosis factor α; 2’-FL, pretreated with antibiotics and 2’-fucosyllactose; 3d2’-FL–Cj+2’-FL, pretreated with 2’-fucosyllactose before inoculating with C. jejuni + 2’-fucosyllactose.

infected the intestinal mucosa, which exhibited extensive signs of colitis analogous to C. jejuni–associated colitis in humans. Inoculated mice were protected when 2’-FL feeding was simultaneous with the C. jejuni inoculation, and even more so when the treatment was initiated before inoculation. The protection before or with the inoculum, with no further protection post-treatment, suggests that part of the protection by 2’-FL involves restoration of the microbiota through a prebiotic mechanism. Prebiotics, indigestible dietary glycans that confer health benefits, include diversity and numbers of intestinal microbiota. HMOSs, which are prebiotics, promote the colonization of the distal gut by mutualistic bacteria that selectively ferment HMOSs to produce organic acids, reduce ambient pH, and inhibit growth virulence of pathogens (40). Such changes would reduce C. jejuni infection and mucosal inflammatory responses.

In vivo models integrate multiple phenomena, including those mediated by the microbiota, whereas human cell culture models reflect the response to C. jejuni infection per se. Elevated synthesis and release of IL-8 is a characteristic innate immune response of HECs to enteric infection (5, 41–44). A virulent strain of S. typhimurium (American Type Culture Collection 14028 wild-type), but not the attenuated strain sapA−, induces Il8 mRNA synthesis and p38 MAPK within 6 h of infection in intestinal epithelial cells. The inhibition of p38 kinases prevents this pathogen-induced IL-8 induction (45). The data herein showed that C. jejuni infection also induced IL-6 and IL-8 synthesis and release from cultured HECs accompanied by induction of p38 kinases; these were abrogated by 2’-FL treatment. C. jejuni infection of mouse intestinal mucosa also elevated mRNA levels and protein release of IL-6, TNF-α, IL-1β, and MIP-2 and induced p38, and these changes were attenuated in 2’-FL–treated mice. The involvement of the p38 signaling pathway in C. jejuni infection, and attenuation of this pathway and its downstream proinflammatory mediators by 2’-FL, does not preclude the potential involvement of other signaling webs, such as those linked to protein kinase B (Akt), c-Jun N-terminal kinase (ERK), and c-Jun N-terminal kinase (JNK). The identification of other potential signaling pathways and putative cellular receptors for 2’-FL is of interest for future studies. The increase in IL-6 and TNF-α in mice was interpreted as an acute-phase response to pathogen infection, IL-1β as reflecting inflammasome activation, and MIP-2 as induction of a neutrophil chemottractant gradient (44, 46). In contrast to these innate immune responses, the lack of change in IL-17 concentrations suggested limited involvement by the adaptive immune response during the early phase of the immune response in this murine model of acute C. jejuni infection.

Treatment with 2’-FL attenuated the increase in overall mucosal response and inflammasome response elicited by C. jejuni infection, which is consistent with reduced C. jejuni infection in 2’-FL–treated mice. Not shown is an additional group fed 2’-FL after the inoculation for an additional 3 d, whose weight changes and DAIa were essentially similar to those whose 2’-FL treatment was only that contained in the inoculum. However, only prophylactic treatment of 2’-FL for the 3 d before infection significantly reduced MIP-2. This suggests a change in the microbiota community composition through a prebiotic
Effect of 2'-FL, thereby influencing inter-kingdom communication between mutualistic bacteria and mammalian intestinal mucosa. Such communication would be through signaling pathways that are distinct from, but intersect with, pathways used for inflammatory signaling (25). The data herein could be interpreted as 2'-FL exhibiting antiadhesive, prebiotic, and anti-inflammatory activities, but the experiments do not differentiate among these overlapping mechanisms of protection. Yet, the further protection afforded by 3 d of pretreatment with 2'-FL before inoculation, but not by 3 d of supplemental 2'-FL postinoculum (data not shown), strongly implicates an additional prebiotic effect as prophylactic to *C. jejuni* exposure.

This modified murine model, wherein microbiota disruption preceded *C. jejuni* inoculation, recapitulated several known principal clinical responses to infection: acute transient enteric infection, immune response, and spontaneous clearing. This provides confidence in the potential clinical relevance of the inflammatory mechanisms of pathogenesis measured and their response to oral agents that protect against *C. jejuni* infection. Furthermore, because it is based on antibiotic pretreatment, this model is relevant to other human diseases associated with antibiotic use. This in vivo model, combined with the HEC in vitro models, confirmed the identification of 2'-FL as the HMOS most strongly associated with inhibition of *C. jejuni*. The inhibition by 2'-FL now shown for *C. jejuni* strain 81-176, yet another independent clinical isolate, further reinforces the conclusion that 2'-FL inhibits a common virulence factor critical for *C. jejuni* pathogenesis. Three putative mechanisms of 2'-FL inhibition are as follows: binding inhibition, which would diminish the ability of *C. jejuni* to infect; prebiotic activity, which would accelerate recovery of the microbiota disrupted by antibiotics, allowing the beneficial microbes to compete better with *C. jejuni*; and inhibition of inflammatory signaling. The confluence of in vitro and in vivo results supports binding inhibition and inhibition of inflammation (direct, indirect, or both); the efficacy of pretreatment by 2'-FL in vivo supports the prebiotic mechanism. These *C. jejuni* infection models could now be used to differentiate among these potential mechanisms of 2'-FL for each of these overlapping phenomena. Feeding pure 2'-FL may prove useful as a novel clinical prophylactic and therapeutic agent against *C. jejuni* and other enteric pathogens.

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