Campylobacter jejuni Colonization Is Associated with a Dysbiosis in the Cecal Microbiota of Mice in the Absence of Prominent Inflammation

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Abstract

Background: *Campylobacter jejuni* causes enterocolitis in humans, but does not incite disease in asymptomatic carrier animals. To survive in the intestine, *C. jejuni* must successfully compete with the microbiota and overcome the host immune defense. *Campylobacter jejuni* colonization success varies considerably amongst individual mice, and we examined the degree to which the intestinal microbiota was affected in mice (i.e. a model carrier animal) colonized by *C. jejuni* at high relative to low densities.

Methods: Mice were inoculated with *C. jejuni* or buffer, and pathogen shedding and intestinal colonization were measured. Histopathologic scoring and quantification of mRNA expression for α-defensins, toll-like receptors, and cytokine genes were conducted. Mucosa-associated bacterial communities were characterized by two approaches: multiplexed barcoded pyrosequencing and terminal restriction fragment length polymorphism analysis.

Results: Two *C. jejuni* treatments were established based on the degree of cecal and colonic colonization; *C. jejuni* Group A animals were colonized at high cell densities, and *C. jejuni* Group B animals were colonized at lower cell densities. Histological examination of cecal and colonic tissues indicated that *C. jejuni* did not incite visible pathologic changes. Although there was no significant difference among treatments in expression of mRNA for α-defensins, toll-like receptors, or cytokine genes, a trend for increased expression of toll-like receptors and cytokine genes was observed for *C. jejuni* Group A. The results of the two methods to characterize bacterial communities indicated that the composition of the cecal microbiota of *C. jejuni* Group A mice differed significantly from *C. jejuni* Group B and Control mice. This difference was due to a reduction in load, diversity and richness of bacteria associated with the cecal mucosa of *C. jejuni* Group A mice.

Conclusions: High density colonization by *C. jejuni* is associated with a dysbiosis in the cecal microbiota independent of prominent inflammation.

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Introduction

Campylobacter jejuni is a curved gram-negative motile bacterium, which is a common cause of foodborne enteritis in humans in the developed world [1,2,3]. Campylobacteriosis is characterized by fever, abdominal pain, watery to bloody diarrhea. In some instances, infected individuals may subsequently develop reactive arthritis, neurological disorders, or inflammatory bowel disease [4,5,6]. The bacterium readily colonizes a wide variety of animals asymptomatically (e.g. wildlife and livestock), and these animals may serve as a reservoir of infectious cells to humans [3,7,8]. Although *C. jejuni* is not considered to be a normal constituent of the intestinal microbiota of humans, a large number of asymptomatic humans were positive for the bacterium in developing countries [9]. Furthermore, a high number of individuals may be colonized by *C. jejuni* without exhibiting any clinical symptoms during outbreaks of the disease [1,10,11].

The mammalian intestinal tract harbours large numbers of bacterial cells (≈100 trillion) and hundreds of different species which are thought to prevent colonization and growth of many intestinal pathogens including *C. jejuni*, although the

mechanisms are poorly understood at present [6,12]. This phenomenon of microbiota-imparted resistance to colonization against pathogens is commonly known as "colonization resistance". In addition to colonization resistance, re-establishing eubiosis following host damage is also essential for pathogen clearance [13]. To successfully colonize a host, some intestinal pathogens alter the composition of host microbiota and this change in the microbiota is believed to be primarily due to the host inflammatory response [6,14,15,16,17]. However, an alteration in microbiota composition by means other than host inflammation (e.g. due to antibiotic administration or physiological stress) also facilitates colonization by intestinal pathogens [18,19,20,21]. The mechanisms by which the microbiota prevents colonization by bacterial pathogens is believed to be due the production of inhibitory substances (e.g. bacterial metabolites, bacteriocins), depletion of nutrients (which will be efficient in highly diverse and rich bacterial communities), and/or stimulation of the host immune system [22]. Conversely, it is also possible that a bacterial pathogen like *C. jejuni*, which readily colonizes the intestines of a diverse number of non-human mammals and avian species at high cell densities without inciting prominent inflammation [7,23,24], is able to affect the microbiota composition to allow it to persist in these hosts.

It is not currently known whether *C. jejuni* influences the composition of microbiota to facilitate colonization in asymptomatic animals or whether the microbiota from particular animal species is naturally amenable to high density colonization by *C. jejuni*. To examine the association between *C. jejuni* colonization in relation to the intestinal microbiota in an asymptomatic host, we chose mice as a mammalian model. *Campylobacter jejuni* typically colonizes mice without causing any illness [14,25,26,27,28,29,30], and like humans, mice are not consistently colonized by *C. jejuni*. However, once mice become colonized by *C. jejuni*, the bacterium can remain in high numbers within the intestine for prolonged periods similarly to other mammals and birds [14,29]. Furthermore, the intestinal microbiota of mice is often used as a model for the human enteric microbiota [22]. Ascertaining the degree to which the intestinal microbiota is altered subsequent to colonization by *C. jejuni* in an asymptomatic host is an important step toward elucidating the mechanisms by which this important enteric pathogen colonizes the intestines of mammals. We hypothesized that the enteric microbiota will differ in mice colonized by *C. jejuni* at high densities in the absence of inflammation.

Materials and Methods

Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research Centre (LRC) Animal Care Committee (Animal Use Protocol Review 0703) and the LRC Biosafety and Biosecurity Committee before commencement of the research. The stool sample of the human infected by *C. jejuni* NCTC 11168 was donated by the afflicted individual, and written informed consent was provided by the infected individual to isolate *C. jejuni* from their stool sample, and to genotype and utilize the recovered *C. jejuni* isolates in subsequent research.

Animals

Parent mice (C57/6J) were obtained from Jackson Laboratories (Bar Harbor, ME) and the mice were bred and reared using standard protocols. Twenty-two F_1 offspring at *ca.* 5 weeks of age were used in the experiment, and individual mice were randomly assigned to treatments. Mice were individually maintained in ventilated cages (Techniplast, Exton, PA), and provided with autoclaved feed (Prolab RM 3500, LabDiet, ON, Canada); animals were permitted to feed and drink *ad libitum*.

Inoculum

Campylobacter jejuni NCTC 11168 passed through a human was used. A human who had been working with NCTC 11168 developed severe enteritis, *C. jejuni* was isolated from a stool sample obtained from the afflicted individual, and it was genotyped using a 40 locus comparative genomic fingerprint method [31] which showed that it possessed an identical fingerprint pattern to NCTC 11168 (data not presented). Further, the strain of *C. jejuni* recovered from the stool sample was whole genome sequenced and compared to the whole genome sequence of the NCTC 11168 (i.e. the strain with which the human had been working), and was confirmed to be the same strain (data not presented). To produce inoculum, *C. jejuni* was grown in a microaerobic environment (10% CO_2, 3% H_2, 5% O_2, 82% N_2) at 37°C on Columbia agar (Oxoid, Nepean, ON) supplemented with 5% sheep blood for 16 hr. Cells were harvested in phosphate buffered saline (pH 7.2; PBS), and cell densities were adjusted to a final optical density (OD_{600}) of 0.5. This OD corresponded to a cell density of between 10^8 to 10^9 colony forming units (CFU) per ml. Cells were maintained on ice until used.

Inoculation of mice

To confirm that mice were free of *C. jejuni*, freshly voided feces was collected, genomic DNA was extracted, and *C. jejuni*-specific PCR targeting the *map*A gene was conducted [32]. Mice were arbitrarily divided into two groups. The first group consisted of 14 animals and they were inoculated with *C. jejuni* (*C. jejuni* group). The second group consisted of six animals that were inoculated with buffer alone (i.e. Control). The *C. jejuni*-inoculated group contained more animals to ensure that a sufficient number of animals were colonized by the bacterium. Mice were gavage inoculated with 100 µl of the suspension of *C. jejuni* or PBS alone. Mice were inoculated within 30 min of the collection of *C. jejuni* cells. To confirm densities of viable *C. jejuni* cells, inoculum was diluted in a 10-fold dilution series, 100 µl of each dilution was spread in duplicate onto Karmali agar (Oxoid), cultures were incubated at 37°C in a microaerobic atmosphere, and the number of *C. jejuni* colonies were counted at the dilution yielding 30 to 300 CFU after 48 and 72 hr of incubation. Aliquots of the inoculum were also examined microscopically for the presence of highly motile *C. jejuni* cells. After inoculation, mice were observed at

least once per day for behavioural signs of disease, weight loss, dehydration, fecal consistency, or any other clinical signs of disease.

Collection of feces and weighing of animals

Freshly voided fecal pellets were collected from each mouse 0.5, 7, 14, and 21 days post-inoculation (p.i.). Each sample was weighed, homogenized in 1 ml of PBS, the homogenate diluted in a 10-fold dilution series, and 100 µl of each dilution was spread in duplicate onto Karmali agar containing selective supplement SR167 (Oxoid). Cultures were maintained at 37°C in a microaerobic atmosphere, and the number of CFU determined as described above. Animals were also weighed each time feces were collected. Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC), and collection time was treated as a repeated measure. The appropriate covariance structure was utilized according to the lowest Akaike's Information Criterion.

Tissue collection and gross pathology

Mice were humanely euthanized 21 days p.i. Mice were first anesthetized with isofluorane gas (Halocarbon Products Corporation, River Edge, NJ) and then euthanized with an overdose of CO_2. Immediately after death, a midline incision was made, and the gastrointestinal tract (GIT) and associated tissues were exteriorized and observed for gross abnormalities (e.g. increased intestinal wall thickness, enlarged mesenteric lymph node). The entire stomach, the distal jejunum, and the entire ileum, cecum, and colon were aseptically removed from each mouse. The distal portion of each tissue segment (≈0.5 cm) was removed and processed for RNA extraction, DNA extraction, and histopathology. Residual ingesta was removed by gently submerging the tissues in sterile phosphate buffered saline, and bacteria retained after the gentle rinse were considered "mucosa-associated". Each tissue type (≈10 mg) was aseptically removed for DNA extraction, placed in 2.0 ml tubes on ice, and samples stored at -20°C (within 30 min of animal euthanization) until processed. For RNA extraction, cecal tissues were immediately immersed in RNAlater™ (Life Technologies, Burlington, ON) in 2.0 ml tubes (within 10 min of animal euthanization), and stored at -80°C until processed. For histopathologic examination, tissue segments were placed in 10% phosphate buffered formalin (Surgipath Canada Inc., Winnipeg, MB), gently agitated to remove ingesta, transferred to histological cassettes, and submerged in a fresh solution of phosphate buffered formalin. The proximal segment of each tissue segment was opened longitudinally, and examined closely for gross pathologic changes (e.g. congestion, presence of blood or abnormal quantities of mucus) after tissues had been collected for DNA and RNA extraction, and histopathologic examination.

Histopathology

Cecal and colonic samples were maintained in 10% buffered formalin for a maximum of 2 weeks. Tissues were dehydrated with ethanol and Histoclear (Fisher Scientific Inc, Toronto, ON, Canada), and paraffinized with Paraplast Plus (Fisher Scientific Inc.) for 2 hr at 60°C in a vacuum oven. Tissues were embedded using a Shandon Histocentre III (Fisher Scientific Inc.), sectioned (≈4 µm) using a Finesse 325 microtome (Fisher Scientific Inc.), and sections were placed on Superfrost Plus Gold slides (Fisher Scientific Inc.). Sections were de-paraffinized with xylene, stained with hematoxylin and eosin following a standard protocol, and examined for congestion, mucosal necrosis, neutrophils, macrophages and lymphocyte infiltration, goblet cell size and number, tissue congestion, lympholysis, and fibrosis. Histological inflammation scoring was performed in a "blinded" fashion (i.e. as to treatment) by a veterinary pathologist (RREU), with scoring criteria adapted from previously described methods [33,34]. With the exception of goblet cell size and number (scored 0 to 3), cecal and colonic tissues were graded from 0 (normal) to 4 (marked changes). In addition, a total score was calculated by summing scores of mucosal necrosis, neutrophil, macrophage and lymphocyte infiltration, goblet cell size and number, and fibrosis. As the data was categorical, treatments were compared non-parametrically using the NPAR1WAY procedure of SAS (SAS Institute Inc.) with the Wilcoxon rank-sum test. Total histopathologic scores were categorized as no effect to negligible changes (score of 0 to 4), mild to moderate changes (score of 5 to 9), and marked changes (score of 10 to 19).

RNA extraction and quantification of α-defensin, toll-like receptor and cytokine mRNA expression

Total RNA was extracted from cecal tissue using the RNeasy mini protocol for isolation of total RNA from animal tissues and the RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer's recommendations. RNA was checked for quality and quantity by electrophoresis. Any contaminating DNA was removed by DNase digestion (Qiagen Inc.). RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen Inc.). An in-house qPCR array (384 well format) was used to quantify the mRNA expression of the following genes: cryptdin peptide (cryptdin) 4; cryptdin 5; cryptdin 20; toll-like receptor (TLR) 2; TLR4; TLR5; TLR9; interferon (INF)-γ; interleukin (IL)-1β; IL-4; IL-5; IL-6; IL-10; IL-17A; IL-22; tumor necrosis factor (TNF)-α; and TNF-β. The housekeeping genes Actb (β-actin), B2m (β-2-microglobulin), GusB (β-glucuronidase), Ldha16a (lactate dehydrogenase A), and Ppia (peptidylprolyl isomerase A) were evaluated. Of these genes, three stably expressed housekeeping genes (Actb, B2m, and Ppia) were selected via geometric averaging [35]. Published primers for IL-10 were used [36]. All other primers were designed using Primer 3 and reference gene sequences within the National Center for Biotechnology Information (NCBI) website. Primers were designed to produce a single amplicon between 140-160 base pairs (bp) in size, and to have a Tm of 60°C. Quantitech SYBRgreen (Qiagen Inc.) real-time PCR was completed using an ABI 7900HT thermocycler (Applied Biosystems, Burlington, ON). PCR conditions were 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec. Reverse transcription and genomic DNA controls were included. A four point five-fold standard curve for each gene was included for the calculation of amplification efficiencies. Following amplification, melt curve analysis was conducted to confirm amplification specificity. All reactions

were run in triplicate, and the mean value of the observations was used for analysis. Normalized gene expression was calculated using qbasePLUS (Biogazelle, Zwijnaarde, Belgium) based on geNorm and qBase quantification models [35,37], and $\log_{10}$-transformed data were analyzed using the one-way analysis of variance feature within the program.

DNA extraction

DNA was extracted from cecal tissue using a RTP Bacteria DNA Mini Kit (Invitek, Berlin, Germany), according to the manufacturer's instructions. Concentrations of DNA were quantified spectrophotometrically. DNA was stored at -80°C until utilized.

Intestinal colonization by *Campylobcter jejuni*

Densities of *C. jejuni* and total bacteria associated with mucosa of the stomach, jejunum, ileum, distal colon were determined by quantitative PCR targeting the *map*A and 16S rRNA gene, respectively; PCR efficiency, optimum primer concentration, and dynamic range were determined in advance. DNA was diluted to reduce the concentration of any PCR inhibitors present. The SYBR Green-based standard curve method for quantification of DNA was carried out using Power SYBR® Green PCR (Life Technologies). Each 20 µl PCR reaction contained 2 µl of DNA (20-50 ng), 10 µl of the 2X Power SYBR® Green PCR Master Mix, and 200 nmol of each of the forward and reverse primers. For the quantification of *C. jejuni*, the QCjmapANF and QCjmapANR primers were used [38]. For quantification of total bacteria, the HDA1 and HDA2 primers were used [39]. Standard curves were established using genomic DNA from *C. jejuni* or *Escherichia coli* (ATCC 25922). DNA copy number varied from 10^1 to 10^7; as there are seven copies of the 16S rRNA gene in *E. coli* ATCC 259229, the number of 16S rRNA gene copies in the standard curve were adjusted accordingly. Samples were amplified as follows: one cycle at 95°C for 10 min; and 40 cycles at 95°C for 15 sec, and at 60°C for 60 sec. A Stratagene Mx 3005 (Stratagene Products, La Jolla, CA) was used. All reactions were run in triplicate, and the mean value of the observations was used for analysis. The number of bacteria was expressed as copy number per gram of tissue. For all reactions, melt curve analysis was conducted to confirm amplification specificity. Data were analyzed using a one-way analysis of variance using the MIXED procedure of SAS (SAS Institute Inc.). In conjunction with a significant F-test, the lsmeans function of SAS was used to compare treatments.

Sequence-Based Bacterial Community Analysis

The basic bacterial tag-encoded FLX 454-pyroseqencing (bTEFAP) procedure was performed as described previously [20,40,41]. Briefly, DNA from each cecal sample was diluted to a final concentration 2 ng μl^{-1}. An initial 30-cycle PCR was performed to amplify a 512 bp region of the 16S rRNA gene spanning variable regions V1 to V3 using Gray28F (5′-GAGTTTGATCNTGGCTCAG -3') and Gray519r (5′-GTNTTACNGCGGCKGCTG-3') [41] with HotStar high fidelity Taq polymerase (Qiagen, Valencia, CA). The resulting PCR product was used as template in a second PCR reaction with fusion primers [42]. PCR products from different samples were barcoded and bTEFAP was completed using a Roche 454 FLX instrument (Roche, Nutley, NJ) with Titanium reagents at the Research and Testing Laboratory (Lubbock, TX). Raw data was processed to remove sequences less than 200 bp, and sequences containing homopolymers greater than 8 bp, mismatches in the barcode or primer, one or more ambiguous bases, or an average quality score below 30 over a moving window of 50 bp. The remaining sequences were aligned to the SILVA-based bacterial reference alignment [43] using of Needleman-Wunsch algorithm [44]. Potential chimeric sequences were removed using UCHIME [45], sequencing noise was reduced by applying a preclustering step, and sequences assigned to the Cyanobacteria lineage were removed. The cleaned pyrotag data was processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline [46]. The 'uclust' method within QIIME was used to cluster Operational Taxonomic Units (OTUs) at a 97% similarity level, and the RDP classifier was applied to classify OTU's at an 80% confidence level. Richness, Chao1 estimates, Shannon's index, and phylogenetic diversity were calculated by sample and treatment. For calculation of alpha diversity metrics, the lowest number of sequences per sample within individual treatment groups was used; 2892, 1916, and 2057 for *C. jejuni* Group A, *C. jejuni* Group B, and Control samples, respectively. For pairwise t-tests, data was normalized to 1916 for all samples. The heat map was generated for each sample by treatment; only OTUs for which ten or more sequences were observed were included in the heat map. Principal coordinate cluster analysis (PCoA) was conducted using the Unifrac distance metric on weighted (normalized abundance values) and unweighted datasets that were subsampled to an even depth. The R package (Available: http://www.r-project.org/. Accessed 2013 Jul 09) was used for data visualization. To statistically compare community compositions between treatments, pairwise analysis of similarity (ANOSIM) was performed (i.e. weighted and unweighted) using Vegan (999 permutations) [47]. Sequences were accessioned in GenBank (NCBI) under: SRR933603 (A1); SRR933605 (A2); SRR933606 (A3); SRR933608 (A4); SRR933609 (A5); SRR934203 (A6); SRR934204 (B1); SRR934205 (B2); SRR934206 (B3); SRR934207 (B4); SRR934208 (B5); SRR934209 (B6); SRR934210 (C1); SRR934211 (C2); SRR934212 (C3); SRR934213 (C4); SRR934214 (C5); and SRR934215 (C6).

Fingerprint-based bacterial community analysis

The basic terminal restriction fragment length polymorphism (T-RFLP) and analysis protocol described by Costa et al. [48] was used. The primers 27f and 1492r [49] were used to amplify the 16S rRNA gene in 10 ng of cecal DNA. The forward primer was labeled with FAM (FAM27f). Each reaction consisted of 2 µl of genomic DNA (≈10 ng), 2.0 µl of 1X PCR buffer, 0.1 µl of each deoxynucleoside triphosphate (0.2 mM), 2.0 µl of acetylated bovine serum albumin (BSA; Promega, Madison, WI; 0.1 µg μl^{-1}), 0.1 µl of Taq DNA polymerase (Qiagen, Inc.; 5 units μl^{-1}), 1.0 µl each of the bacterial primers (0.5 µM), and 11.5 µl Optima water (Fisher Scientific, Ottawa, ON). PCR

conditions were 95°C for 15 min, 25 cycles consisting of 94°C for 30 sec, 53°C for 60 sec, and 72°C for 60 sec, and a final extension period at 72°C for 10 min. All PCR reactions were performed in triplicate, and pooled before restriction digestion. All amplicons were electrophoresed in a 1% TAE agarose gel relative to a 100 bp DNA ladder (Promega). The target amplicon of ~1500 bp was purified using a QIAquick PCR purification Kit (Qiagen, Inc.), and DNA concentrations were quantified using a TD 360 Mini Fluorometer (Turner Designs, Sunnyvale, CA) and TNE / Hoescht dye buffer. If required, DNA concentrations were also quantified by agarose gel electrophoresis. Concentrations of DNA in all samples were standardized to 25 ng μl^{-1} using Optima water. Restriction digestions were carried out in duplicate in a mixture containing 75 ng of the purified PCR product, 3 units of HaeIII (Life Technologies), 2.5 µl of enzyme buffer, and Optima water to a final volume of 25 µl. Samples were incubated at 37°C for 2 hr in the dark, and ethanol precipitation was performed to stop the reaction by adding 50 µl of 95% ethanol and 2 µl of sodium acetate (pH 5.2) to each sample. Samples were incubated for 20 min at 20°C, and centrifuged for 20 min (13,200 x g) to pellet DNA. Nucleic acids were washed by adding 500 µl of 70% ethanol, followed by centrifugation at 13,200 x g for 5 min. After ethanol precipitation, samples were air dried overnight in the dark, re-suspended in 9.25 µl of Hi Di formamide (Applied Biosystems Canada, Streetsville, ON) and 0.25 µl of LIZ600 size standard marker (Applied Biosystems Canada), denatured at 95°C for 3 min, and immediately placed on ice. Fluorescent labeled terminal restriction fragments (T-RFs) were separated in POP7 polymer using a 3130 Genetic Analyzer (Applied Biosystems Canada), and analyses were performed on T-RFs ranging in size from 50 to 580 bp covering V1 to V3 of the 16S rRNA gene. Electropherograms were analyzed using GeneMapper software version 4.0 with the Local Southern size calling method (Applied Biosystems Canada) as described previously [48]. Euclidean distance, and Pearson and Dice coefficients were calculated to cluster animals into groups, and the clusters were linked together by unweighted pair-group using the centroid average (UPGMA) and Ward's method within the Bionumerics software (Applied Maths, Austin, TX). The statistical significance of each group was tested by comparing between group similarities with randomization tests using 1000 iterations (Applied Maths Inc.) [48]. To further explore the composition of bacterial communities, non-metric multi-dimensional scaling (NMS) was applied using SAS (SAS Institute Inc., Cary, NC), and three dimensional NMS plots were graphed using SigmaPlot (Systat Software Inc., Chicago, IL).

Results

Two groups of mice were observed based on Intestinal colonization by *Campylobacter jejuni*

The group of mice gavaged with *C. jejuni* consisted of more animals than the *C. jejuni*-free Control treatment to account for inconsistent intestinal colonization by *C. jejuni* amongst individual mice [30,50]. Four animals in the *C. jejuni*-inoculated group were either not colonized or were colonized at very low densities (<100 CFU g^{-1} of feces), and *C. jejuni* was successfully cleared by these animals during the experimental period. Since we were interested in ascertaining the effects of *C. jejuni* colonization on the host microbiota over longer periods (i.e. 21 days), we excluded these four animals from subsequent analysis. In the remaining inoculated mice, *C. jejuni* was detected in the feces of all individuals over the 21-day experimental period (Table 1). The *C. jejuni* inoculated group was divided into two distinct groups based on colonization density (i.e. *C. jejuni* Group A, and *C. jejuni* Group B). Campylobacter jejuni Group A mice shed significantly larger numbers of *C. jejuni* cells (~4 orders of magnitude) in feces than did *C. jejuni* Group B mice throughout the experimental period (Table 1). The Group A mice also had higher numbers (~2 orders of magnitude) of *C. jejuni* cells associated with mucosa within their ceca than *C. jejuni* Group B mice (Table 2). The same pattern of *C. jejuni* colonization was observed in colon (Table 2). Based on colonization patterns, the following three treatment groups were established: (1) *C. jejuni* Group A consisted of six mice colonized by high densities of *C. jejuni*; (2) *C. jejuni* Group B consisted of six mice colonized by lower densities of the bacterium; and (3) the Control consisted of six animals devoid of *C. jejuni* (18 mice total).

Table 1. Log_{10} *C. jejuni* CFU g^{-1} of mice feces (mean ± standard error of the means).

Group	7 days p.i.	14 days p.i.	21 days p.i.
C. jejuni Group A[a]	8.91 ± 0.09 a[b]	8.28 ± 0.36 a	7.88 ± 0.43 a
C. jejuni Group B	4.72 ± 0.26 b	4.41 ± 0.17 b	3.16 ± 0.28 b
Control	0.0	0.0	0.0

a. Group A mice and Group B mice were inoculated with *C. jejuni*, whereas Control mice were gavaged with buffer alone.

b. Means not followed by the same letter within columns differ (P<0.05).

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Table 2. Copy number (Log_{10} g^{-1}) of mucosa-associated *C. jejuni* in ceca and colons, and total bacteria in ceca of mice (mean ± standard error of the means).

Group	Cecum – *C. jejuni*	Colon – *C. jejuni*	Cecum - Total
C. jejuni Group A[a]	8.80 ± 0.07 a[b]	7.07 ± 0.11 a	10.43 ± 0.11 a
C. jejuni Group B	6.42 ± 0.19 b	5.41 ± 0.15 b	10.78 ± 0.12 b
Control	0.0	0.0	10.79 ± 0.06 b

a. Group A mice and Group B mice were inoculated with *C. jejuni*, whereas Control mice were gavaged with buffer alone.

b. Means not followed by the same letter within columns differ (P≤0.05).

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Absence of inflammation and no impact on growth of mice colonized by *Campylobacter jejuni*

No clinical signs of illness (e.g. diarrhea, malaise), increased mucus production, intestinal distension, or gross evidence of inflammation or lesions were observed in any of the mice regardless of whether they were inoculated with *C. jejuni*.

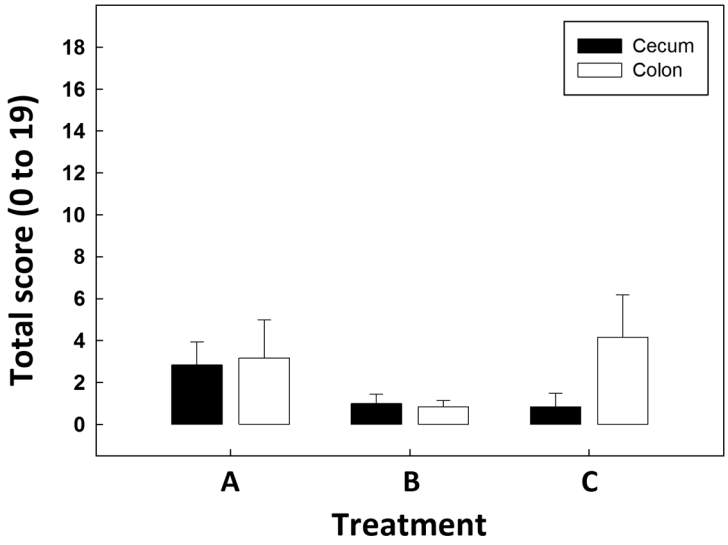

Figure 1. Histopathologic changes. Mean histological scores of cecal and colonic tissues for combined necrosis, neutrophils, macrophages and lymphocytes, and fibrosis, and goblet cell size and number. Treatments are: (A) *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); (B) *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and (C) control (not inoculated with *C. jejuni*). Vertical lines associated with histogram bars are standard error of the means (n=6). There were no significant differences for cecal (P≥0.20) or colonic (P≥0.34) tissues among treatments. Scores were categorized as no effect to negligible changes (score of 0 to 4), mild to moderate changes (score of 5 to 9), and marked changes (score of 10 to 19).
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Table 3. Weekly increase in body weight (g) of mice (mean ± standard error of the means).

Group[a]	7 days p.i.	14 days p.i.	21 days p.i.
Group A	2.39 ± 0.41 a[b]	1.56 ± 0.63 b	0.82 ± 0.23 c
Group B	2.60 ± 0.34 a	1.18 ± 0.33 b	0.71 ± 0.19 c
Control	2.74 ± 0.41 a	0.92 ± 0.29 b	0.64 ± 0.18 c

a. Group A and Group B mice were inoculated with *C. jejuni*, whereas Control mice were gavaged with buffer alone.

b. Means not followed by the same letter within columns differ (P<0.05).

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Microscopically, very low scores (≤1) were observed for congestion, changes in goblet cell size and number, tissue congestion, and lympholysis (data not shown). There were no significant differences (P≥0.12) in mucosal necrosis, neutrophil infiltration, macrophage and lymphocyte infiltration, or fibrosis in the cecum or colon among the three treatments (data not shown). Furthermore, there was no difference (P≥0.20) in total histopathology scores among treatments for either location (Figure 1). Mean total histopathology scores were ≤2.8 ± 1.1 and ≤4.2 ± 2.0 for the cecum and distal colon, respectively; a score of 4.0 or less indicates negligible changes. In addition, no significant difference (P>0.05) was observed in growth, measured as weekly increase in body weight among treatments (Table 3).

Cecal colonization by *C. jejuni* did not significantly affect α-defensin, toll-like receptor, or cytokine mRNA expression

No amplification of cryptdin 4 was detected, and no difference (P≥0.52) was observed in mRNA expression of the α-defensins, cryptdin 5, and cryptdin 20 among the three treatments. There also was no difference (P≥0.12) among treatments in the regulation of mRNA for toll-like receptor genes (TLR2, TLR4, TLR5, and TLR9) (Figure 2). However, a trend for increased expression of mRNA was observed in cecal tissue from *C. jejuni* Group A mice for TLR4 and TLR9. No amplification of mRNA for the Treg cytokine, TNF-β, the Th17 cytokines, IL-17A and IL-22, or the Th2 cytokines, IL-4 and IL-5 was observed. Although not significant (P≥0.056), a trend for up regulation of IL-1β, IL-6, IL-10, INF-γ, and TNF-α mRNA was observed for *C. jejuni* Group A mice (Figure 3).

Campylobacter jejuni colonization was associated with a dysbiosis in the cecal microbiota

In the current study, analyses of the microbiota were limited to the mucosa-associated microbiota of the cecum. A decision was made to focus on the cecum because *C. jejuni* readily and persistently colonizes this region of the GIT in mice [29]. The total bacterial load in cecum was significantly (P<0.05) reduced in *C. jejuni* Group A mice. In contrast, no difference (P<0.05) in total number of bacteria associated with the cecal mucosa of *C. jejuni* Group B and Control mice was observed (Table 2).

Two methods were used to characterize the mucosa-associated microbiota of the cecum. Pyrosequence analysis targeting the variable region of the 16S rRNA gene spanning

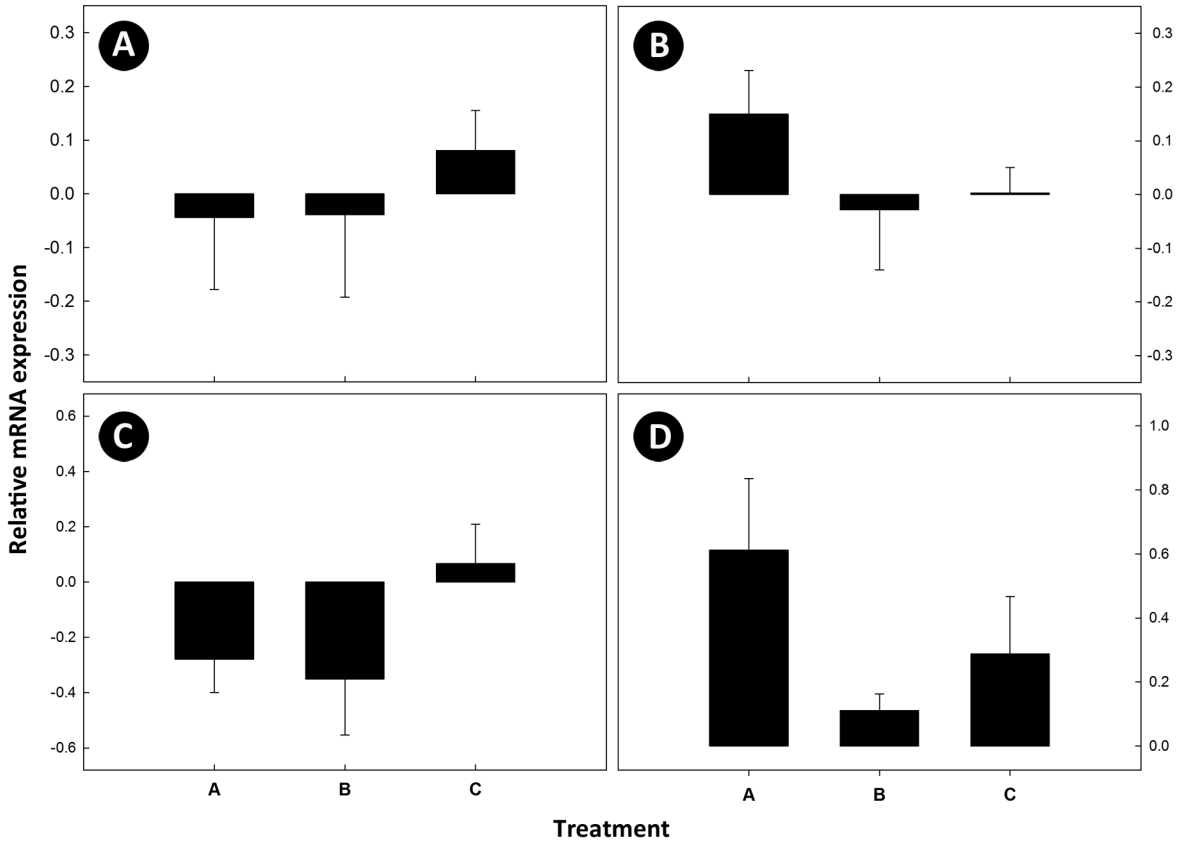

Figure 2. Messenger RNA expression of toll-like receptors. Relative mRNA expression of toll-like receptors (TLR) in cecal tissue where: (A) TLR2; (B) TLR4; (C) TLR5; and (D) TLR9. Treatments are: (A) *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); (B) *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and (C) control (not inoculated with *C. jejuni*). Vertical lines associated with histogram bars are standard error of the means (n=6).
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the V1, V2, and V3 regions was applied. At a 97% sequence identity delineation for species [20,51], 571 OTUs were observed for the 87,492 total sequences processed (i.e. before normalization). For all animals, rarefaction curves did not asymptote (Figure 4). The richness of communities associated with the cecal mucosa of *C. jejuni* Group A mice was reduced (P=0.015) relative to *C. jejuni* Group B and Control mice (Table 4; Figure 4). A trend for decreased diversity of bacterial communities was also observed in *C. jejuni* Group A mice (Figure 4). The composition of the mucosa-associated cecal microbiota differed conspicuously between the three treatments (Figure 5), and bacterial communities in the ceca of *C. jejuni* group A mice formed distinct clades (P ≤ 0.005) from *C. jejuni* group B and Control mice (Table 5). Consistent with culture- and qPCR-based enumeration results (Tables 1-2), substantially more *C. jejuni* OTU were measured in DNA from the cecal mucosa of *C. jejuni* Group A (494.2 ± 395.5) relative to *C. jejuni* Group B (10.5 ± 21.9) mice (Figure 6). Firmicutes were the most prevalent (66.2-90.4%) group of bacteria detected. A comparison of OTU prevalence by treatment revealed a decrease in the occurrence of OTU 2, 10, 14, 29, 40, 44, 59, 76, 106, 109, 129, 148, 193, 224, 237, 252, 281, 293, 317, 334, 335, 371, 394, 460, 470, 496, 513, 514, 539, 551, and 563 in *C. jejuni* Group A relative to *C. jejuni* group B and Control mice (Figure 7; Table S1); with the exception of OTU 40, 109, 252, and 334 which was unidentified beyond the Kingdom level, all of these OTU were clostridia (*Coriobacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae*). An increase in the frequency of a number of OTU was also observed in *C. jejuni* Group A mice (4, 15, 21, 34, 42, 57, 143, 206, 216, 221, 312, 323, 340, 346, 368, 374, 390, 438, 439, 440, 441, and 560). With the exception of OTU 390 (*C. jejuni*) and 206 (unidentified), all were Firmicutes (Clostridia and Erysipelotrichi).

A second method (T-RFLP fingerprint analysis) was applied to characterize bacterial communities associated with the mucosa of mice ceca. Similarly to pyrosequence-based analysis, T-RFLP analysis indicated that the cecal microbiota of *C. jejuni* Group A mice clustered separately (P≤0.005) from both *C. jejuni* Group B and Control mice (Table 5; Figure 8).

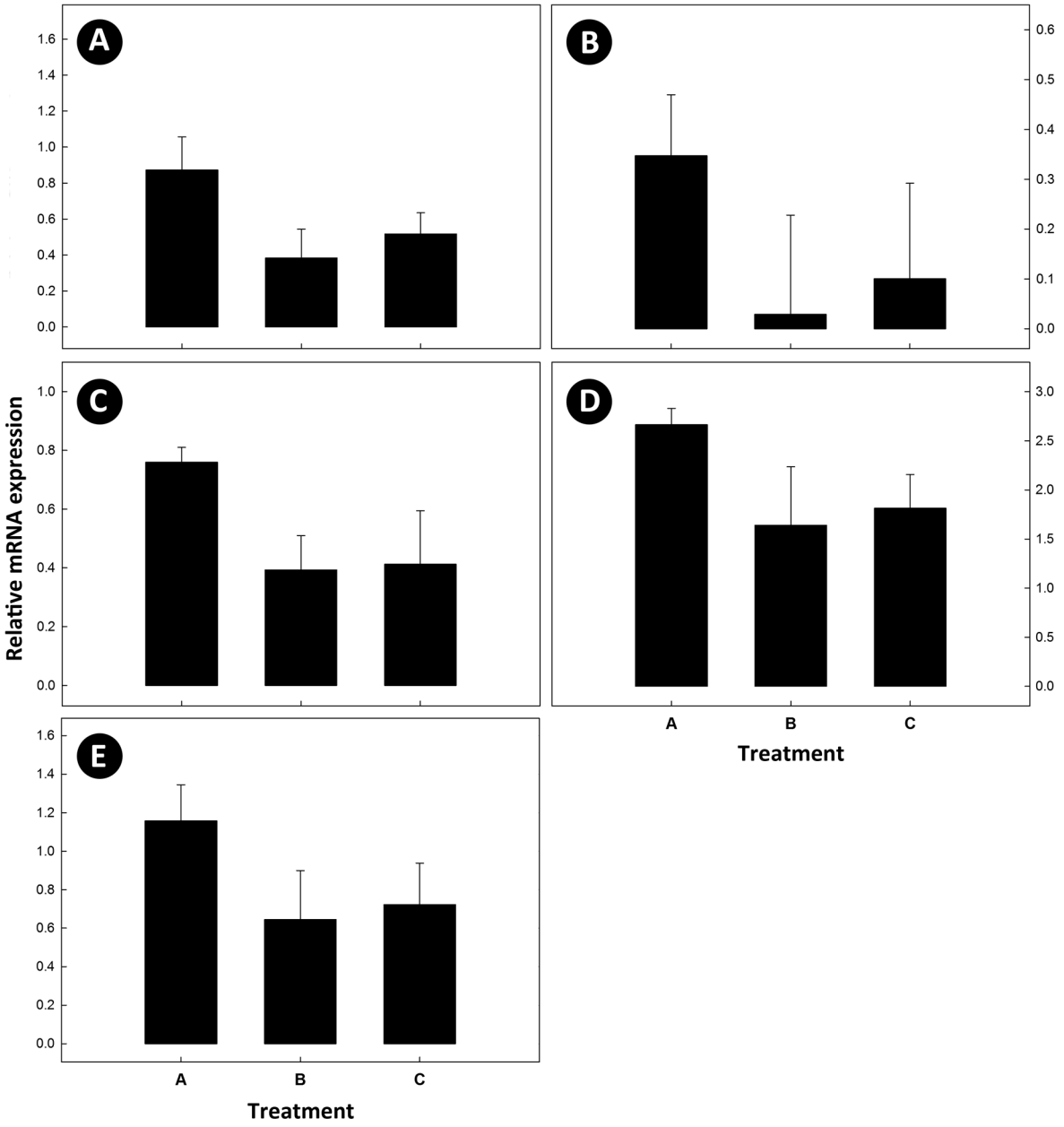

Figure 3. Messenger RNA expression of cytokines. Relative mRNA expression of cytokines in cecal tissue where: (A) interleukin (IL)-1β; (B) IL-6; (C) IL-10; (D) interferon-γ; and (E) tumor necrosis factor-α. Treatments are: (A) *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); (B) *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and (C) control (not inoculated with *C. jejuni*). Vertical lines associated with histogram bars are standard error of the means (n=6).
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Discussion

The role of the intestinal microbiota on *C. jejuni* colonization is poorly understood, particularly in asymptomatic *C. jejuni* carrier animals. We chose to use mice as a model given *C. jejuni* does not naturally colonize the intestine of mice, and hence it is not adapted to the murine GIT ecosystem [14]. However, *C. jejuni* readily colonizes the intestine of mice with a simplified or altered microbiota [6,26,52,53], and large doses of *C. jejuni* are typically required to experimentally colonize mice possessing a naturally-acquired microbiota [29,50,54]. Both of these observations suggest that *C. jejuni* may affect the composition of the microbiota in order to successfully colonize the GIT of asymptomatic mammals.

To study the *C. jejuni*-microbiota interaction, we examined the microbiota associated with the cecum, specifically the

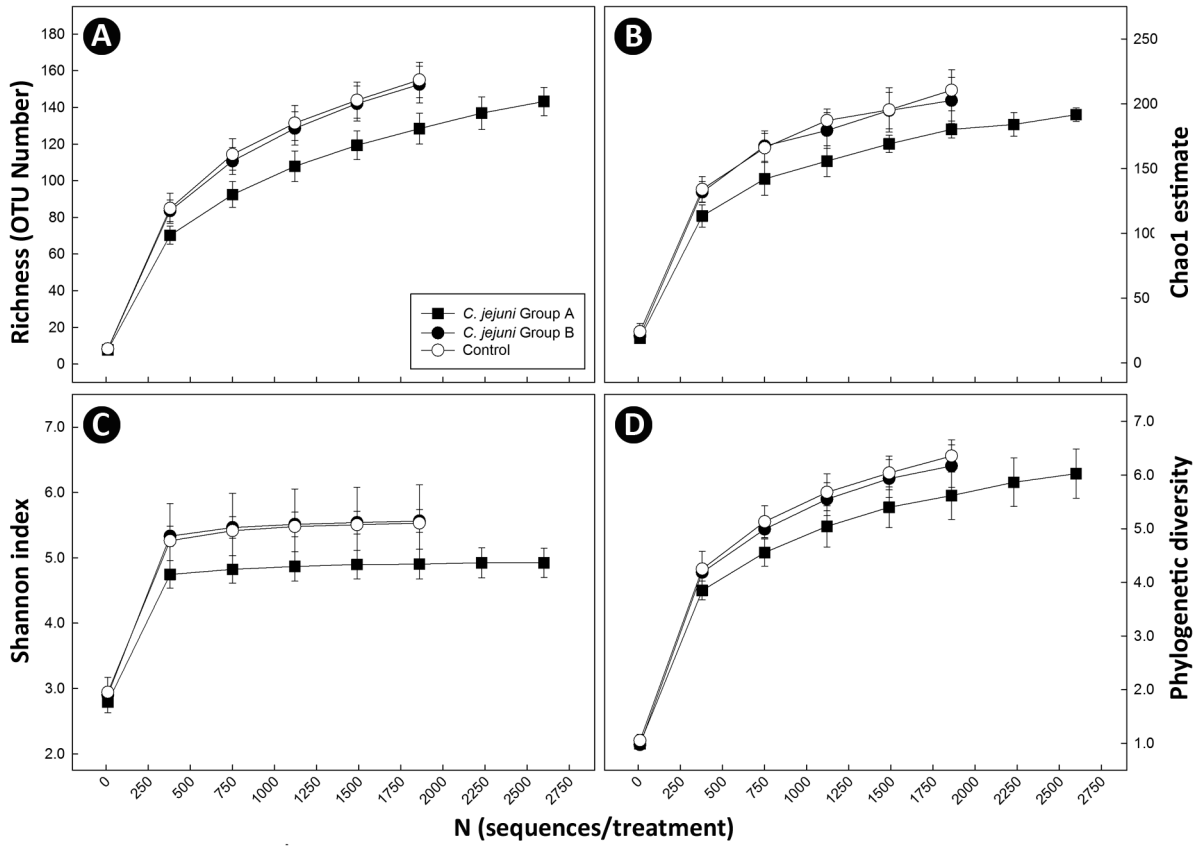

Figure 4. Richness and diversity of bacterial communities. (A) Richness; (B) Chao1 diversity; (C) Shannon diversity; and (D) phylogenetic diversity. Treatments are: (A) *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); (B) *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and (C) control (not inoculated with *C. jejuni*). Vertical lines associated with markers are standard error of the means (n=6).

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mucosa-associated microbiota, because the cecum is a preferred site of colonization by *C. jejuni* in mice [29], as well as in poultry [55]. Substantial densities of *C. jejuni* cells were observed in association with the mucosa of the cecum in all the inoculated mice included in the study. In contrast, *C. jejuni* was not detected or detected at low cell densities in the stomach, jejunum and ileum. The bacterium was also consistently detected in the distal colon, but at lower densities than in the cecum. We specifically targeted the mucosa-associated microbiota because luminal contents (ingesta) are not necessarily representative of the localized microbiota, and at any particular location within the intestinal lumen, ingesta carries microorganisms from the proximal regions of the GIT. Thus, examination of the luminal microbiota may provide an inaccurate representation of the localized microbiota. Additionally, bacteria within ingesta in the intestinal lumen encounter a different micro-environment than bacteria closely associated with the mucosal surface which are influenced by host factors to a much greater degree [56].

We observed two distinct groups of mice based on the density of *C. jejuni* cells shed in feces and associated with the cecal and colonic mucosa; one group of mice was colonized by *C. jejuni* at a much higher density than the second group. *Campylobacter jejuni* colonization in mice varies amongst individuals, and *C. jejuni* strains exhibit inconsistent and highly variable colonization ability [30,57]. For example, ≈10^5 fold variation in *C. jejuni* NCTC 11168 densities was observed within cecal ingesta amongst C57BL/6J IL-10 deficient mice 7 days p.i. [30]. Similarly, we frequently observe variable

Table 4. Probability values from pairwise comparisons of bacterial richness and diversity[a].

Treatment group[b]	Richness	Chao1	Shannon	Phylogenetic
Group A vs Group B	0.015	0.096	0.003	0.228
Group A vs Control	0.015	0.012	0.156	0.042
Group B vs Control	1.000	1.000	1.000	1.000

a. Pairwise t-tests were conducted on data was normalized to 1916 sequences per sample.

b. Group A and Group B mice were inoculated with *C. jejuni*, whereas Control mice were gavaged with buffer alone

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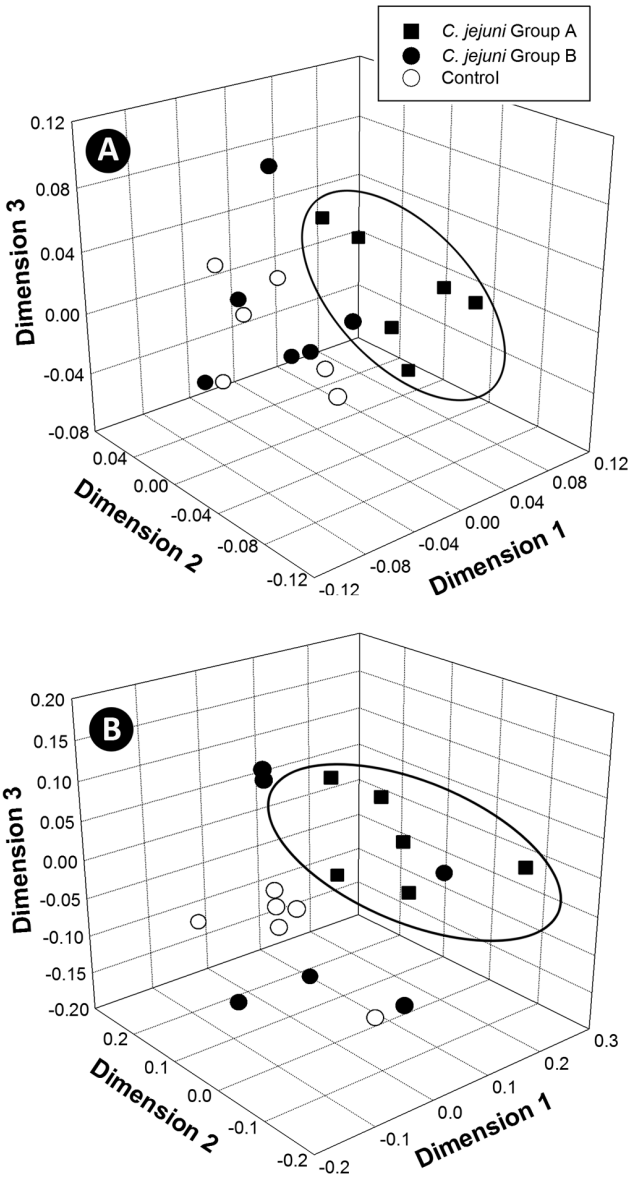

Figure 5. Principal coordinate cluster plots of bacterial communities. Plots depict community composition similarities based on pyrosequence analysis of: (A) weighted; and (B) unweighted datasets subsampled to an even depth. Treatments are: *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and control (not inoculated with *C. jejuni*). The ellipsoids show predominant clustering of bacterial communities in *C. jejuni* Group A mice relative to *C. jejuni* Group B and control mice.

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colonization of C57BL/6J mice between and within *C. jejuni* strains, including NCTC 11168 (unpublished).

All methods to characterize the intestinal microbiota possess strengths as well as weaknesses [58]. Thus, we applied two methods to characterize the mucosa-associated microbiota within the cecum of mice (pyrosequence and T-RFLP). Sequence-based analysis of microbial community composition showed that bacterial diversity was high in all samples, and rarefaction curves did not asymptote indicating that not all the taxa present in the community were represented. However, microbial community rarefaction curves, particularly for diverse communities within the GIT typically do not saturate even at high levels of coverage provided by pyrosequencing [59,60].

Table 5. Probability values from pairwise cluster analyses of bacterial communities.

Treatment group[a]	Pyrosequence[b]		T-RFLP
	(Weighted)	(Unweighted)	(T-RF Presence)
Group A vs Group B	0.001	0.005	0.002
Group A vs Control	0.001	0.003	0.005
Group B vs Control	0.442	0.303	0.106

a. Group A and Group B mice were inoculated with *C. jejuni*, whereas Control mice were gavaged with buffer alone.

b. Analysis of similarity was performed on weighted and unweighted pyrosequence datasets (subsampled to an even depth).

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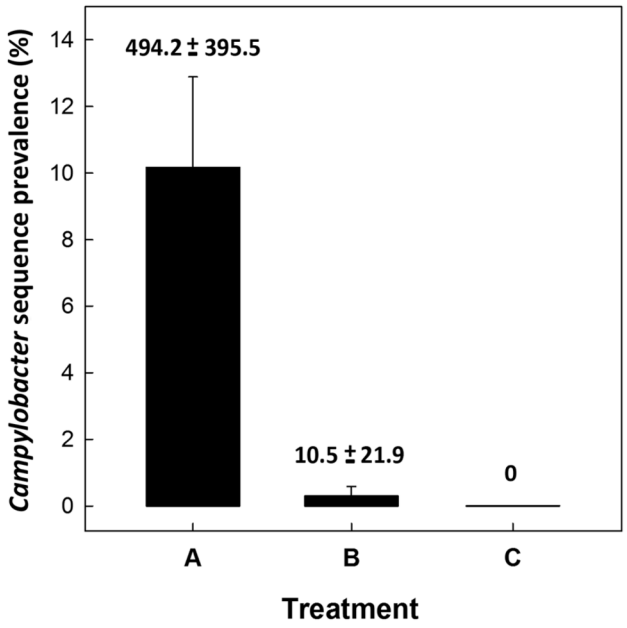

Figure 6. Prevalence of *Campylobacter jejuni* sequences. Prevalence of sequences (%) identified as *C. jejuni* by treatment. Treatments are: (A) *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); (B) *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and (C) control (not inoculated with *C. jejuni*). Vertical lines associated with histogram bars are standard error of the means (n=6). Number associated with histogram bars are the mean number of sequences (± standard error of the means) that were identified as *C. jejuni* by treatment.

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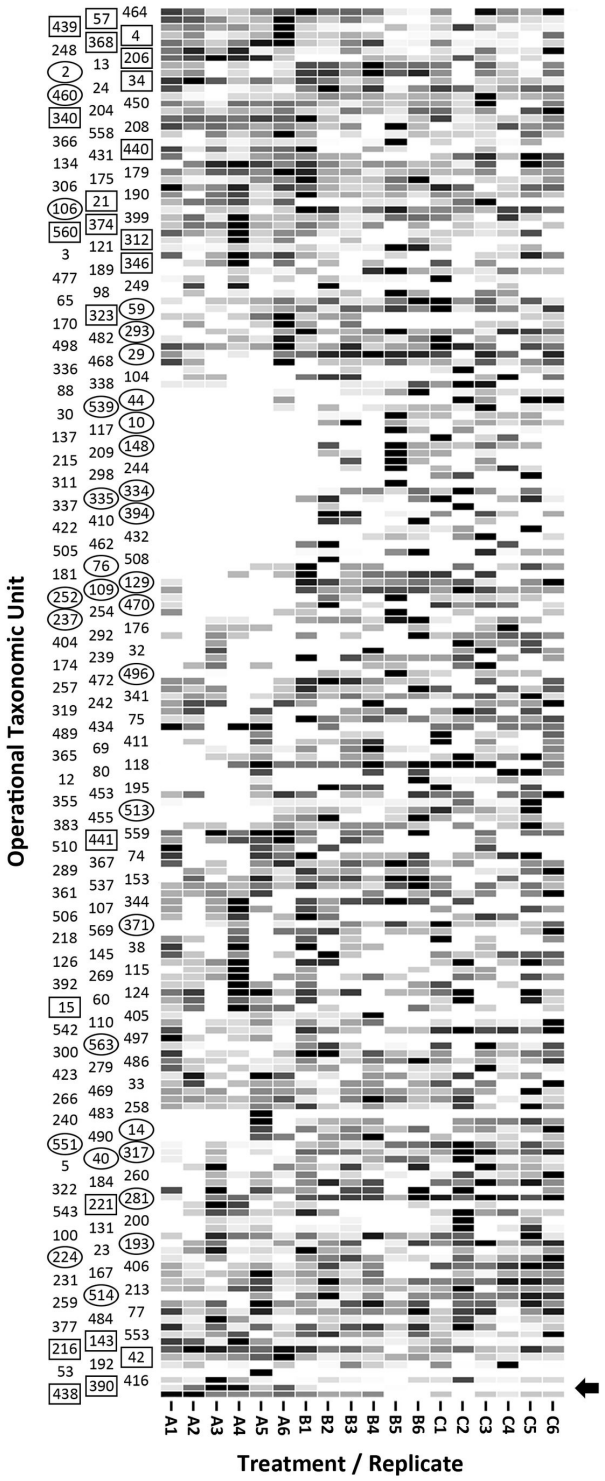

Figure 7. Heat map of sequence frequencies. The map shows the relative prevalence of 183 OTUs by mouse within treatment (i.e. OTUs in which ten or more sequences were observed). Treatments are: (A) *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); (B) *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and (C) control (not inoculated with *C. jejuni*). The arrow indicates the OTU corresponding to *C. jejuni* (i.e. #390). OTU within circles represent OTU that occur conspicuously less frequently in *C. jejuni* Group A relative to *C. jejuni* Group B and Control mice. OTU within boxes represent OTU that occur conspicuously more frequently in *C. jejuni* Group A relative to *C. jejuni* Group B and Control mice. A list of taxonomic classifications by OTU is available in Table S1.

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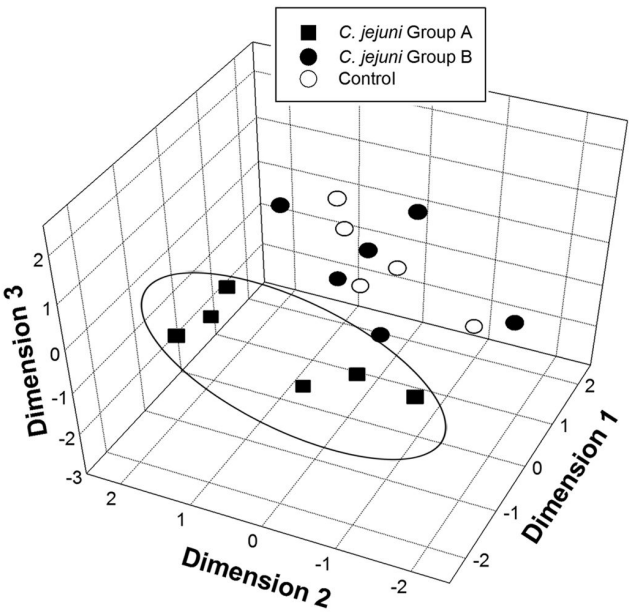

Figure 8. Non-metric multi-dimensional scaling plots of bacterial communities. Plots depict community terminal restriction fragment (T-RF) T-RF presence/absence of bacteria associated with mucosa within the cecum of mice. Treatments are: *C. jejuni* Group A (8.8 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); *C. jejuni* Group B (6.4 $\log_{10}$ copy number of *C. jejuni* g^{-1} of cecal tissue); and control (not inoculated with *C. jejuni*). The ellipsoid shows clustering of bacterial communities in *C. jejuni* Group A mice relative to *C. jejuni* Group B and control mice.

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Sequence based-analysis grouped the cecal mucosa-associated microbiota of mice colonized at a high cell density of *C. jejuni* cells (Group A) as a distinct cluster. In contrast, there was no difference in the composition of bacterial community between mice colonized by *C. jejuni* at the lower density (Group B) and *C. jejuni*-free (Control) animals. Thus, the composition of the cecal microbiota of *C. jejuni* Group A mice was distinct from the other two treatments. The particular composition of bacterial communities might be responsible for differential colonization of *C. jejuni* in dissimilar animal species, but the high degree of inter-individual variability typically observed within a particular species is problematic [61]. Our data contrasts with an earlier report that concluded that *C. jejuni* colonization of the mouse intestine did not significantly affect bacterial load or the composition of the enteric microbiota within the colons of IL-10 deficient mice [14]; however, *C. jejuni* colonization density was not specifically considered, and characterization of the microbiota was limited to enumeration of a limited number of bacterial groups using fluorescence *in situ* hybridization. Although not significant, they did observe a trend for reduced bacterial load [14]. We observed that the mucosa-associated bacterial community was dominated by Firmicutes, and a number of Clostridia OTU (primarily *Coriobacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae*) were either less or more frequently observed in mice colonized by *C. jejuni* at high densities. The role of these bacteria in colonization resistance warrants further investigation.

To confirm the pyrosequence-based community composition results, T-RFLP analysis was applied as an alternate method (e.g. to address the possibility that the high frequency measurement of *C. jejuni* sequences in *C. jejuni* Group A mice skewed richness, diversity, and composition metrics). Although the T-RFLP method is not capable of identifying constituents of the community, it is a method that generates highly reproducible community fingerprints that facilitates rapid and cost-effective comparative characterization of communities [62,63]. In this regard, Pilloni et al. [64] observed that T-RFLP analysis was able to recover the same amplicon pools from environmental samples, and yielded highly comparable overall microbial community patterns to pyrosequencing, but may underestimate diversity. The application of the T-RFLP method confirmed that the composition of the mucosa-associated microbiota in ceca of mice colonized by a high density of *C. jejuni* cells (Group A) was distinct. The findings of our study clearly demonstrate that high density colonization by *C. jejuni* was associated with a dysbiosis in the cecal microbiota.

The dysbiosis that we observed may have been incited by *C. jejuni*, or may have resulted from unknown event(s) that caused a dysbiosis thereby permitting *C. jejuni* to colonize the cecal mucosa at high densities. Host factors, including differential immune competence can influence the composition of the microbiota within individual animals [65,66]. We exercised care to ensure that mice were treated identically and randomly assigned to treatments. Furthermore, the composition of the microbiota of all Control mice grouped together. Collectively, this suggests that *C. jejuni* directly affected the cecal microbiota. Inflammation incited by other enteric pathogens has been documented to influence the composition of the intestinal microbiota [14,17,67]. However, *C. jejuni* did not incite prominent inflammation in the current study. This was evident by the absence of clinical signs, and gross and microscopic indications of intestinal inflammation. In addition, we did not detect a statistically significant difference in growth rate over the experimental period between *C. jejuni*-infected and Control mice. Furthermore, we did not observe an increase in the prevalence of *Enterobacteriaceae* bacteria which is often observed in association with inflamed intestines [6,14]. Similarly to previous reports [14,27,28,29,30], we observed that *C. jejuni* did not incite prominent intestinal inflammation in mice (e.g. histopathologic changes). Total histopathologic scores were ≤4.2 in the current study, whereas total scores exceeding 10 (i.e. marked changes) are recorded in C57/6J mice with acute enteritis [33]. Consistent with this observation, non-significant differences were observed among treatments in the expression of mRNA for α-defensins, toll-like receptors, or cytokines. Of note, increases in expression of cytokine mRNA (e.g. INF-ɣ, TNF-α, IL-2) exceeding five-fold are typically observed in mice with acute enteritis [33,68,69]. Although α-defensins (termed cryptdins in mice) are primarily expressed in Paneth cells concentrated in the small intestine, Paneth cells can also be present in cecum and colon [70]. Expression of α-defensins can modulate intestinal inflammation

and tissue injury, as well as the microbiota [71,72,73]. Our data showed that Cryptdin 4, 5, or 20 are not induced by *C. jejuni*, and that they do not play a role in facilitating cecal colonization in mice. Although non-significant, we observed trends of differential regulation of toll-like receptor and cytokine genes in mice colonized at a high cell density by *C. jejuni* (Group A) relative to mice in other treatments. The toll-like receptors, TLR4 and TLR9 are important molecules for the recognition of lipopolysaccharide moieties of Gram negative bacteria and unmethylated CpG rich regions of bacterial DNA respectively, and the modest increase in gene expression observed in the current study is consistent with other research that identified enhanced TLR signalling with the induction of immunopathology due to *C. jejuni*-infection in knockout mice [53]. Furthermore, it is known that *C. jejuni* modulates the expression of proinflammatory cytokines in a variety of murine models consistent with our findings [6,53,74,75,76]. *Salmonella enterica* has been shown to exploit the inflammatory response to compete with the enteric microbiota [16,17,77,78,79]. Our data suggests that *C. jejuni* incites a low-grade inflammation response as a colonization strategy in asymptomatic hosts, but the bacterium is unable to do so in all individuals due to unknown factors. Besides exploiting the host, it is also possible that *C. jejuni* affects the microbiota or that constituents of the microbiota affect *C. jejuni* independent of the host, as has been observed for other pathogenic bacteria [80,81,82,83]. In conclusion, we observed that high density colonization of the cecum by *C. jejuni* was associated with a dysbiosis in the cecal microbiota independent of prominent inflammation. Although our research identifies a unique aspect by which *C. jejuni* impacts on the host and the intestinal microbiota, future research to elucidate the mechanisms is warranted.

Supporting Information

Table S1. Identities of operational taxonomic units (OTUs). See Figure 6 for the relative frequency of individual OTUs by sample within treatments (i.e. OTUs for which ten sequences or more were observed).
(DOCX)

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Author Contributions

Conceived and designed the experiments: AGL LBS GDI. Performed the experiments: AGL RREU GDI. Analyzed the data: AGL RREU YX GDI. Contributed reagents/materials/ analysis tools: LBS GDI. Wrote the manuscript: AGL RREU YX GDI. Maintained mice, collected tissues, quantified C. jejuni colonization, and conducted microbiota characterizations: AGL. Scored histopathologic changes: RREU. Conducted immunologic analyses: RREU GDI. Conducted statistical analyses: AGL GDI. Conducted pyrosequence data analyses: YX. Critically reviewed and revised the manuscript for important intellectual content: LBS RREU GDI. Obtained animal care committee and biosafety approval, provided technical support, and supervised the study: GDI.

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