The Intestinal Microbiota Influences *Campylobacter jejuni* Colonization and Extraintestinal Dissemination in Mice

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Campylobacter jejuni is a leading cause of human foodborne gastroenteritis worldwide. The interactions between this pathogen and the intestinal microbiome within a host are of interest as endogenous intestinal microbiota mediates a form of resistance to the pathogen. This resistance, termed colonization resistance, is the ability of commensal microbiota to prevent colonization by exogenous pathogens or opportunistic commensals. Although mice normally demonstrate colonization resistance to *C. jejuni*, we found that mice treated with ampicillin are colonized by *C. jejuni*, with recovery of *Campylobacter* from the colon, mesenteric lymph nodes, and spleen. Furthermore, there was a significant reduction in recovery of *C. jejuni* from ampicillin-treated mice inoculated with a *C. jejuni* virulence mutant (Δ*flgL* strain) compared to recovery of mice inoculated with the *C. jejuni* wild-type strain or the *C. jejuni* complemented isolate (Δ*flgL/flgL*). Comparative analysis of the microbiota from nontreated and ampicillin-treated CBA/J mice led to the identification of a lactic acid-fermenting isolate of *Enterococcus faecalis* that prevented *C. jejuni* growth *in vitro* and limited *C. jejuni* colonization of mice. Next-generation sequencing of DNA from fecal pellets that were collected from ampicillin-treated CBA/J mice revealed a significant decrease in diversity of operational taxonomic units (OTUs) compared to that in control (nontreated) mice. Taken together, we have demonstrated that treatment of mice with ampicillin alters the intestinal microbiota and permits *C. jejuni* colonization. These findings provide valuable insights for researchers using mice to investigate *C. jejuni* colonization factors, virulence determinants, or the mechanistic basis of probiotics.

Campylobacter jejuni is a leading cause of human gastroenteritis worldwide. *C. jejuni* is a Gram-negative pathogen that grows in low-oxygen (3 to 5%) environments, including the digestive tracts of animals. *C. jejuni* regularly colonizes commercial chicken flocks, and human disease is usually linked to the ingestion of food cross-contaminated with raw or undercooked poultry. The clinical symptoms for *C. jejuni*-mediated enteritis in humans include diarrhea with blood and leukocytes in the stool, leading to exsiccosis and electrolyte loss, fever, nausea, and abdominal cramps (1, 2). Individuals infected with *C. jejuni* may be treated with antibiotics, including erythromycin or ciprofloxacin (1, 2). Infection also increases the risk of developing Guillain-Barré syndrome, which is currently the leading cause of flaccid paralysis (3).

The human intestinal microbiota is comprised of hundreds of distinct bacterial species, bacteriophages, archaea, and fungi (4). The intestinal microbiota of healthy mammals is typically dominated by organisms from the phyla *Firmicutes* (Gram-positive bacteria) and *Bacteroidetes* (Gram-negative bacteria) (5). Collectively, the intestinal commensal microbiota provides the host with numerous physiological benefits, including vitamin synthesis, tissue integrity, digestion, fermentation of proteins and polysaccharides, bile salt metabolism, and stimulation of the immune system (6). One additional physiological benefit of the intestinal microbiota is the enhancement of host immune defenses by inhibiting growth of potentially pathogenic microorganisms (colonization resistance). Colonization resistance prevents pathogens from establishing a niche and inhibits the outgrowth of opportunistic pathogens (7).

Mice vary in their susceptibilities to *C. jejuni* and can be either completely resistant to colonization or only transiently infected.

Mice devoid of intestinal microbiota (germfree) and mice with a defined microbiota (gnotobiotic) have been shown to be more susceptible to *C. jejuni* colonization than mice with normal intestinal microbiota. For example, *C. jejuni* effectively colonizes germfree mice and disseminates to immune tissues, including the mesenteric lymph nodes (MLN) (8–10). However, germfree mice demonstrate altered lymphoid development, resulting in an impaired immune response (11–13). There are documented instances that mice are susceptible to colonization with *C. jejuni* (14, 15). However, many researchers have experienced difficulty in obtaining *C. jejuni* colonization of mice unless the animals have been treated with an antibiotic prior to challenge to alter the intestinal microbiota (8, 10). To this end, mice treated with a five-antibiotic cocktail over the course of 6 weeks have been shown to be more susceptible to *C. jejuni* (16). Transplanting fecal material

containing either human or mouse microbiota into these germ-free mice demonstrated that mice given human microbiota were more susceptible to *C. jejuni*-mediated disease than mice given mouse microbiota (16). Thus, it is known that the murine intestinal microbiota impacts *C. jejuni* colonization as mice with limited flora are also more susceptible to *C. jejuni* (14). Collectively, these results suggest that the murine intestinal microbiota is comprised of microorganisms that specifically inhibit *C. jejuni* colonization.

In this study, we evaluated the contribution of resident microbiota in CBA/J mice. We found that all animals were colonized with *C. jejuni* following treatment with a single antibiotic (ampicillin), as measured by *C. jejuni* burden in the colon, spleen, and mesenteric lymph nodes (MLN). The intestinal microbiota of untreated and ampicillin-treated animals was examined by both culture-dependent and culture-independent methods. We recovered an isolate of *Enterococcus faecalis* from the murine intestine that inhibited *C. jejuni* growth *in vitro* and reduced *C. jejuni* colonization of mice. Additionally, deep sequencing of DNA extracted from murine fecal pellets revealed that the microbial community of the intestine influenced resistance to *C. jejuni* colonization, as a decrease in representatives of the *Firmicutes* phylum and an increase in representatives of the *Bacteroidetes* phylum were found in the animals treated with ampicillin. This study provides a simple method to alter murine intestinal microorganisms, thereby changing susceptibility to *C. jejuni* colonization of mice, and may be applicable for additional *in vivo Campylobacter* models.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *C. jejuni* F38011 wild-type clinical strain, the Δ*flgL* mutant, and the Δ*flgL/flgL* complemented isolate were used throughout this study. The strains were cultured under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) on Mueller-Hinton (MH) agar plates or in MH broth (Thermo Fisher Scientific, Hanover Park, IL) supplemented with 5% citrated bovine blood at 37°C and passaged to a fresh plate at least once each 48 h. *Lactobacillus acidophilus* NCFM and the murine-isolated *E. faecalis* were cultured on deMan-Rogosa-Sharpe (MRS) agar plates or in MRS broth (Thermo Fisher Scientific) under anaerobic conditions (86% N_2, >13% CO_2, <0.7% O_2) at 37°C. Isolates recovered from intestinal samples were serially diluted and plated on MH agar plates or Luria-Bertani (LB) agar plates (Thermo Fisher Scientific) under microaerobic conditions or on MRS agar plates under anaerobic conditions at 37°C.

***C. jejuni* inoculation of mice, necropsy, and bacterial quantification.** Animal experiments were conducted according to National Institutes of Health (NIH) guidelines under supervision by the Washington State University (WSU) Animal Care and Use Committee (ASAF 04313). CBA/J mice (females, 6 to 8 weeks old) were obtained commercially from Harlan (Harlan Laboratories, Indianapolis, IN) and maintained in a specific-pathogen-free colony in microisolator cages at WSU (≤5 animals/cage). Mice were inoculated with *C. jejuni* (~10^{10} CFU/ml in 200 μl of phosphate-buffered saline [PBS]), and control animals were given sterile PBS (uninfected) by oral gavage and observed daily for clinical signs of disease. In preliminary experiments, animals were administered 200 μl of antibiotics by oral gavage at 48 and 24 h prior to oral challenge with *C. jejuni* at the following concentrations: 1 mg/ml ampicillin (Fisher BioReagents, Fair Lawn, NJ), 1 mg/ml metronidazole (Acros Organics, Morris Plains, NJ), 50 μg/ml novobiocin (Sigma, St. Louis, MO), 20 μg/ml streptomycin (Sigma), and 15 μg/ml trimethoprim (Sigma). Based on the data obtained from preliminary experiments, ampicillin treatment was chosen for the remainder of the experiments. Mice were euthanized and necropsied promptly when clinical signs of disease developed or at 2, 7, and 35 days postinfection (dpi). The colon, spleen, and MLN of mice were re-moved aseptically and resuspended in 1 ml of MH broth. The number of *C. jejuni* bacteria in each organ was determined by plating serial dilutions on Campy Cefex plates. Intestinal samples were collected by excising 1-in. ileum sections. Each section was initially put in a tube containing 0.5 ml of RPMI culture medium supplemented with 1× penicillin and streptomycin (Life Technologies Corporation, Carlsbad, CA). All samples were adjusted to obtain equal weight per unit volume prior to cytokine/chemokine analysis. The second sample was placed in 10% formaldehyde for histopathological analysis. For experiments using CBA/J mice reconstituted with inhibitory bacteria, mice were treated with ampicillin at 48 h and 24 h prior to administration of inhibitory bacteria, as described above. Following ampicillin treatment, 200 μl (~10^9 CFU/ml) of *E. faecalis* bacteria was administered to a group of mice, and *L. acidophilus* was administered to another group of mice by oral gavage at 24 h and 12 h prior to challenge with *C. jejuni*.

Cytokine/chemokine analysis. Cytokines/chemokines were assessed in the *ex vivo* distal ileum sections following 24 h of incubation at 37°C with 5% CO_2. Interleukin-6 (IL-6) and CXCL2/MIP-2 were assessed using a mouse IL-6 OptEIA kit (BD Biosciences, San Jose, CA) and mouse CXCL2/MIP-2 DuoSet (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions, respectively.

Gross pathology and histopathology. Assessment of gross pathology was performed using a rubric, as described elsewhere (17). Histopathology was assessed from paraffin-embedded tissue sections (5 μm) mounted on slides and visualized by microscopy by a board-certified pathologist blinded to the samples using a Nikon Eclipse at a ×200 magnification at Washington State University's Washington Animal Disease Diagnostic Laboratory.

Isolation and sequence typing of inhibitory microbiota in CBA/J mice. Culture-dependent microbiota analysis was performed using the bacteria recovered from the intestinal contents of mice with and without ampicillin treatment. DNA was extracted from individual colony isolates, and PCR of the 16S rRNA subunit was performed using the 27F primer (5′-AGAGTTTGATCMTGGCTCAGAACG-3′) (18) and 1435R primer (5′-CGATTACTAGCGATTCCRRCTTCA-3′) (Kelly A. Brayton, personal communication), where M is A or C and R is A or G. The primers used for sequencing included the 27F primer, 1435R primer, 533F primer (5′-GTGCCAGCMGCCGCGGTAA-3′) (19), and 519R primer (5′-GTA TTACCGCGGCTGCTGG-3′) (20). Sequences were trimmed and analyzed by BLAST for identification and submitted to the National Center for Biotechnology Information (NCBI).

Multilocus sequence typing of *E. faecalis* was performed using primers for *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL*, as described elsewhere (21).

HPLC analysis of lactic acid production. A chromatographic analysis method was used to determine lactic acid levels in MH broth (22, 23). The samples were analyzed via high-performance liquid chromatography (HPLC) by a standard addition method using a Hitachi L-6220 pump system with an AS-4000 auto-sampler and a PerkinElmer LC-85B UV-visible light (Vis) detector (analysis wavelength of 212 nm) controlled by Hitachi D-6000 HPLC manager software (Hitachi High Tech Americas, Inc., Schaumburg, IL). A total of 250 μl of the respective sample of growth medium was diluted with MiliQ water alone or with an addition of an appropriate aliquot of lactic acid standard to a final volume of 1.5 ml. Three samples of each growth medium were prepared for the standard addition: a diluted medium, a diluted medium with 500 mg/liter lactic acid, and a diluted medium with 1,000 mg/liter lactic acid. Each individual solution was measured in triplicate. For each analysis, 10 μl was injected onto an Acclaim Organic Acid column (5-μm particle size, 120-Å pore size, 4.0 by 120 mm) from Dionex (Thermo Scientific, Waltham, MA). An isocratic elution method was used with a mobile phase of 0.100 M Na_2SO_4 at a pH of 2.65 (adjusted by addition of methane sulfonic acid) and a flow rate of 0.6 ml/min at room temperature.

Determining the microbiota in CBA/J mice. Culture-independent intestinal microbiome analysis was performed using fresh fecal pellets collected prior to and following antibiotic treatment of 15 individual

CBA/J mice. The pellets were flash frozen. Genomic DNA extraction was performed using a MoBio UltraClean soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) and an MP FastPrep instrument (MP Biomedicals, Solon, OH) set at 40 s for rapid cell lysis. Each of the individually extracted DNA samples was quantified using a high-sensitivity Qubit kit (Life Technologies), and 15 ng of DNA was PCR amplified using specific bar-coded primers around the V3-V5 16S rRNA, as described previously (24). Amplified samples were sequenced using a 16S rRNA high-throughput next-generation Illumina MiSeq (Illumina, San Diego, CA) sequencing platform. A total of 42,537,848 sequence reads were generated and found suitable for further analysis. Sequence reads were aligned using the Illinois-Mayo Taxon Operations for RNA Data Set Organization (IM_TORNADO), and paired-end reads were used for determination of operational taxonomical units (OTU), as previously described (25). The OTUs were then analyzed using QIIME software for taxonomy comparison between treatment groups (26). Specific analyses included rarefaction on all samples to ensure equality of sequence number for subsequent comparisons of beta diversity (principal-component analysis [PCoA], Shannon's index, and taxonomical summary).

Statistical analysis. All *in vitro* experiments consisted of at least three replicates performed on separate days to demonstrate reproducibility. Within each experiment, the samples were measured in triplicate. Results are presented as means ± standard errors of the means (SEM). Statistical analyses were performed using GraphPad Prism, version 6 (La Jolla, CA), and statistical significance was measured at a P value of ≤0.05. A nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) followed by *post hoc* Dunn's multiple comparisons or Tukey's test of the means was used for all *in vivo* assays. Results are presented as means ± SEM, and statistical significance was measured at a P value of ≤0.05. To determine differences between animals treated with ampicillin, permutational multivariate analysis of variance (PERMANOVA) or Student's t test was applied to determine statistical significance and to calculate a two-tailed P value, with a P value of ≤0.05 considered significant, using Primer, version 6, statistical software (Primer-E, Ltd., United Kingdom).

Ethics statement. All animal work was performed using protocols approved by the Institutional Animal Care and Use Committee (IACUC; protocol no. 04313) at WSU. All efforts were made to raise and euthanize the animals humanely.

Sequence data accession numbers. The 16S rRNA sequence of *E. faecalis* KLC3001 is available in GenBank under accession number KP942841. The 16S rRNA gene sequences used to determine the microbiota in CBA/J mice are available in the NCBI Sequence Read Archive (SRA) under accession number SRP057511.

RESULTS

Ampicillin-treated CBA/J mice are efficiently colonized with *C. jejuni*. The intestinal microbiota of mice provide colonization resistance to *C. jejuni*. We tested whether antibiotics could alter the community of microorganisms to allow *C. jejuni* to colonize more efficiently. In preliminary experiments, mice were treated with a panel of antibiotics, including ampicillin, metronidazole, novobiocin, streptomycin, and trimethoprim, and tested for colonization resistance to *C. jejuni*. At 7 days postinfection (dpi), we were able to recover *C. jejuni* from mice treated only with ampicillin (data not shown). Furthermore, *C. jejuni* bacteria were not recovered from mice not given antibiotics.

Based on our preliminary findings, we investigated *C. jejuni* colonization in ampicillin-treated CBA/J mice. CBA/J mice are widely used as a general-purpose strain and have been shown to elicit an inflammatory response to enteric pathogens (27, 28). Ampicillin-treated CBA/J mice were challenged with the *C. jejuni* wild-type strain, a Δ*flgL* mutant, the Δ*flgL*/*flgL* complemented mutant or PBS (uninfected). Bacterial burden and markers of intestinal inflammation were assessed at 2, 7, and 35 dpi (Fig. 1). In

C. jejuni, *flgL* encodes a flagellar hook-filament junction protein. We included the *C. jejuni* Δ*flgL* mutant in our analysis because it does not produce a functional flagellum. This mutant should be attenuated in mice as *C. jejuni* utilizes the flagellum for both motility and secretion of virulence proteins that are delivered to host epithelial cells (29, 30).

The *C. jejuni* wild-type strain was recovered from the colon, spleen, and MLN at all time points, with the exception that no organisms were recovered from the spleen at 35 dpi, indicating that ampicillin treatment promotes stable colonization in CBA/J mice. Although not all of the mice inoculated with the *C. jejuni* Δ*flgL* mutant completely cleared the infection, there were significantly fewer *C. jejuni* bacteria recovered from the colons of mice infected with the mutant than from the colons of those infected with the wild-type strain (Fig. 1). The colons of animals infected with either the *C. jejuni* wild-type strain or the Δ*flgL*/*flgL* complemented isolate showed signs of edema and apparent stool softening in contrast to uninfected mice or mice given the Δ*flgL* mutant (see Fig. S1 in the supplemental material). However, histopathological differences in ileocecocolic junction sections between animals infected with *C. jejuni* and uninfected controls were not obvious (see Fig. S2 in the supplemental material). Inflammatory cytokines, including the pleiotropic cytokine IL-6 and the chemotactic cytokine MIP-2 (the murine counterpart of IL-8), were assessed in *ex vivo* distal ileum sections to determine local inflammation within the intestinal tract. IL-6 levels were not significantly different between mice infected with the *C. jejuni* wild-type strain and the uninfected control at 2 dpi (2.9 ± 1.0 versus 6.7 ± 1.2 ng/g tissue), 7 dpi (9.5 ± 0.5 versus 3.3 ± 2.4 ng/g tissue), or 35 dpi (2.6 ± 1.1 versus 2.6 ± 0.9 ng/g tissue). Similarly, MIP-2 levels were not different at 2 dpi (5.3 ± 1.6 versus 8.9 ± 0.9 ng/g tissue), 7 dpi (4.6 ± 1.2 versus 4.2 ± 2.0 ng/g tissue), or 35 dpi (2.6 ± 0.7 versus 2.5 ± 0.8 ng/g tissue). Taken together, the data indicate that ampicillin-treated CBA/J mice demonstrate stable colonization with high pathogen burden and spread to extraintestinal tissues but lack the parameters of a disease model (limited histopathology and cytokine response). Based on these results, ampicillin-treated CBA/J mice provide an ideal model to investigate and identify inhibitory microbiota that normally prevents *C. jejuni* colonization.

Isolation of *C. jejuni*-inhibitory microbiota in CBA/J mice. Peroral ampicillin treatment permitted *C. jejuni* to colonize CBA/J mice, likely by reducing key bacterial communities that contributed to the colonization resistance to *C. jejuni*. Therefore, experiments were performed to identify the bacteria that inhibit *C. jejuni* colonization. We collected the colonic fecal content from five mice either treated or untreated with ampicillin and serially diluted and plated the intestinal contents on three separate culture media (MHB or LB under microaerobic conditions and MRS under anaerobic conditions). We intentionally sought to recover lactic acid bacteria to demonstrate proof of principal (recovery of an ampicillin-sensitive bacterium that might inhibit *C. jejuni* colonization of mice) as we have previously documented that specific *Lactobacillus* strains inhibit *C. jejuni* colonization of animals (31). Thirteen visually distinct colonies were chosen for analysis (see Table S1 in the supplemental material). The viability of *C. jejuni* was tested by coculture with each of the individual isolates, as described elsewhere (31). Five intestinal isolates limited *C. jejuni* growth *in vitro* during coculture, and their identification and classification were determined by sequencing PCR-amplified regions of the 16S rRNA. DNA typing revealed that the three inhibitory
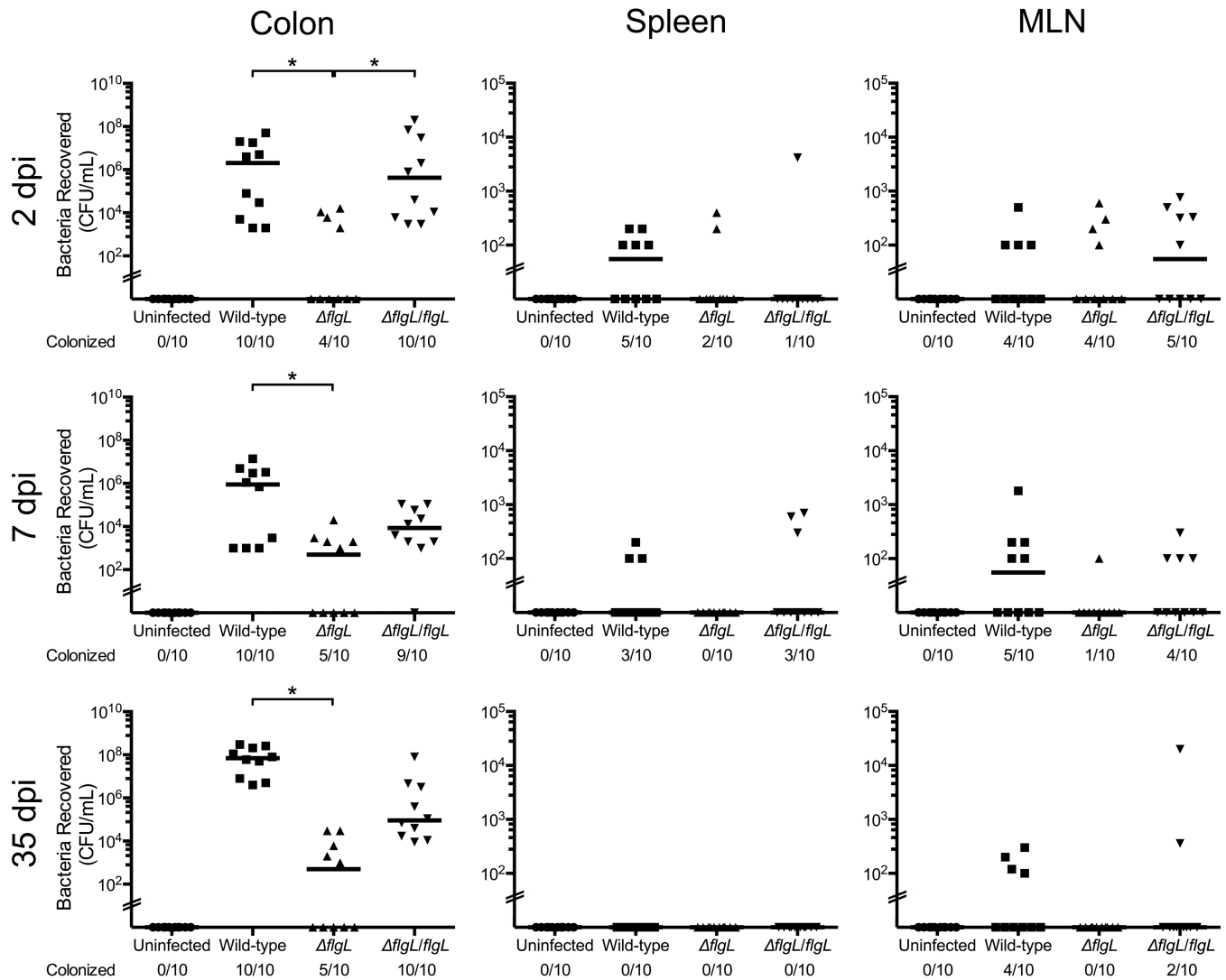
FIG 1 *C. jejuni* burden in the colon, spleen, and MLN of CBA/J mice treated with ampicillin at 2, 7, and 35 dpi. CBA/J mice were treated with ampicillin and inoculated by oral gavage with a *C. jejuni* wild-type strain, a Δ*flgL* mutant, the Δ*flgL*/*flgL* isolate, or PBS (uninfected). The colon, spleen, and MLN were removed aseptically and homogenized, and serial dilutions were plated to determine CFU counts. Each data point represents the CFU/ml of *C. jejuni* recovered from the tissue of a single mouse. The median for each group is indicated. *, $P < 0.05$ for the indicated comparison (by ANOVA with Kruskal-Wallis posttest). The limit of detection was 10^2 CFU. MLN, mesenteric lymph nodes.

isolates (MEK1, MEK2, and MEK3) were identified as *Enterococcus faecalis* (see Table S2 in the supplemental material), while MEK4 matched sequences of *Rothia* species and MEK5 matched *Staphylococcus epidermidis*. Although *Rothia* sp. and *S. epidermidis* have been isolated from the upper gastrointestinal tract, neither is considered to be a major constituent in the intestinal microbiota (32). However, *E. faecalis* is a major constituent of the human and murine intestinal tract. Each recovered isolate of *E. faecalis* was determined to be sequence type 55 (ST55), as determined by multilocus sequence typing (MLST) analysis (see Table S3 in the supplemental material). Given that the three *E. faecalis* isolates were indistinguishable from one another, we chose to further characterize only MEK1 (redesignated *E. faecalis* KLC3001). *E. faecalis* KLC3001 was susceptible (≥17-mm diameter) to ampicillin (10-μg susceptibility disk) in a Kirby-Bauer disk diffusion assay, with an average zone of inhibition of 33.3 ± 5.2 mm. Considering

that MEK1 was sensitive to ampicillin and would likely be reduced in ampicillin-treated mice, we assessed this strain further for anti-*Campylobacter* activity using both *in vitro* and *in vivo* assays.

The ability of *E. faecalis* to mediate resistance to *C. jejuni* during *in vitro* growth assays was assessed by inoculating *C. jejuni* into pasteurized milk either in monoculture or coculture with *E. faecalis* and monitoring growth for 24 h. The reason for using pasteurized milk for this assay is because both *C. jejuni* and lactic acid bacteria are metabolically active in this medium (31). We also included *Lactobacillus acidophilus* in this assay as this bacterium has previously been shown to inhibit *C. jejuni* growth *in vitro* through production of organic acids (31). While *C. jejuni* was able to survive in monoculture for 24 h, the growth of *C. jejuni* in coculture with *E. faecalis* or *L. acidophilus* was significantly inhibited. More specifically, no viable *C. jejuni* bacteria were recovered at 24 h, with a detection limit of 10^2 CFU/ml (Fig. 2A). *C. jejuni*
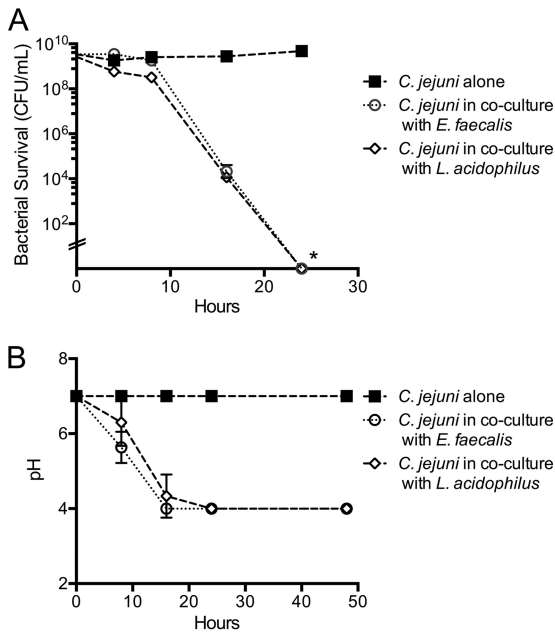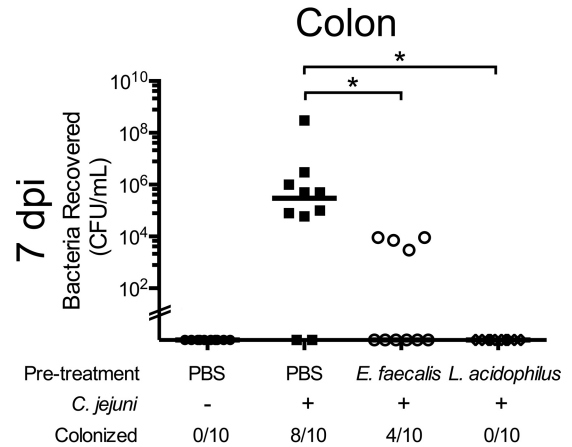
FIG 2 *E. faecalis* inhibits *C. jejuni* growth during *in vitro* coculture. (A) *C. jejuni* strain F3011 was inoculated into pasteurized milk samples either in monoculture (*C. jejuni* alone) or in coculture with *E. faecalis* KLC3001 and *L. acidophilus* NCFM (control) and incubated for 24 h. The average number of CFU $\pm$ standard deviation is shown for each time point for at least three independent replicates. *, $P < 0.05$ for the comparison between results for *C. jejuni* alone and in coculture with either *E. faecalis* or *L. acidophilus* at 24 h (by one-way ANOVA). (B) Concurrently, the pH of each milk sample was assessed during the experiment shown in panel A.

FIG 3 *E. faecalis* prevents *C. jejuni* colonization of CBA/J mice. CBA/J mice were treated with ampicillin, followed by oral gavage with PBS, *E. faecalis* KLC3001, and *L. acidophilus* NCFM, and then challenged with either PBS (uninfected) or *C. jejuni* after 48 h. At 7 dpi, the mice were euthanized, the colons were removed aseptically and homogenized, and serial dilutions were plated to determine CFU counts. Each data point represents the CFU/ml of *C. jejuni* recovered from the colon of a single mouse. The median for each group is indicated. *, $P < 0.05$ for the indicated comparison (by ANOVA with Kruskal-Wallis posttest).

viability correlated with acidification of the medium and with the production of lactic acid by the homofermentative *E. faecalis* KLC3001 and *L. acidophilus* NCFM strains (Fig. 2B). Lactic acid production in supernatants harvested from overnight broth cultures for *E. faecalis* KLC3001 (210 mM $\pm$ 30 mM) and *L. acidophilus* NCFM (180 mM $\pm$ 25 mM) was elevated compared to the level from broth alone but not significantly different, as judged by HPLC analysis. In summary, these experiments demonstrated that *E. faecalis* KLC3001 inhibits the growth of the *C. jejuni* F38011 strain *in vitro*.

Murine-isolated *Enterococcus faecalis* inhibits *C. jejuni* colonization of CBA/J mice. To test the hypothesis that specific bacteria mediate colonization resistance, we assessed whether the *E. faecalis* KLC3001 isolate could inhibit *C. jejuni* colonization of mice. We developed a colonization resistance model in which ampicillin-treated CBA/J mice were given oral inoculations with *E. faecalis* prior to infection with *C. jejuni*. *L. acidophilus* was used as a test control, and PBS-treated (uninfected) mice were used to demonstrate *C. jejuni* colonization. At 7 dpi, *Campylobacter* burden in the colon was assessed. The results indicated that there was a significant reduction in *C. jejuni* bacteria recovered from the colons of animals inoculated with *E. faecalis* prior to *C. jejuni* challenge compared to levels in mice given PBS (Fig. 3). Additionally, mice inoculated with *L. acidophilus* exhibited colonization resistance to *C. jejuni*. These results indicate that the *E. faecalis* KLC3001 strain limits *C. jejuni* colonization of mice.

Ampicillin treatment alters the intestinal microbiota in CBA/J mice. Considering the complexity of the intestinal micro-

biota and the limitations of culture-dependent analysis, we also assessed the culture-independent diversity of the intestinal bacterial community in mice treated or untreated with ampicillin. Fresh fecal pellets were collected from CBA/J mice treated or untreated with ampicillin, DNA was extracted, and the V3-V5 16S rRNA was amplified and sequenced using an Illumina MiSeq sequencing platform. Sequence reads were aligned and clustered into operational taxonomic units (OTUs), and differences between taxonomies were compared for each treatment group (Fig. 4A). The results indicated that ampicillin treatment of mice decreased the overall taxonomical complexity of the intestinal microbiota, with an observed increase in representatives of the *Bacteroidetes* (compare 52.8% of OTUs in untreated animals with 72.1% in ampicillin-treated animals), namely, *Barnesiella* sp. (71.9%). Furthermore, we observed a decrease in representatives of the *Firmicutes* in ampicillin-treated animals compared to levels in untreated mice (compare 47.2% in untreated animals with 25.9% in ampicillin-treated animals). Complete taxon identifications for all OTUs identified are listed in Table S4 in the supplemental material. In addition to taxonomic structure analysis, we assessed the OTUs common to all animals within a treatment group (i.e., the core microbiome) (see Table S5 in the supplemental material). This analysis revealed two genera present in the intestines of all untreated and ampicillin-treated CBA/J mice. This analysis also revealed seven genera present in all of the untreated mice and three genera present only in the ampicillin-treated mice. Microbial community structures were clearly impacted by ampicillin treatment, as judged by principal coordinate analysis (Fig. 4B), and this change was likely due to a significant decrease in overall diversity among community structures following ampicillin treatment (Fig. 4C). The intestinal microbial composition of animals was more similar between mice that received ampicillin treatment than between control mice (untreated). There was no significant alpha diversity (within-community diversity). These results indicate that ampicillin treatment reduces the complexity
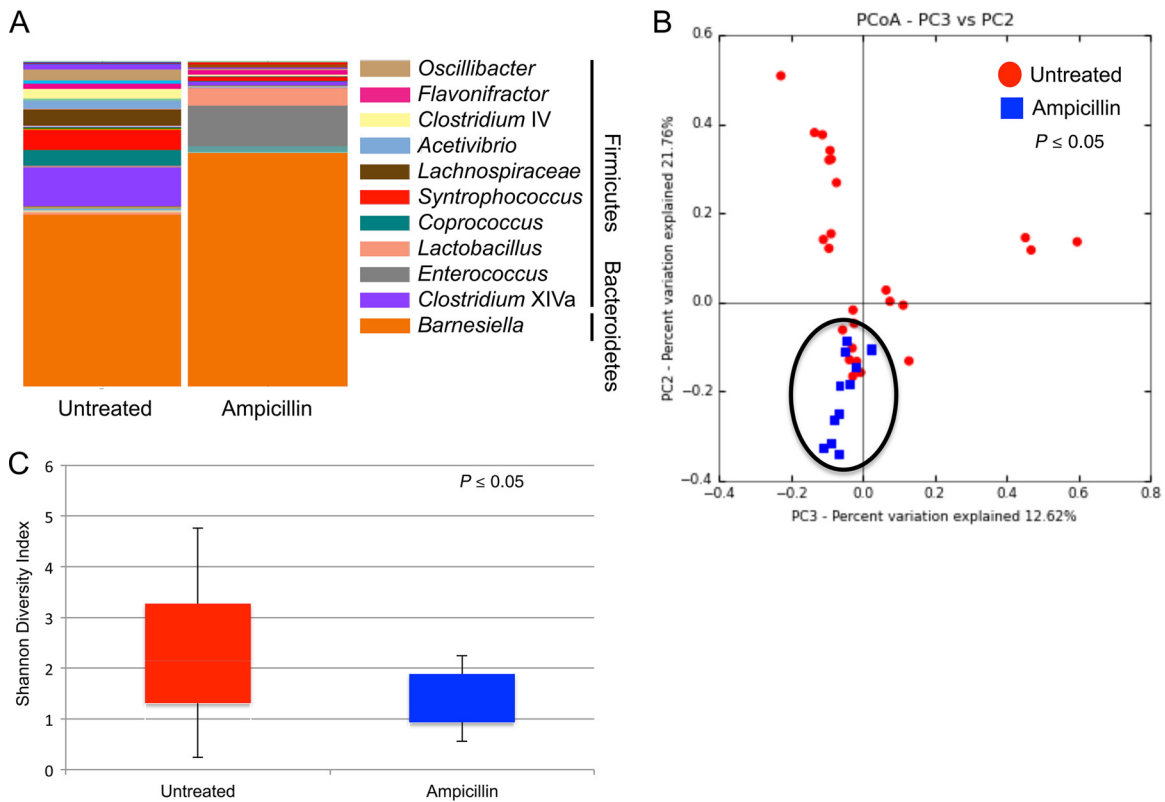
FIG 4 Taxonomy summary and microbial diversity of OTUs from fecal samples of CBA/J mice with and without peroral ampicillin treatment. DNA extracted from fecal samples was used for 16S rRNA PCR amplification, sequenced, and clustered into OTUs. The taxonomy summary (A), principal coordinate analysis (B), and Shannon diversity index (C) were analyzed using QIIME software to determine differences in microbial community structures. The sequences from at least 15 animals per treatment are shown in each analysis. The taxonomy summary in panel A represents OTUs with greater than 1% total representation; OTUs with less than 1% representation are listed in Table S4 in the supplemental material. Statistical difference ($P \leq 0.05$) in panel C was determined by PERMANOVA.

of the intestinal microbiota, including shifting the total community toward a single *Bacteroidetes* genus, and this shift corresponded to the observed disruption of the murine colonization resistance to *C. jejuni*. Ampicillin treatment changes the composition of the murine intestinal microbiota, resulting in increased *C. jejuni* colonization susceptibility, and enables dissemination beyond the intestine.

DISCUSSION

The intestinal tract of animals is colonized by a large number of commensal and symbiotic microorganisms, collectively known as microbiota, with levels in the colon exceeding 10^{11} bacterial cells per gram of fecal content (33). Colonization resistance, or the ability of the commensal microbiota to prevent colonization by exogenous pathogens or opportunistic commensals, is an important part of the host defense (34). Intestinal microbiota provide colonization resistance to pathogens in multiple ways, including competition for niches by preventing pathogens from attaching to their target sites, depleting essential nutrients for pathogen viability, producing bacteriocins or other metabolites that inhibit pathogen function, producing organic acids that alter intraluminal pH levels to acidic conditions unfavorable to pathogens, or utilizing the limited oxygen available in the gut contributing to the anaerobic and capnophilic environment (35–38). Furthermore, the commensal microbiota provides immune-mediated colonization resistance to pathogens by stimulating the development of

immune cell populations involved in innate and adaptive immune processes as well as the production of antimicrobial and proinflammatory factors (39, 40). The goal of this study was to develop a simple and reproducible method that allows *C. jejuni* to colonize mice.

Bereswill and colleagues recently published an article that shows that mice treated with a quintuple-antibiotic cocktail develop clinical signs of *C. jejuni* disease (16). We first attempted to reproduce this model, with the idea of possibly refining the methods to identify a simpler method of altering the intestinal microbiota in a manner to permit *C. jejuni* colonization (16). However, no animals were colonized by *C. jejuni* (data not shown). Our results may be attributed to differences in the *C. jejuni* strain used or to variations in the human intestinal microbiota used to reconstitute the animals. In support of this notion, human susceptibility to infection by *Campylobacter* is associated with the species composition of the human intestinal microbiome (41). We then performed studies using the streptomycin *ad libitum* model, which has been used to investigate *Salmonella* pathogenesis (42). However, the animals in this model had cleared *C. jejuni* by 2 dpi, demonstrating that *Campylobacter* differs from *Salmonella* in this model (data not shown). Based on these findings, we initiated studies to determine whether the treatment of mice with various antibiotics would permit *C. jejuni* colonization. We treated mice with one of five different antibiotics (ampicillin, metronidazole, novobiocin, streptomycin, and trimethoprim). Most of these an-

tibiotics were chosen because they have a limited bacterial spectrum of activity; the idea was to not completely disrupt the intestinal microbiota. In our preliminary experiments, ampicillin treatment of mice resulted in the greatest change (increase) in *C. jejuni* colonization. More specifically, we found that the treatment of CBA/J mice with ampicillin resulted in *C. jejuni* colonization with recovery of the pathogen from extraintestinal tissues (Fig. 1). These results are in agreement with the findings of Stahl et al., demonstrating that SIGIRR (single immunoglobulin IL-1R-related molecule)-deficient mice treated with a single antibiotic (vancomycin) can be colonized with *C. jejuni* (43). We also found that a functional flagellum was necessary for *in vivo* colonization. More specifically, we found that a Δ*flgL* mutant shows a significant reduction in its ability to colonize mice compared to the ability of a *C. jejuni* wild-type isolate. This finding is consistent with work performed with *Salmonella enterica* serovar Typhimurium, whereby flagellar defects were found to impair the fitness of a pathogen due to its inability to utilize the nutrients released in the inflamed intestine (44). In summary, our results demonstrate that the administration of ampicillin prior to infection is sufficient to permit *C. jejuni* colonization of all treated mice.

To demonstrate that ampicillin treatment of mice permits *C. jejuni* colonization by altering the intestinal microbiota, we performed a proof-of-concept experiment. We purposely biased our selection of microbes that might inhibit *C. jejuni* growth by plating the intestinal contents from mice treated with ampicillin on MRS plates (MRS medium supports the growth of lactic acid bacteria, including enterococci and lactobacilli). The *E. faecalis* KLC3001 isolate recovered from mice was indeed found to be sensitive to ampicillin and inhibited *C. jejuni* growth *in vitro* (Fig. 2). We also found that *E. faecalis* KLC3001 contributes to the *in vivo* colonization resistance to *C. jejuni*. More specifically, inoculation of ampicillin-treated mice with *E. faecalis* KLC3001 showed reduced *C. jejuni* colonization levels compared to levels in *C. jejuni*-inoculated mice not administered *E. faecalis* (Fig. 3). In comparison to the *E. faecalis*-inoculated mice, the bacteria recovered from the control mice at 7 dpi showed more diversity in colony morphology and fewer *Enterococcus*-like colonies (data not shown). Of additional interest is that the inhibitory effects of *E. faecalis* and *L. acidophilus* on *C. jejuni in vitro* were indistinguishable from one another, but *L. acidophilus* provided greater resistance to *C. jejuni* colonization of mice.

Given that ampicillin treatment of mice permits *C. jejuni* colonization by altering the intestinal microbiota, we wanted to identify the changes in the intestinal microbiota following treatment with ampicillin using deep sequencing. The principal coordinate analysis of total microbiota indicated that the microbial communities in the intestines were more similar in animals treated with ampicillin than in animals not receiving treatment (Fig. 4). Of note, the relative abundances of the two phyla (*Bacteroidetes* and *Firmicutes*) found in both ampicillin-treated and untreated CBA/J mice were significantly impacted by ampicillin treatment. Specifically, we observed that the treatment of mice with ampicillin resulted in a significant increase in representatives of the *Bacteroidetes* (genus *Barnesiella*) and a decrease in representatives of the *Firmicutes* (genus *Clostridium* XIVa). *Bacteroidetes* and *Firmicutes* are the two most prominent phyla that comprise the mouse intestinal microbiota, as well as the human intestinal microbiota, and a shift in the ratio of these phyla has been associated with many disease conditions (45–47). The influence of *Barnesiella* in *C. je-*

juni colonization of mice is not known. Nevertheless, it is clear that the microbiota can influence pathogen clearance or susceptibility. For example, infant mice with elevated *Escherichia coli* levels are more susceptible to *C. jejuni* (48). The decrease in *Clostridium* XIVa bacteria in ampicillin-treated animals is also of interest given the findings of Atarashi and colleagues (49). The investigators reported that colonization of gnotobiotic mice with mouse-derived clostridial XIVa bacteria enhances anti-inflammatory signaling by directing the expansion of lamina propria and systemic regulatory T cells associated with secretion of the anti-inflammatory protein IL-10. Considering that *C. jejuni* colonizes animals with increased inflammation, including mice with an altered genetic background (IL-10$^{-/-}$), the decrease in *Clostridium* XIVa bacteria may contribute to colonization by facilitating a favorable intestinal environment (49). Taking these results together, the overall complexity of the intestinal environment was reduced in ampicillin-treated animals.

Based on the recovery of the *E. faecalis* KLC3001 isolate from mice treated with ampicillin, the observation of an increase in *Enterococcus* taxa following ampicillin treatment by deep sequencing was enigmatic. However, isolates of *E. faecalis* vary considerably in their phenotypic properties, including resistance to antibiotics. Moreover, *E. faecalis* strains have been used as probiotics to balance the intestinal microbiota or used in dairy products (50, 51), whereas other strains are opportunistic pathogens associated with nosocomial infections (52). Consistent with our work to recover ampicillin-sensitive bacteria that could demonstrate anti-*Campylobacter* activity, Robyn and coworkers found that *E. faecalis* (strain MB 5229) has anti-*Campylobacter* activity *in vitro*. However, the anti-*Campylobacter* activity demonstrated by this strain was ineffective in broiler chickens (53). Our results indicate that *E. faecalis* KLC3001 has anti-*Campylobacter* activity *in vivo*. Based on the striking differences in the intestinal microbiota from the untreated and ampicillin-treated mice, as judged by deep-sequence analysis, there are undoubtedly additional culturable and nonculturable microorganisms that influence *Campylobacter* colonization.

The effects of antibiotics on intestinal microbiota are profound. Although our results demonstrate that ampicillin treatment is associated with an increase in *C. jejuni* colonization, the exact mechanism is unknown. The direct and indirect effects of antibiotic treatment on the intestinal community structure can be long lasting and results in an alteration in the total number of bacteria as well as a change in the composition and balance of specific bacteria in the community (54, 55). It is well known that a shift in community structure following antibiotic treatment can alter the function of the community, including disease susceptibility and nutrient acquisition (56). For example, antibiotic treatment has been shown to promote *Clostridium difficile* colonization (57, 58). In contrast, the administration of a cocktail of six microorganisms to mice has been shown to provide *C. difficile* colonization resistance and to inhibit stable residence (58). Reeves and colleagues also found that mice precolonized with a murine *Lachnospiraceae* isolate had significantly decreased *C. difficile* colonization compared to mice colonized with *E. coli* (57). In addition to altering the intestinal community, antibiotic treatment may cause bacterial lysis and the release of carbon sources as well as an increase in the level of bile acid that could be critical for pathogen colonization (59). It is likely that if two organisms are trying to occupy the same niche, the organism that most efficiently

competes for the limiting nutrient will be successful (59). Moreover, if an organism already occupies a niche, an invading organism will be at a disadvantage to compete for nutrients. Furthermore, antibiotic treatment disrupts this relationship, resulting in an alteration in the ability of the endogenous community to limit colonization of a pathogen.

Our results demonstrate that ampicillin alters the intestinal microbiota, thereby allowing *C. jejuni* to colonize the intestinal tract. Additionally, we show that ampicillin-treated mice given *E. faecalis* have restored colonization resistance to *C. jejuni*. It is likely that there are other bacterial species involved in the murine colonization resistance to *C. jejuni*, as evidenced by the efficacy of *L. acidophilus* to prevent *C. jejuni* colonization. A potential next step is to look at different *C. jejuni* strains and their susceptibility or resistance to colonization. Genetically modified mice may also benefit from ampicillin treatment, as IL-10$^{-/-}$ mice cleared of microbiota with a multiantibiotic cocktail demonstrated increased *C. jejuni*-mediated disease leading to death (60). In conclusion, we believe our findings will allow researchers to better explore *C. jejuni*-host interactions, whether the focus is probiotic inhibition or virulence assessment.

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