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Research paper

Intestinal colonization and acute immune response in commercial turkeys following inoculation with *Campylobacter jejuni* constructs encoding antibiotic-resistance markers

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ABSTRACT

Consumption of contaminated poultry products is one of the main sources of human campylobacteriosis, of which *Campylobacter jejuni* subsp. *jejuni* (*C. jejuni*) is responsible for approximately 90% of the cases. At slaughter, the ceca of commercial chickens and turkeys are the main anatomical site where *C. jejuni* asymptomatically colonizes. We have previously colonized commercial turkey poults with different isolates of *C. jejuni* and evaluated different media to best enumerate *Campylobacter* from intestinal samples, but the host-response is unknown in turkeys. Enumeration of *Campylobacter* (colony forming units (cfu)/gram of intestinal contents) can be challenging, and can be confounded if animals are colonized with multiple species of *Campylobacter*. In order to precisely enumerate the *C. jejuni* isolate used to experimentally colonize turkeys, constructs of *C. jejuni* (NCTC 11,168) were tagged with different antibiotic resistance markers at the *CmeF* locus (chloramphenicol (CjCm) or kanamycin (CjK)). We sought to examine the kinetics of intestinal colonization using the antibiotic resistant constructs, and characterize the immune response in cecal tissue of turkeys. *In vitro* analysis of the tagged antibiotic-resistant constructs demonstrated no changes in motility, morphology, or adherence and invasion of INT-407 cells compared to the parent isolate NCTC 11,168. Two animal experiments were completed to evaluate intestinal colonization by the constructs. In experiment 1, three-week old poults were colonized after oral gavage for 14 days, and CjCm and CjK cfu were recovered from cecal, but not ileal contents. In experiment 2, nine-week old poults were orally inoculated with CjCm, and the abundance of CjCm cfu/g of cecal contents significantly decreased beyond 14 days after inoculation. Significant lesions were detected in CjCm colonized poults at day 2 post-colonization. Using immunohistochemistry, *Campylobacter* antigen was detected in between cecal villi by day 7 of CjCm colonized poults. Quantitative RT-PCR of CjCm-colonized cecal tissue demonstrated significant down-regulation of *IL-1β*, *IL-10* and *IL-13* mRNA, and significant up-regulation of *IL-6, IL-8, IL-17 A, IL-22* and *IFN*γ mRNA on day 2, and for some on day 7 post-colonization. All differentially expressed genes were similar to mock-infected poults by day 14. These data suggest that *C. jejuni* induced a brief inflammatory response in the cecum of poults that quickly resolved. Results from this study provide valuable insight into host-response and persistent colonization of the turkey cecum. These findings will help to develop and test strategies to promote food safety in commercial turkeys.

1. Introduction

Campylobacteriosis is the most prevalent bacterial foodborne disease in humans worldwide, with over 90% of cases caused by *Campylobacter jejuni* subsp. *jejuni* (*C. jejuni*). Consumption of contaminated poultry is the main source of human exposure (Humphrey et al., 2007). Per capita, Americans consumed 58.7 pounds of chicken per person in 2014, compared to 12.4 pounds of turkey (Bentley, 2017).

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Turkey is an emerging human protein source, but the role of turkeys as a source of human campylobacteriosis is not well documented. Thermophillic Campylobacters (*e.g.*, *C. jejuni, C. coli* and others) persistently colonize the cecum of chickens, approaching 10^9 colony forming units (cfu)/g of cecal contents (Buckley et al., 2010; Kobierecka et al., 2016; Nothaft et al., 2016; Wyszynska et al., 2004), often asymptomatic and producing mild morbidity in young chicks (Sanyal et al., 1984; Welkos, 1984). After colonization in the chicken cecum, *C. jejuni* remain in the mucus layer in between villi (Beery et al., 1988). A transient pro-inflammatory response is produced (Awad et al., 2018; Connerton et al., 2018; Reid et al., 2016; Smith et al., 2008), and is followed by a tolerogenic mucosal response in the cecum (Hermans et al., 2012; Li et al., 2010). The host-response to *Campylobacter jejuni* colonization is unknown in turkeys. Experimental colonization of young turkey poults with *C. jejuni* produced morbidity (*e.g.*, 20% weight loss), but no mortalities (Lam et al., 1992). Initial work demonstrated that the cecum of turkeys harbored the greatest number of *C. jejuni*, which could be recovered for several weeks after experimental colonization (Wallace et al., 1998). Commercial turkeys, prior to and at slaughter, and retail meat products, have tested positive for *C. jejuni* or *C. coli* (Alter et al., 2005; Kashoma et al., 2014; Logue et al., 2003; Noormohamed and Fakhr, 2014; Thorsness et al., 2008; Wallace et al., 1998; Wesley et al., 2005, 2009; Wright et al., 2008).

We have previously colonized turkey poults with wild-type isolates of *C. jejuni*, and demonstrated persistent colonization of the cecum for up to 3 weeks after inoculation (Sylte et al., 2018). Accurate enumeration of wild-type *Campylobacter* spp. from intestinal contents of animals can be difficult, especially if experimental animals are already colonized with separate *Campylobacter*. We previously demonstrated that some selective *Campylobacter* media are beneficial to enumerate *Campylobacter* from turkey intestinal contents (Sylte et al., 2018), however these media are unable to differentiate the challenge strain from endogenous *Campylobacter*. In this study, we generated *C. jejuni* with antibiotic-resistance markers and hypothesized that enumeration of the challenge *C. jejuni* strain would be improved compared to previous studies with wild-type *C. jejuni*, as well as assessing the host-response after colonization.

2. Materials and methods

2.1. Generation of Campylobacter jejuni constructs with antibiotic resistance

The *CmeF* locus was selected as the insertion site of chloramphenicol or kanamycin antibiotic resistant cassettes into *Campylobacter jejuni* subsp. *jejuni* (*C. jejuni*) strain NCTC 11,168. Two sets of primers were designed to amplify both ends of the *CmeF* gene and were used as the flanking sequences to help integrate the antibiotic resistant cassettes into the *C. jejuni* chromosome by homogenous recombination (Pair one: Cme-1F: 5′−CCTAAGGAAAGATCATTCACTCCAGCTGTG-3′, Cme-1R: 5′-GATATATTGATAAGCGGGATCCGCGTGCAGGCATTGATGATC CCG-3′; Pair two: Cme-2F:5′-GTCTTAGCATTATCCTGCAGTTCGCAGCT TGTAAAGGCGGAT-3′, Cme-2R: 5′-GAACTTAGCAATCTTCGCATAAAA ACAGGAG-3′). The kanamycin-resistance cassette (Kan) was amplified from pMW10 (Wosten et al., 1998) by the following primers: kanF 5′-CGCGGATCCCGCTTATCAATATATCTATAGAATGG-3′, kanR 5′-GAA CTGCAGGATAATGCTAAGACAATCACTAAAG-3′. An overlap PCR was performed to get a three-fragment-ligated product (CmeF-part1_-Kan_CmeF-part2). The *CmeF* fragment, inserted by chloramphenicol-resistance cassette (Cm), was directly amplified from our previously generated strain 11,168 F (NCTC 11,168; *CmeF*::*Cm*) (Akiba et al., 2006) using primers CmeF-1 F and CmeF-2R. The CmeF-antibiotic resistance cassette fragments were introduced into *C. jejuni* NCTC 11,168 using an electroporator (Gene Pulser Xcell System; Bio-Rad Laboratories, Richmond, CA, USA). Transformants *CmeF*::*Cm* and *CmeF*::*Kan* were selected on MH agar containing chloramphenicol (15 μg/L) or kanamycin (30 μg/L) at 42 °C in a microaerophilic environment (5% O_2, 10% CO_2 and 85% N_2 gas) for 24 h, and were confirmed using PCR for the chloramphenicol or kanamycin resistance cassettes. In order to determine the resistance of different transformants, individual colonies were cultured on Campy Line agar (CLA) (Line, 2001) containing different concentrations of antibiotic for selection (chloramphenicol 5, 10 and 20 μg/mL) or kanamycin (25, 50 and 100 μg/mL). *CmeF*::*Cm* transformants (CjCm) were considered chloramphenicol resistant if they grew on CLA containing at least 10 μg/mL chloramphenicol and failed to grow on CLA containing at least 50 μg/mL kanamycin. *CmeF*::*Kan* transformants (CjK) were considered kanamycin resistant if they grew on CLA containing at least 50 μg/mL kanamycin, and failed to grow on CLA containing at least 10 μg/mL chloramphenicol. The parent strain failed to grow on CLA containing at least 50 μg/mL kanamycin or 10 μg/mL chloramphenicol. Once antibiotic resistance was characterized, aliquots of each isolate were stored at −80 °C in MH broth containing 10% (v/v) sterile glycerol.

2.2. Campylobacter jejuni motility and growth curves

The motility of the wild-type *C. jejuni* NCTC 11,168 and antibiotic-resistant constructs CjCm and CjK were compared to an amotile *C. jejuni* isolate (NADC 14,103) by stab inoculating into *Campylobacter* motility agar tube (MH agar (0.4% w/v) and incubating at 42 °C for 18 h in a microaerophilic environment as described previously (Golden and Acheson, 2002). Additionally, the same *C. jejuni* isolates were cultured in MH broth, incubating at 42 °C for 18 h, shaking (100 rpm) in a microaerophilic environment. Ten μL of each broth culture was cover slipped on a clean microscope slide and visualized at 400X magnification using a Nikon Eclipse Ni dark-field microscope (Nikon Instruments Inc., Melville, NY). Videos were captured to document motility using NIS-Elements Basic Research software v4.13 (Nikon Instruments Inc.). Isolates were considered motile if they had a positive motility agar test or visual assessment of dark field microscopy showed approximately 90% of the organisms were actively moving. If no motility was seen on the agar stab, or < 90% were motile, the culture was not used as inoculum.

Preparation of the growth curve inocula were performed by statically culturing NCTC 11,168, CjCm and CjK at 42 °C in the broth phase of biphasic MH broth and agar (2% w/v) in a microaerophilic environment, as described previously (Davis and DiRita, 2008). Growth curves were performed in octuplet using microplates in a Bioscreen C plate reader (Growth Curves USA, Piscataway, NJ), measuring OD_{600} every 2 h for 48 h. To limit aggregation, which may impact optical density values, microplates were shaken for 30 s prior to each OD_{600} reading. Two hundred μL of each isolate were cultured in MH or Bolton's broth base at 42 °C in a microaerophilic glove box. Uninoculated media served as a control to subtract OD_{600} background. Growth curve data were analyzed using the R package growthcurver (Sprouffske and Wagner, 2016), which measured the logistical area under the curve, growth rate and generation time.

2.3. Scanning electron microscopy

Parent strain NCTC 11,168 and antibiotic constructs CjCm and CjK were cultured in Bolton's broth base broth statically at 42 °C for 24 h in a microaerophilic environment. One mL of each broth culture was passed through a 0.22 μm nucleopore filter using a Swinney filter holder (Millipore Sigma, Burlington, MA). Filters were fixed in 2.5% glutaraldehyde in 0.1 M Cacodylate buffer and stained by sequential exposure to osmium and thiocarbohydrazide. Filters were dehydrated through graded alcohols and chemically dried with hexamethyldisilizane (Nation, 1983). Samples were decorated with a thin coating of gold and palladium mixture and viewed using a Hitachi TM3030Plus scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.4. Campylobacter adherence and intracellular survival assay

The adherence and intracellular survival of NCTC 11,168, CjCm or CjK in human embryonic INT-407 intestinal epithelial cells was evaluated as described previously (Negretti and Konkel, 2017), with the following modifications. INT-407 cells were cultured at 37 °C with 5% CO_2 gas incubator in Dulbeco's Modified Eagle's medium containing glucose (4.5 g/L), sodium pyruvate (110 mg/mL) and L-glutamine (200 mM) (DMEM; Gibco, Carlsbad, CA), 10% fetal bovine serum (FBS), 20 mM HEPES buffer (Gibco) and a 1X antibiotic solution of streptomycin and penicillin (Gibco). The day before the adherence and invasion assay, INT-407 cells were seeded at 1×10^5 viable INT-407/well in a 24-well plate. Triplicate wells were seeded for each treatment, and duplicate plates were prepared for adherence and invasion assays. Broth cultures of NCTC 11,168, CjCm or CjK were initiated the night before by selecting 5 colonies of each isolate from a freshly grown MH agar plate, and spreading over a new MH plate to form a lawn. The plate was cultured at 42 °C for 18 h, in a microaerophilic environment. The next morning, the lawn of bacteria for each isolate was harvested by adding 10 mL of sterile PBS. Motility was assessed using dark-field microscopy, as described above. Using a Bioscreen C plate reader, each inocula was adjusted to OD_{540} of 0.3 in sterile PBS, and further diluted in DMEM supplemented with 1% FBS without antibiotics to achieve an OD_{540} of 0.03. Exact counts of each inocula were determined by serial dilution of inocula and plating, in duplicate, on MH agar plates to determine cfu/ml. INT-407 cells were approximately 90% confluent after overnight culture. Adherent cells were washed 3X with 1 mL of antibiotic-free DMEM medium. For adherence and intracellular survival assays, INT-407 cells were infected by adding 0.5 mL of 0.03 OD_{540} inoculum (MOI approximately 100:1). Infection was synchronized by centrifuging the cells and bacteria at $800 \times g$ for 5 min at room temperature. Cells were incubated for 3 h at 37 °C with 5% CO_2. Adherent *C. jejuni* was assessed from the first of two duplicate plates by washing each well 3X in 1 mL of Hanks balanced salt solution containing Ca^{2+} and Mg^{2+} (HBSS; Gibco). Cells were lysed by the addition of 200 µL of 0.1% (v/v) Triton X-100 diluted in sterile PBS for 5 min at 37 °C. The volume per well was adjusted to 1 mL in sterile PBS and cell-associated (adherent and invaded) *C. jejuni* were enumerated by serial dilution and culture on MH plates. Intracellular survival was assessed in the second duplicate plate. After a 3 h incubation with respective *Campylobacter*, media was removed, cells were washed 3X with 1 mL of HBSS and 1 mL of DMEM containing 1% FBS and 250 µg/mL gentamicin was added to each well. Cells were incubated for an additional 3 h at 37 °C with 5% CO_2. Cells were washed 3X in HBSS, lysed with Triton X-100 and invaded bacteria were enumerated, as described above. For each *C. jejuni* isolate tested, the percent of inoculum cell-associated was calculated by dividing the mean of adherent *C. jejuni*/the mean of inoculum added and multiplying this number by 100. The percent of inoculum internalized was calculated by dividing the mean of invading *C. jejuni*/the mean of inoculum added and multiplying this number by 100.

2.5. Experimental design of animal studies

Experiment 1 was conducted according to protocol ARS-2016-489 which was approved by the NADC Institutional Animal Care and Use Committee (IACUC). Day of hatch Hybrid poults (n = 80), lacking vaccinations or treatment with antibiotics or probiotics, were obtained from a commercial breeder. Poults were group housed in a single ABSL-2 room with clean pine shavings seeded with approximately 2 kg of litter from an on-site flock of SPF adult small Beltsville-white turkeys. Poults were fed a turkey poult starter ration and had water available *ad libitum*. Prior to day 15 of age, 5 poults from the group were randomly selected and euthanized by intravenous barbiturate overdose and cervical dislocation to confirm negative *Campylobacter* status. From each poult, 1 g of cecal contents was cultured in Bolton's selective *Campylobacter* enrichment broth (Neogen Corporation, Lansing, MI) for 48 h at 42 °C in a microaerophilic environment. One hundred µL was cultured for 48 h on CLA agar containing 2.5 µg/mL sulfamethoxazole (CLA-S) (Line et al., 2008) to determine the flock's *Campylobacter* colonization status (Hunt et al., 2001). Poults were considered free of *Campylobacter* colonization if no small 1 mm diameter dark red to magenta-colored colonies (Line et al., 2008), resembling pure cultures of *C. jejuni* or *C. coli*, were cultured. At 20 days of age, the remaining 75 poults were split into three ABSL-2 rooms, each containing approximately 25 poults. *C. jejuni* inocula were prepared by culturing at least 5 colonies of each construct into sterile Bolton's broth base shaking at 100 rpm at 42 °C in a microaerophilic environment. On the day of challenge, broth cultures were adjusted to an OD_{600} value of 0.4, and cfu/mL were enumerated using serial dilution on CLA-S, supplemented with either 10 µg/mL chloramphenicol for CjCm or 100 µg/mL of kanamycin for CjK. Motility was assessed using dark-field microscopy, as described above. At day 21 of age, poults were inoculated by oral gavage with 1 mL of Bolton's broth base containing 2×10^8 cfu of CjCm and 3×10^8 cfu of CjK. Mock inoculated poults were administered 1 mL of sterile Bolton's broth base by oral gavage. At days 2, 7 and 14 post-colonization, 7 poults from the CjCm, CjK, and mock-inoculated rooms were euthanized, as described above. Necropsy was performed and contents from the ileum and cecum were aseptically harvested by squeezing the abluminal surface of each organ into sterile conical tubes for quantitative culture of CjCm and CjK from each animal.

Experiment 2 was conducted according to protocol ARS-2016-567 which was approved by the NADC IACUC. Day of hatch Hybrid poults (n = 53), lacking vaccinations or treatment with antibiotics or probiotics, were obtained from a commercial breeder. Poults were group housed in a single ABSL-2 room with clean pine shavings seeded with approximately 2 kg of litter from an on-site flock of SPF adult small Beltsville-white turkeys. At day 15 of age, 5 poults were randomly selected and euthanized to determine the *Campylobacter* status of the flock, as described above. Poults were fed *ad libitum* a turkey poult starter ration for the first 6 weeks, and a grower ration up to 13-weeks of age. Water was available *ad libitum*. At 9 weeks of age, the remaining 48 poults were split into two rooms consisting of 28 poults for CjCm colonization and 20 for mock challenge. At 9 weeks of age, poults were inoculated by oral gavage with 1 mL of Bolton's broth containing 5×10^8 cfu of CjCm. Mock inoculated poults received 1 mL of sterile Bolton's broth base by oral gavage. Inocula preparation and motility was assessed using dark-field microscopy, as described above. At weeks 10, 11, 12 and 13 of age, 7 poults from the CjCm inoculated and 5 from the mock-inoculated rooms were euthanized, and necropsy was performed to aseptically harvest cecal contents for quantitative culture of CjCm.

2.6. Enumeration of C. jejuni constructs from intestinal samples

Freshly isolated intestinal contents were stored on ice prior to transport to the laboratory for culture. One gram of cecal or ileal contents was diluted in 9 mL of sterile PBS, vortexed for 5 s and serially diluted up to 10^{-6}. Using the track-plating dilution method (Jett et al., 1997; Siragusa, 1999; Sylte et al., 2018), 10 µL of each dilution was plated in duplicate on CLA-S (Line et al., 2008), which was modified to contain chloramphenicol (10 µg/mL) or kanamycin (100 µg/mL). Serially diluted intestinal samples were also plated on Campy Cefex agar plates containing chloramphenicol (10 µg/mL) or kanamycin (100 µg/mL). Plates were incubated at 42 °C in a microaerophilic environment for 48 h, and cfu resembling magenta 1 mm in diameter colonies from a pure culture of constructs CjCm or CjK were enumerated. For statistical purposes, samples with no detectable CjCm or CjK cfu were assigned value of 10^3 cfu/gram of contents, the limit of detection by culture.

2.7. RNA preservation

At necropsy, up to 2 cm of proximal cecum from each bird was

stored on ice in 10 mL of RNALater stabilizer solution (Life Technologies, Carlsbad, CA). Samples were incubated at 4 °C for 24 h. Cecal tissue was removed from RNA Later solution and intestinal contents were removed from the luminal surface. Approximately 500 mg of tissue was cut into 3 pieces and transferred to internally threaded cryovial. RNALater preserved tissues were snap frozen by fully immersing cryovials in liquid nitrogen for 1 min. Cryovials were then stored indefinitely in a −80 °C freezer.

2.8. RNA extraction and cDNA synthesis

Cryovials containing RNALater preserved cecal tissues were removed from −80 °C freezer and stored on dry ice. Up to 150 mg of cecum was placed into an gentleMACS M tube (Miltenyi Biotec Inc., San Diego, CA) containing 1 mL of TRIZOL reagent (Life Technologies). Cecum was homogenized using a gentleMACS Octo Dissociator (Miltenyi Biotec Inc.) using the gentleMACS program RNA_02. The homogenate was centrifuged in the M tube for 30 s at 800xg at room temperature, and supernatant was transferred to nuclease-free 1.5 mL microfuge tubes. Two hundred µL of chloroform was added and up to 300 µL of the aqueous phase was harvested after centrifugation at 12,000 x g for 15 min at 4 °C. The aqueous phase was further processed using mirVana miRNA isolation kit without phenol, per the manufacturer's protocol (Ambion, Carlsbad, CA). The quantity of eluted total RNA was spectrophotometrically estimated using a NanoDrop-2000 instrument (Thermo Fisher Scientific, Waltham, MA), and up to 500 ng of total RNA was evaluated for RNA integrity using an RNA ScreenTape and 2200 TapeStation instrument (Agilent Technologies Inc., Santa Clara, CA), per the manufacturer's protocol. Total RNA was stored at −80 °C until cDNA synthesis. All total RNA samples from cecal tissue had RINe values ≥7, demonstrating the high quality of isolated RNA. Contaminating Genomic DNA was removed by treating 500 ng of total RNA with DNAse prior to synthesizing cDNA using the iScript gDNA Clear cDNA Synthesis Kit (BioRad), following the manufacturers protocol. As a control, some samples were not treated with reverse transcriptase, to assess potential non-specific amplification from genomic DNA contamination. Samples of cDNA were stored at −20 °C.

2.9. qPCR

Exon-spanning primer sets for the turkey-specific genes in cecal tissue were designed using the Primer Quest Tool program (https://www.idtdna.com/Primerquest/Home/Index; Integrated DNA Technologies, Coraville, IA, USA). Gene, accession numbers, primer sets, amplicon length are listed in Table 1. The qPCR reactions were performed using BioRad SSO Advanced SYBR green master mix (BioRad). Each reaction was performed at a volume of 15 µL, containing 7.5 µL of SSO Advanced SYBR green master mix, 1 µL of F primer (10 µM), 1 µL of R primer (10 µM), 4.5 µL of nuclease free water and 1 µL of cDNA. Reactions were performed in triplicates (cDNA replicates). No-template controls (NTC) were included to detect non-specific amplification and no-reverse transcriptase controls were included to test for genomic DNA contamination. Amplification and detection of specific products were performed using the BioRad C1000 thermal cycler 384-well system with the following cycle profile: one cycle of 95 °C for 30 s and 40 cycles of 95 °C for 15 s and 60 °C for 30 s, measuring SYBR green fluorescence after each cycle. Melting curve analysis was performed for each sample and primer pair. Gene expression (Cq value) was normalized using turkey Ribosomal Protein S13 (RPS13) as a reference gene (Borowska et al., 2016). Fold change were analyzed by comparing age matched inoculated to non-inoculated samples using the ΔΔCt method using the CFX manager software v3.1 (BioRad). Gene expression was considered significantly different if $p < 0.05$ and the relative expression was greater or less than 2 fold.

2.10. Histopathological analysis

At necropsy, cecum, colon and ileum tissues were stapled to bibulous paper, and cecal tonsil was immersed and fixed in 10% buffered neutral formalin for 48 h. Then, tissues were stored in 70% ethanol before cutting into histology cassettes and embedding in paraffin for thin sectioning (5 µm) and hematoxalin and eosin staining. Slides were single-blinded analyzed and scored by a board certified veterinary pathologist (LS) for lesions. Because *Campylobacter* are commensals of the poultry intestinal microbiota, and severe lesions were not observed, a unique grading score was used to evaluate changes of the intestinal histomorphology, including: 1) number of heterophils in the epithelium, lamina propria, and submucosa/muscularis as a possible indicator of inflammation and immune activation, 2) presence of small granulomas within the lamina propria, 3) crypt ectasia with or without heterophilic accumulation, 4) presence of apoptotic cells in the lamina propria, 5) areas with attenuated epithelium. Intermediate scores for each category (1 to 5) were obtained for the intestinal sections (*e.g.*, ileum, cecal tonsils, ceca, and colon) of each bird. The final histological score of one poult was the sum of each intermediate scores. For assessment of the heterophilic infiltrate in the lamina propria, for each section of the intestine the number of heterophils was counted in 9 fields at 600X magnification with the resulting average used to formulate a severity grade (severity = 0, if < 3 average heterophils; severity = 1, ≥3-5; severity = 2, ≥5-10; severity = 3, ≥10). Rare granulomas were also found in the lamina propria of the intestine of a few poults: the extent and severity of these lesions was captured by

Table 1
- Primers used for qRT-PCR.

Gene	Acession number/gene ID	F primer (5'-3')	R primer (5'-3')	Amplicon (bp)
IL-1β	Q393271.1	CCGACACGCAGGGACTTT	GAAGGTGACGGGCTCAAAAA	72
IL-6	XM_003207130.1	GATCCGGCAGATGGTGATAAA	CTATCCACGCCTTATCTGACTTC	101
IL-8	DQ393276.1	GGTTTCAGCAGCTCTGTCACA	TGGCACCGCAGCTCGTT	63
IL-10	AM493432.1	CTGGGCCTGAAGATGACAAT	CTCATCCATCTTCTCGAACGTC	105
IL-13	AM493431.1	TCTGTCTGGCAGAGCTCATT	AAGGGACCTGCTCTCCTTTG	117
IL-17 A	XM_003204633.2	TCCCTTGTTCTCCTTTGTTCAG	GCTCAAGTCCTGGCCATATC	102
IL-22	XM_003202049.1	AAAGGGACAGGGATGGTTTC	GATCTGAGACTCTGGCCATTTC	121
IFNγ	AJ000725.1	GCCGCACATCAAACACATATC	GTCATTCATCTGAAGCTTTGCC	112
TGFβ2	XM_010706695.1	AGCTGTACCAGGTTCTGAAATC	CCATTCTCCTTCAGCTCTTGT	95
MUC-2	100545480	TGGTGTTACCACAACGGTAAT	GGACAACCTGGAATTGGTGTA	108
pIgR	XM_010724183.1	GCAAGCTTAAACCCAGTGTTC	CAGTACTTCCTATCGTGTCTGTTC	116
RPS13	XM_010711118.1	CCCTCACAAATAGGTGTCATCC	GGGCCAGTCCCTTTGATTTA	106

IL-1β = turkey interleukin 1 beta; IL-6 = turkey interleukin 6; IL-8 = turkey interleukin 8.
IL-10 = turkey interleukin 10; IL-13 = turkey interleukin 13; IL-17 A = turkey interleukin 17 A.
IL-22 = turkey interleukin 22; IFNg = turkey interferon gamma; TGFβ2 = turkey transforming growth factor beta 2; MUC-2 = turkey mucin-2; pIgR = turkey polymeric immunoglobulin receptor; RPS13 = turkey 40S Ribosomal Protein S13.

assessing the actual number (severity = 0, no granulomas; severity = 1, < 3; severity = 2, ≥3-5; score = 3, ≥ 5), with an additional modifier based on the diameter of the largest ones (score up to 6). For the other categories (heterophils in the epithelium and muscularis, crypt ectasia, attenuated epithelium), severity assessment was semi-quantitative, based on subjective assessment of the extent of the lesions (score = 0, < 10% of sections; score = 1, ≥10-25%; score 2, ≥25-75; score = 3, ≥75). The possible scores ranged from 0 (lowest score) – 27 (highest score). For each group, the final scores were averaged, and differences between means were determined using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Differences were considered to be significant with $p < 0.05$.

2.11. Immunohistochemistry

Immunohistochemistry was performed to detect *Campylobacter* antigen in tissues, as described previously (Burrough et al., 2009). Deparafinized slides with cecal tissue from CjCm or mock-infected poults (experiment 1) were probed with rabbit anti-*Campylobacter* outer membrane protein polyclonal sera diluted at 1:200 (Zhang et al., 2000). Primary antibody binding was detected using VECTASTAIN Elite ABC-HRP Kit (Vector Laboratories, Burlingame, CA) and VECTOR NoveRED HRP substrate kit, per manufacturer instructions. Slides were visualized at 400X magnification using a Nikon Eclipse Ni microscope (Nikon Instruments Inc.) and images were captured using NIS-Elements Basic Research software v4.13 (Nikon Instruments Inc.).

2.12. Statistical analysis

Data for host-cell adherence and survival, bacterial enumeration, histological scoring and fold-gene expression were analyzed using Prism 7.03 statistical software (GraphPad Inc.) Growth curve data were analyzed for logistical area under the curve, generation time and growth rate using the R package growthcurver (https://github.com/cran/growthcurver). One- or 2-way ANOVA followed by a post-hoc multiple comparisons test (Tukey) were used to detect significant differences between groups or treatment days. Results were considered significant at values of $p \leq 0.05$.

3. Results and discussion

3.1. Growth, motility, adherence and invasion of intestinal epithelial cells by wild-type and antibiotic-resistant constructs of C. jejuni

Antibiotic-resistant constructs of *C. jejuni* strain NCTC 11,168 were generated by inserting the chloramphenicol or kanamycin-resistance cassettes into the *CmeF* locus in the *C. jejuni* chromosome. The chloramphenicol resistant NCTC 11,168 mutant (*CmeF::Cm*), referred to as CjCm, grew on CLA with sulfamethoxazole and containing 5 to 20 μg/mL of chloramphenicol, and was sensitive to kanamycin (> 25 μg/mL). CjCm failed to grow if chloramphenicol was > 20 μg /mL. The kanamycin resistant NCTC 11,168 mutant (*CmeF:Kan*), referred to as CjK, grew on CLA-S containing 25–100 μg/mL of kanamycin, but was sensitive to chloramphenicol (> 5 μg/mL). NCTC 11,168, the parent strain, was sensitive to chloramphenicol (≥5 μg/mL) or kanamycin (≥25 μg/mL). The growth of antibiotic-resistant constructs CjCm and CjK were compared to the parent strain of NCTC 11,168 in Muller Hinton and Bolton's broth base cultures for 48 h. In Bolton's broth base, both constructs grew to higher terminal OD_{600} values compared to MH broth (Fig. 1AB), The logistical area under curve, doubling time and growth rate of both CjCm or CjK were statistically different ($p < 0.05$) from that of the parent strain when grown in Bolton's broth base (Supplemental Figure S1). Motility is essential for *Campylobacter* colonization in different animal models (Guerry, 2007), and was assessed using motility agar and dark field microscopy. There was no detectable difference in motility between CjCm or CjK and the parent strain, and no change in bacterial morphology was detected using scanning electron microscopy (Supplemental Figure S2). Antibiotic-resistant constructs CjCm and CjK and parent strain were lastly compared for adherence and intracellular survival in INT-407 cells, a human embryonic intestinal epithelial cell line, previously used to identify genes vital for *Campylobacter* adherence to host cells (Monteville et al., 2003). No significant difference in adherence, invasion or survival were detected for CjCm or CjK compared to NCTC 11,168 (Fig. 2). Overall, these data demonstrate that constructs CjCm and CjK were conferred with the specified antibiotic resistance phenotype that didn't affect motility, morphology or adherence and invasion to host-cells. Because growth of both constructs was superior in Bolton's broth base, compared to MH broth (Fig. 1AB), it was used to prepare the inocula for animal experiments 1 and 2.

3.2. Enumeration of antibiotic-resistant C. jejuni constructs from intestinal contents of turkeys

In animal experiment 1, poults were free of *Campylobacter* colonization prior to oral gavage of three-week-old turkey poults with CjCm (1 mL containing 2×10^8 cfu) or CjK (1 mL containing 3×10^8 cfu) in order to evaluate their ability to colonize the turkey intestinal tract. The ideal age to inoculate poults with *C. jejuni* to induce sustained colonization is unknown. Inoculation age data from chickens is controversial, with some studies suggesting that chicks ≤21 days of age are less susceptible to colonization (Sahin et al., 2001), and others suggesting 21-day old chicks are the least susceptible (Han et al., 2016). We previously inoculated poults at 21 days of age with different wild type *C. jejuni* isolates and produced persistent cecal colonization (Sylte et al., 2018). Based on these data, we elected to inoculate poults at 21 days of age in experiment 1. Oral inoculation of turkey poults with constructs CjCm or CjK induced mild morbidity (*e.g.*, watery diarrhea seen on days 2 and 7 in some inoculated poults) and no mortalities. Both constructs were detected in cecal contents from 2 to 14 days post-inoculation (Fig. 3), and cross-contamination was not detected (Table 2). At day 7 post-colonization, the amount of recoverable CjCm was significantly different ($p < 0.01$), relative to days 2 and 14 (Fig. 3). In spite of the addition of chloramphenicol or kanamycin to Campy Cefex agar, excessive background bacteria grew from cecal samples and confounded *C. jejuni* enumeration. Recent identification of extended spectrum beta lactamase producing *E. coli* isolated from poultry or poultry products (Casella et al., 2017; Chishimba et al., 2016) may explain excessive background growth from turkey samples in cefoperazone-containing Campy Cefex agar. Similar to a previous study (Sylte et al., 2018), the use of Campy Cefex agar was discontinued after day 2 post-inoculation, and enumeration was determined solely by culture on CLA-S with the appropriate antibiotic to recover the different constructs. In the case of CjK at day 7 post-inoculation and CjCm at day14, the number of highly colonized poults (≥10^7 cfu/g of cecal contents) began to drop (Fig. 3). The ileum was poorly colonized with CjCm or CjK. At day 2 post-colonization, only a single poult had detectable CjK (1.5×10^4 cfu/g of ileal contents), but all other ileal samples, CjCm or CjK, were below the level of culture detection (Table 2).

Spontaneous *Campylobacter* colonization in commercial turkeys may be influenced by the changes in intestinal microbiota between 4–12 weeks of age (Scupham, 2007), with great flux and increased susceptibility to *Campylobacter* colonization between 11–12 weeks of age (Scupham, 2009). We elected to see if intestinal colonization would improve if poults were older before inoculating (experiment 2). CjCm colonization in experiment 1 resulted in higher terminal cfu/g of cecal contents than CjK in experiment 1 (Fig. 3); therefore, CjCm was used in experiment 2. Nine-week-old turkeys were inoculated by oral gavage with 1 mL containing 5×10^8 cfu and cecal colonization was evaluated for 4 subsequent weeks. The number of CjCm recovered from cecal contents at 21 and 28 days post-inoculation significantly differed ($p < 0.01$) compared to the day 7 and 14 samples (Fig. 4). In spite of

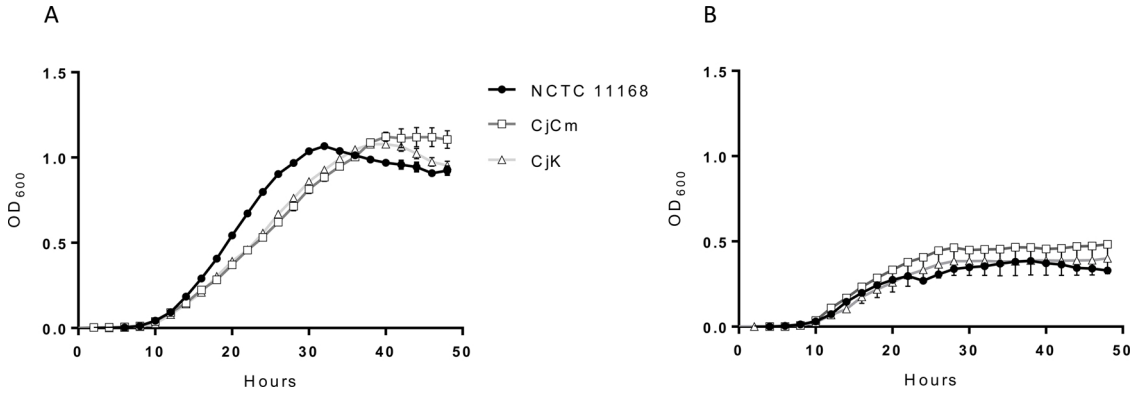

Fig. 1. Growth characteristics of *C. jejuni* parent strain NCTC 11,168, CjCm and CjK antibiotic resistant constructs. Growth curves were performed in A) Bolton's broth base or B) Mueller Hinton broth for 48 h at 42 °C in a microaerophilic environment (5% O_2, 10% CO_2 and 85% N_2). Data represent the mean ± SEM OD_{600} of 8 cultures in Bolton's broth measured every 2 h for a total of 48 h with the background value of unincolated media subtracted.

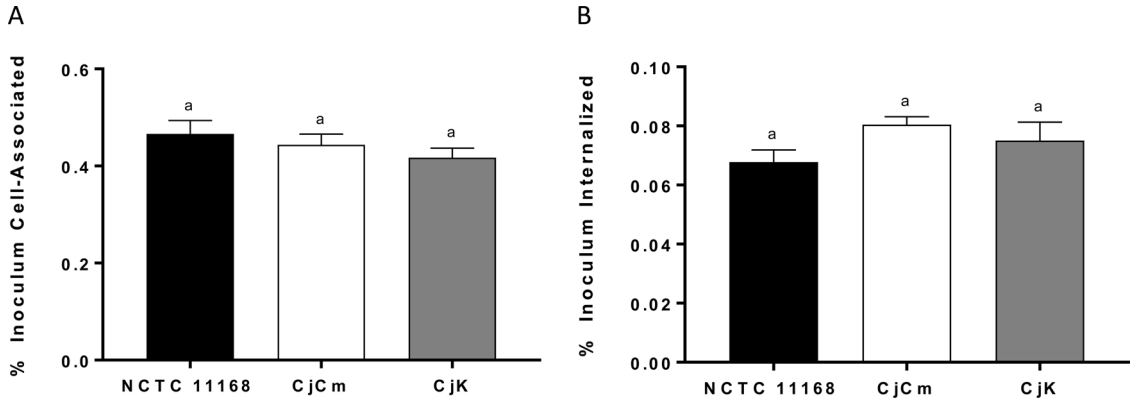

Fig. 2. Adherence and invasion of *C. jejuni* parent strain NCTC 11,168 and antibiotic resistant constructs CjCm or CjK in INT-407 cells. Inocula were diluted an OD_{540} of 0.03 and 0.5 mL (MOI of 100:1) was incubated with 1.5×10^5 adherent INT-407 cells (90% confluent) for 3 h at 37 °C with 5% CO_2 to measure cell-association, and for an additional 3 h with gentamicin (250 μg/mL) to measure internalization. The MOI was approximately 100:1. Data represent the mean ± SEM% cell-associated (A) or internalized (B) of three replicates, compared to the inoculum. Data were statistically analyzed using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($p < 0.05$) between treatments are represented by different letters.

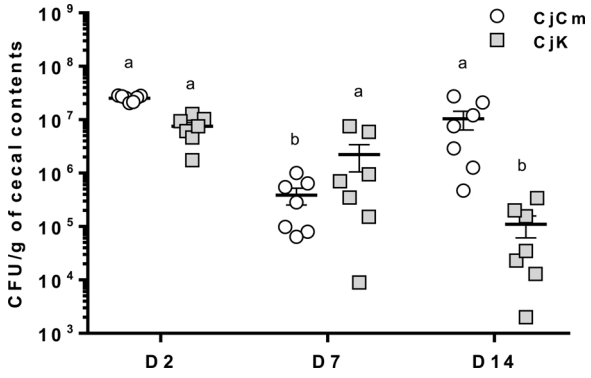

Fig. 3. Enumeration of antibiotic resistant constructs CjCm and CjK from cecal contents of turkey poults from experiment 1. Data represent the mean *Campylobacter* cfu/g of cecal contents from each poult and the mean (solid bar) ± SEM for CjCm or CjK at days 2, 7 and 14 post-colonization. Statistical differences in the number of enumerated *Campylobacter* cfu/g of cecal contents were determined using two-way ANOVA followed by a post-hoc multiple comparisons test (Sidak). Significant differences ($p < 0.05$) between treatments are represented by different letters.

Table 2
Summary of enumeration of *C. jejuni* isolates in animal experiment 1.

			Number of poults *C. jejuni* positive by culture	
Challenge isolate	Sample	Days post-colonization	CLA-S with Chloramphenicol	CLA-S with Kanamycin
Mock	Cecum	2	0 of 5†	0 of 5†
		7	0 of 5†	0 of 5†
		14	0 of 5†	0 of 5†
CjCm	Ileum	2	8 of 8	0 of 8
		7	8 of 8	0 of 8
		14	8 of 8	0 of 8
	Cecum	2	8 of 8	0 of 8
		7	8 of 8	0 of 8
		14	8 of 8	0 of 8
CjK	Ileum	2	0 of 8	1 of 8
		7	0 of 8	0 of 8
		14	0 of 8	0 of 8
	Cecum	2	0 of 8	8 of 8
		7	0 of 8	8 of 8
		14	0 of 8	8 of 8

† = No *C. jejuni* were isolated.

inoculating older poults, the level of CjCm enumerated from turkey cecal samples was substantially lower than recovered in experiment 1 or what we reported in younger poults colonized with wild-type isolates of *C. jejuni* (Sylte et al., 2018), or what is reported in chickens, where recovery of 10^8-10^9 *C. jejuni*/g of cecal contents was common (Buckley et al., 2010; Kobierecka et al., 2016; Nothaft et al., 2016; Wyszynska et al., 2004). Because animal experiments 1 and 2 were performed independently, we cannot exclude the possibility that changes in intestinal microbiota may be responsible for the differences in CjCm or

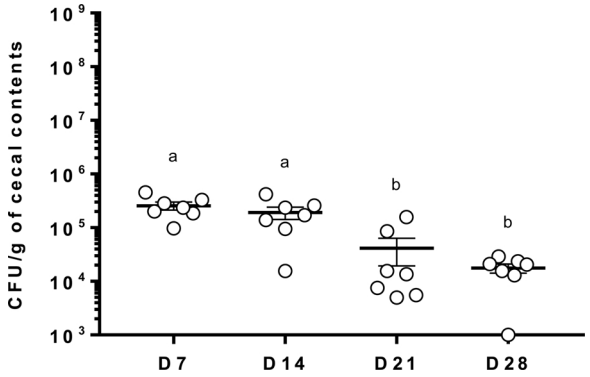

Fig. 4. Enumeration of antibiotic resistant construct CjCm from cecal contents of turkey poults from experiment 2. Data represent the mean *Campylobacter* cfu/g of cecal contents from each poult and the mean (solid bar) ± SEM for CjCm at days 7, 14, 21, and 28 post-colonization. Statistical differences in the number of enumerated *Campylobacter* cfu/g of cecal contents were determined using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($p < 0.05$) between treatments are represented by different letters.

CjK colonization in the different aged poults. It is not clear why both constructs failed to achieve persistent colonization in the cecum of young or older poults, compared to the wild-type strain (Sylte et al., 2018), but may involve an inability of *CmeF* deficient strains to survive stressors in the turkey intestinal tract. Mutants of NCTC 11,168 lacking *CmeF* were not susceptible to killing by bile salts (Akiba et al., 2006), suggesting that bile salts are not responsible for the decreased colonization in the present study. Growth curve analysis demonstrated significant changes ($P < 0.05$) in the area under the curve, generation time and growth rate for both antibiotic constructs grown in Bolton's broth base, when compared to the parent strain (Supplemental Figure S1), which may explain why the constructs colonized less *in vivo* than the parent strain. The antibiotic resistant constructs may be useful to study acute response after inoculation, studies less than 2 weeks in duration, in the composition of intestinal microbiota or host-response, but should not be considered for studies requiring persistent colonization (*e.g.*, testing pre-harvest reduction) in turkeys.

3.3. Acute response of intestinal tissues to CjCm or CjK colonization in turkeys

The immune changes in turkey tissues (*e.g.*, ileum, cecum, colon and cecal tonsil) from experiment 1 were evaluated for histological lesions after mock or colonization by CjCm or CjK. Comparing the changes across mock, CjCm and CjK tissues at different time points, CjCm induced a significant increase ($p < 0.05$) in histological scores at day 2 post-colonization (Fig. 5A), notably an increased number of heterophils in the cecal lamina propria. No significant differences in histological scores were detected within treatment groups on days 2, 7 and 14 post-colonization. Although mild morbidity (*e.g.*, watery or mucoid diarrhea) is reported after inoculating young chicks (1–8 days old) with *C. jejuni* (Sanyal et al., 1984; Welkos, 1984), very few histological lesions are observed in response to *Campylobacter* colonization in chickens. Overall, our histological findings were consistent with other experimental colonization studies in chickens. Accumulations of *Campylobacter*-like organisms were seen in between the cecal villi and the crypts from CjCm and CjK colonized animals (Fig. 5B), which was similar to localization described in experimentally colonized chicks (Beery et al., 1988). Immunohistochemistry for *Campylobacter* outer-membrane protein (Zhang et al., 2000) detected immunoreactivity between cecal villi in proximity to goblet cells, or as deep as the cecal crypt of three-weeks old poults inoculated with for seven days (Fig. 5B). *Campylobacter* immunohistochemical reactivity was less abundant in the cecum at day 2 (data not shown), but was maximal at day 7. Because *Campylobacter* spp. are commensals of the poultry intestinal microbiota, and significant lesion scores were not observed beyond day 2 of colonization, quantitative expression of host-genes may be a more sensitive means to assess host-response.

Quantitative RT-PCR was performed to assess fold changes in turkey gene expression of cytokines IL-1β, IL-6, IL-8, IL-10, IL-13, IL-17 A, IL-22, TGFβ2 and IFNγ, polymeric immunoglobulin receptor (pIgR), and mucin-2 (MUC-2) in cecal tissue of CjCm colonized relative to mock-colonized poults. The acute immune response to *Campylobacter*, within 3 days of colonization, may be critical period in which poultry become susceptible or resistant to *Campylobacter* colonization (Pielsticker et al., 2012). Expression of *IL-1β*, *IL-13* and *IL-10* were significantly down regulated ($p < 0.001$, $p < 0.01$ and $p < 0.01$ respectively) in the cecum 2 days after colonization (Fig. 6), as well as at day 7 after colonization. In contrast, expression of *IL-6*, *IL-8*, *IL-17 A*, *IL-22* and *IFN*γ were significantly up-regulated ($p < 0.05$) in the cecum at days 2 and colonization, and *IL-17 A* expression remained significantly up-regulated after 7 days of colonization (Fig. 6). Our results are similar to chickens, where *C. jejuni* colonization induced transient expression of cytokine genes (Awad et al., 2018; Connerton et al., 2018; Reid et al., 2016; Smith et al., 2008) and histological changes (*e.g.*, heterophil influx to cecal lamina propria) without inducing severe pathology in the cecum (Smith et al., 2008). Although the cellular source of these cytokines is unknown, it is possible that type 1 innate lymphoid cells (Annunziato et al., 2015), NK or γδ T cells may be the source of *IFN*γ expression. Type I innate lymphoid cells have not been described in turkeys, and reagents to detect NK or γδ T cells are not commercially available. Prolonged expression of IFNγ may enhance clearance of *Campylobacter* from chickens (Smith et al., 2008), and serve as a biomarker of protection in poultry. Chickens colonized with *C. jejuni* strain NCTC 11,168 showed a significant induction of *IL-17 A* expression, within 20 h of challenge (Shaughnessy et al., 2011), which is similar to our findings (Fig. 6). Similar to the IFNγ, the duration of *IL-17 A* expression in cecal tissue may help protect against colonization in chickens (Reid et al., 2016). The cellular source of *IL-17 A* expression is not known in turkeys, but is possibly expressed in type 3 innate lymphoid cells (Annunziato et al., 2015), TH_{17} (Harrington et al., 2005) or lymphoid tissue inducers-like cells (Takatori et al., 2009), which have not yet been described in turkeys. We detected significant levels of *IL-6* expression, which is similar to what was detected in *C. jejuni* colonized chickens (Shaughnessy et al., 2009). Overall, these data suggest that *C. jejuni* induced a brief pro-inflammatory response in the ceca of turkeys which resolved by day 14 after inoculation. It is unclear whether the immune response to CjCm in poults was affected by a lack of a persistent infection. The wild-type parental strain of *C. jejuni* (NCTC 11,168) used to make the constructs in the present study persistently infected the ceca of turkey poults for 21 days (Sylte et al., 2018). Future work will evaluate the immune response in poults experimentally colonized with wild-type strain NCTC 11,168 to address this concern. Although we hypothesized that inserting antibiotic resistance genes would enhance the enumeration of *C. jejuni* from turkey intestinal contents, it appears that CjCm and CjK were not well adapted for persistent colonization studies, such as vaccine efficacy. These constructs may be best adapted for *in vitro* work or animal studies where the goal is to study acute changes in host-response.

In conclusion, we report that antibiotic resistant *C. jejuni* constructs CjCm and CjK behave, *in vitro*, quite similar to the parent strain NCTC 11,168. Both constructs colonized the cecum, but not the ileum, after oral inoculation of young and older poults. Cecal colonization diminished over time, and was not persistent, as the organism was much lower at day 28 compared to day 7 post-inoculation. Significant histological changes and changes in cecal cytokine gene expression were detected early after colonization, but were the same as mock-colonized poults by 14 days of colonization. The results from these studies are the first to describe the host-response in turkeys to experimental *C. jejuni*

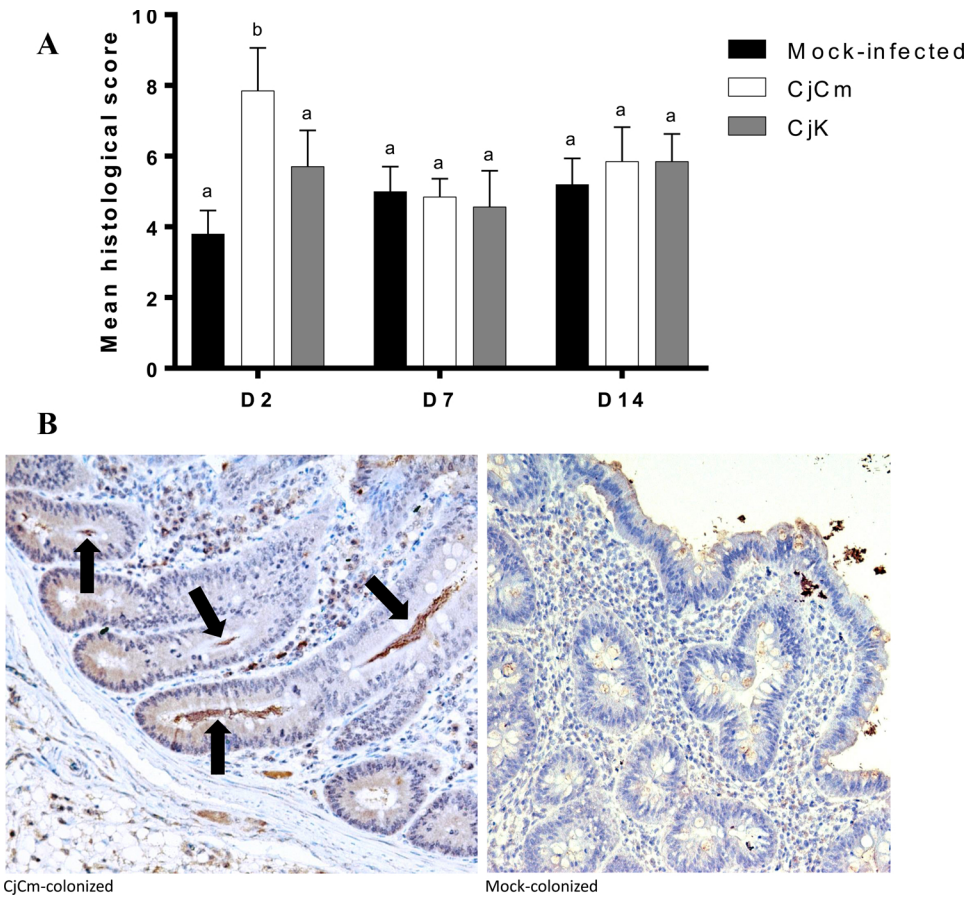

Fig. 5. Histological scoring of intestinal tissues following colonization by CjCm or CjK and *Campylobacter* immunohistochemistry. A) At days 2, 7 and 14 post-colonization in experiment 1, sections of ileum, ceca, cecal tonsils and colon were evaluated from each poult for presence of microscopic lesions. a unique grading score was used to evaluate changes of the intestinal histomorphology, including: 1) number of heterophils in the epithelium, lamina propria, and submucosa/muscularis as a possible indicator of inflammation and immune activation, 2) presence of small granulomas within the lamina propria, 3) crypt ectasia with or without heterophilic accumulation, 4) presence of apoptotic cells in the lamina propria, 5) areas with attenuated epithelium. Intermediate scores for each category (1 to 5) was obtained for the intestinal sections (ileum, cecal tonsils, ceca, and colon) of each bird. The final histological score of one poult was the sum of each intermediate scores. For each group, the final scores were averaged, and differences between means were determined using one-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($p < 0.05$) within time points are represented by different letters. B) Immunohistochemical analysis of *Campylobacter* outer membrane protein antigen in between cecal villi (black arrows) from poults in experiment one 7-days post-colonization from CjCm-colonized (L) or mock-colonization (R) (magnification = 200X). Data are representative of cecal samples from up to 5 different poults, CjCm- or mock-colonized.

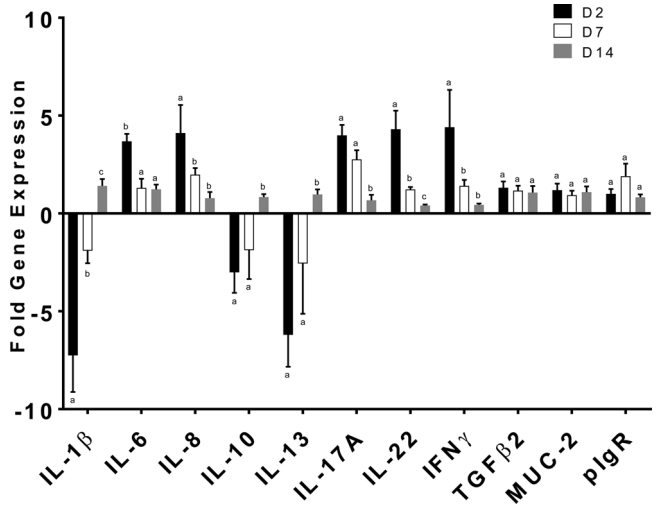

Fig. 6. Host-gene expression from cecal tissue after colonization with CjCm. qRT-PCR was used to determine fold expression of host genes relative to expression of the reference gene Ribosomal Protein S13 (RPS13) for each sample in CjCm and mock-colonized controls at days 2, 7 and 14 post-colonization. Data represent the mean fold ± SEM for 6 CjCm samples, relative to mock-colonized. Reactions for each gene, per time point were performed in triplicate. Statistical differences in the fold expression for each gene was determined using two-way ANOVA followed by a post-hoc multiple comparisons test (Tukey). Significant differences ($p < 0.05$) between different time points are represented by different letters.

colonization. Constructs CjCm and CjK may be best used to evaluate the acute response to *C. jejuni* colonization in turkeys, but may not be ideal for characterizing persistence in turkeys.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.02.003.

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