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Helicobacter bilis Infection Alters Mucosal Bacteria and Modulates Colitis Development in Defined Microbiota Mice

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Abstract

Background: Helicobacter bilis infection of C3H/HeN mice harboring the altered Schaedler flora (ASF) triggers progressive immune responsiveness and the development of colitis. We sought to investigate temporal alterations in community structure of a defined (ASF-colonized) microbiota in normal and inflamed murine intestines and to correlate microbiota changes to histopathologic lesions.

Methods: The colonic mucosal microbiota of healthy mice and ASF mice colonized with H. bilis for 3, 6, or 12 weeks were investigated by fluorescence in situ hybridization targeting the 16S ribosomal RNA genes of total bacteria, group-specific organisms, and individual ASF bacterial species. Microbial profiling of ASF and H. bilis abundance was performed on cecal contents.

Results: Helicobacter bilis–colonized mice developed colitis associated with temporal changes in composition and spatial distribution of the mucosal microbiota. The number of total bacteria, ASF519, and helicobacter-positive bacteria were increased (P , 0.05), whereas ASF360/361-positive bacteria were decreased (P , 0.05) versus controls. Adherent biofilms in colitic mice were most often (P , 0.05) composed of total bacteria, ASF457, and H. bilis. Total numbers of ASF519 and H. bilis bacteria were positively correlated (P ¼ 0.03, r ¼ 0.39 and P , 0.0001, r ¼ 0.73), and total numbers of ASF360/361 bacteria were negatively correlated (P ¼ 0.003, r ¼ 20.53) to histopathologic score. Differences in cecal abundance of ASF members were not observed.

Conclusions: Altered community structure with murine colitis is characterized by distinct ASF bacteria that interact with the colonic mucosa, by formation of an isolating interlaced layer, by attachment, or by invasion, and this interaction is differentially expressed over time.

Keywords

Helicobacter bilis, colitis, altered Schaedler flora, IBD, fluorescence in situ hybridization

Disciplines

Cell and Developmental Biology | Veterinary Microbiology and Immunobiology | Veterinary Pathology and Pathobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

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Background: *Helicobacter bilis* infection of C3H/HeN mice harboring the altered Schaedler flora (ASF) triggers progressive immune responsiveness and the development of colitis. We sought to investigate temporal alterations in community structure of a defined (ASF-colonized) microbiota in normal and inflamed murine intestines and to correlate microbiota changes to histopathologic lesions.

Methods: The colonic mucosal microbiota of healthy mice and ASF mice colonized with *H. bilis* for 3, 6, or 12 weeks were investigated by fluorescence in situ hybridization targeting the 16S ribosomal RNA genes of total bacteria, group-specific organisms, and individual ASF bacterial species. Microbial profiling of ASF *and H. bilis* abundance was performed on cecal contents.

Results: *Helicobacter bilis*–colonized mice developed colitis associated with temporal changes in composition and spatial distribution of the mucosal microbiota. The number of total bacteria, ASF519, and helicobacter-positive bacteria were increased ($P < 0.05$), whereas ASF360/361-positive bacteria were decreased ($P < 0.05$) versus controls. Adherent biofilms in colitic mice were most often ($P < 0.05$) composed of total bacteria, ASF457, and *H. bilis.* Total numbers of ASF519 and *H. bilis* bacteria were positively correlated ($P = 0.03$, $r = 0.39$ and $P < 0.0001$, $r = 0.73$), and total numbers of ASF360/361 bacteria were negatively correlated ($P = 0.003$, $r = -0.53$) to histopathologic score. Differences in cecal abundance of ASF members were not observed.

Conclusions: Altered community structure with murine colitis is characterized by distinct ASF bacteria that interact with the colonic mucosa, by formation of an isolating interlaced layer, by attachment, or by invasion, and this interaction is differentially expressed over time.

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The human inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are chronic, immunologically mediated disorders of the gastrointestinal (GI) tract that are poorly understood. Data from humans and animal model studies of IBD indicate that deleterious host–microbiome interactions can incite and perpetuate intestinal inflammation.[1–3] For example, antibiotics are effective in subsets of human IBD patients,[4,5] and most rodent models of colitis show that inflammation does not occur in a germ-free environment.[6,7] Moreover, bacteria that adhere and invade the intestinal mucosa, including strains of *Escherichia coli*, may be particularly important in disease pathogenesis.[8] Collectively, these observations provide compelling evidence of microbial stimulation of intestinal inflammation in susceptible hosts.

Although there is strong evidence that microbes contribute to development of IBD, the complexity of the gut microbiota makes it difficult to identify the relative contribution of altered microbial composition to the disease process.[9] Efforts to simplify the study of host–microbiota interactions include selective colonization of germ-free mice with a single bacterial species or with a combination of bacteria.[7,10] However, germ-free mice typically have underdeveloped immune systems, which may limit their relevance to human disease.[11] An immunocompetent mouse model colonized with a more complex yet defined microbiota would be helpful in understanding host–microbiota interactions that elicit IBD. We have previously used *Helicobacter bilis* infection in defined microbiota C3H/HeN mice to investigate microbial modulation of IBD.[12,13] A defined microbiota, the altered Schaedler flora (ASF), consisting of 8 murine bacterial species, served as the model bacterial community. We have also shown that *H. bilis* triggers progressive immune responsiveness to ASF bacteria that is associated with the development of mucosal inflammation in defined microbiota C3H/HeN mice.[14,15] Whether perturbations in the composition and distribution of the mucosal microbiota occur and contribute to *Helicobacter*-induced colitis has not been systematically investigated.

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We hypothesized that *H. bilis* infection would alter the spatial distribution of resident mucosal bacteria that induce intestinal inflammation in C3H/HeN mice. To test this hypothesis, we investigated temporal changes in the spatial distribution of a defined (ASF-colonized) microbiota in normal and inflamed murine intestines and correlated these observations to the development of histopathologic lesions of colitis.

MATERIALS AND METHODS

Animals

Male and female gnotobiotic C3H/HeN:Tac mice, 6- to 8-weeks old, possessing a defined microbial community (e.g., ASF consisting of 8 intestinal bacteria) were bred and maintained in a gnotobiotic environment in polypropylene cages within flexible film isolators at the Iowa State University. Members of the ASF include ASF356–*Clostridium* species; ASF360–*Lactobacillus intestinalis*; ASF361–*Lactobacillus murinus*; ASF457–*Mucispirillum schaedleri*; ASF492–*Eubacterium plexicaudatum*; ASF500–*Pseudoflavonifactor* species; ASF502–*Clostridium* species; and ASF519–*Parabacteroides goldsteinii*.[16] All mice were screened for bacterial contaminants (including *Helicobacter* spp.) by culture before the experiments. Mice were fed an irradiated rodent diet (Harlan 2919) and autoclaved water ad libitum and maintained on a 12-hour light–dark cycle.

All animal procedures were approved by the Iowa State University Animal Care and Use Committee (IACUC log #'s 9-04-5755-M and 9-02-5265 M).

Experimental Design

Defined microbiota C3H/HeN mice were assigned to 1 of 2 study groups: (1) control mice or (2) *H. bilis*–colonized mice. Experimental and control mice (4–6 mice per time point) were killed by CO_2 asphyxiation on weeks 0, 3, 6, or 12 after *H. bilis* colonization. A 1-cm section of proximal colon containing full luminal contents for fluorescence in situ hybridization (FISH) studies was collected and placed into 10% neutral-buffered formalin. Colonic tissues were also harvested for evaluation of histopathologic lesions. Sequencing analysis for ASF and *H. bilis* microbial abundance was performed on cecal contents.

Infection with *Helicobacter bilis*

Helicobacter bilis isolate (ATCC strain 51630) was originally provided by Dr. Nancy Lynch (College of Medicine, University of Iowa). Organisms were streaked onto Columbia agar plates supplemented with 5% horse serum and grown under microaerophilic conditions (80% N_2, 10% H_2, and 10% CO_2) and kept at 37°C. Bacteria were collected from 3 to 5 plates and suspended into tryptic soy broth on the day of inoculation. Before inoculation, organisms were collected under sterile conditions and examined for their purity, morphology, and motility by dark-phase microscopy. Organisms were confirmed to be urease positive. Each mouse received 0.3 mL of a fresh inoculum ($\sim 2 \times 10^8$ organisms) by gastric gavage for 3 consecutive days. Confirmation of *H. bilis* infection postinoculum was made by polymerase chain reaction evaluation of DNA isolated from feces.

Histopathologic Analysis

Samples of cecum and proximal colon were placed in 10% neutral-buffered formalin, routinely processed, sectioned, and stained with hematoxylin and eosin. Sections of the cecum and proximal colon were graded for inflammatory lesions by a pathologist (J.H.) who was blinded to the treatment group. Briefly, mucosal inflammation was scored 0 to 9 based on the severity of mucosal epithelial damage, architectural/glandular alterations, and the magnitude/character of lamina propria cellular infiltrate.[14]

DNA Isolation from Cecal Contents

DNA from snap-frozen cecal contents was extracted using an UltraClean Fecal DNA Kit (MoBio, Carlsbad, CA) using a modified protocol. After the addition of 60 μL of Solution S1 (provided with the MoBio kit) to the sample, 20 μL of proteinase K (20 mg/mL) (MoBio) was added, and the mixture was incubated at 55°C for 1 hour. After the 10-minute vortex, the sample was centrifuged for 3 minutes at 10,000*g*. The supernatant was transferred to a clean microcentrifuge tube; 200 μL of Solution IRS was added and the mixture incubated at 4°C for 5 minutes. Before loading the sample onto the column, 900 μL of Solution CB3 (MoBio) was added. After the centrifugation of 300 μL of Solution S4 from the column, 300 μL of freshly prepared 70% ethanol was added to the column and centrifuged for 30 seconds at 10,000*g*. The elution buffer (50 μL of Solution S5) was allowed to sit on the column for 4 minutes before the final spin. DNA was quantified after elution using Quant-iT PicoGreen dsDNA reagent (Invitrogen, Carlsbad, CA).

Microbial Profiling Using 16S ribosomal RNA Library Preparation and Sequencing

Amplification of the V4 region of the 16S ribosomal RNA (rRNA) gene sequence was done using region-specific primers and subsequent sequencing on the Illumina MiSeq platform.[17] Ultrahigh-throughput microbial community analysis on the Illumina MiSeq platform was conducted at the Institute for Genomics & Systems Biology at the Argonne National Laboratory (Chicago, IL). The resulting sequences were analyzed using Quantitative Insights into Microbial Ecology (QIIME).[18] QIIME allows analysis of high-throughput community sequencing data. Reads were demultiplexed and quality filtered. Any reads with homopolymer runs, more than 6 ambiguous bases, nonmatching barcodes, barcode errors, or quality scores less than 25 were removed. Samples with less than 3500 sequences were removed. Operational taxonomic units were picked using uclust in the closed-reference operational taxonomic unit picking strategy in QIIME using an ASF-specific database. Beta diversity, weighted Unifrac principal coordinates plots, analysis of similarity, and Adonis tests were generated using QIIME. Wilcoxon rank–sum tests were performed on taxonomic abundances obtained from the QIIME

pipeline using a custom R script provided by the Institute for Genome Sciences at the University of Maryland School of Medicine.

Fluorescence In Situ Hybridization

Oligonucleotide probes targeting the 16S ribosomal RNA sequence of total bacteria, ASF bacteria, and *H. bilis* were synthesized with a Cy3 or FITC (Life Sciences)-reactive fluorescent dye added at the 5' end. Six group- and species-specific probes were applied to intestinal tissues mounted on glass slides (Table 1). In brief, paraffin-embedded tissue specimens were deparaffinized using an automated system by passage through xylene (3 × 10 min), 100% alcohol (2 × 5 min), 95% ethanol (5 min), and finally 70% ethanol (5 min). Once the slides were air-dried, FISH probes were reconstituted with DNAse-free water and diluted to a working concentration of 5 ng/μL with a hybridization buffer appropriate for the probe. Tissue sections were bathed in 30 μL of DNA probe mix in a hybridization chamber maintained at 54°C overnight (12 hour). Wash buffer was used for washing (hybridization buffer without sodium dodecyl sulfate), the slides were rinsed with sterile water, then allowed to air-dry, and mounted with SlowFade Gold mounting media (Life Technologies, Carlsbad, CA) and 25X25-1 coverglass (Fisher Scientific, Pittsburgh, PA).

The specific hybridization conditions, including buffer design, temperature, and formamide concentration, for each probe were optimized in pilot studies using pure bacterial cultures and fresh tissues. A Eub338 Cy3 probe, specific for all bacteria (Table 1), was combined with the nonsense probe non–Eub338-FAM (ACT CCT ACG GGA GGC AGC) to assess for nonspecific hybridization.[19] Archived sections of gastric mucosa from a dog diagnosed with *Helicobacter* infection were used as positive control for helicobacter FISH.[20]

In Situ Quantification of Mucosal Bacteria

The bacteria were visualized by FISH and 4,6-diamidino-2-phenylindole (DAPI) staining using a ×60 Plan Apo oil objective in conjunction with an optional ×1.5 multiplier lens on an Eclipse TE2000-E fluorescence microscope (Nikon Instruments Inc., Melville, NY) and photographed with a CoolSnap EZ camera (Photometrics, Tuscon, AZ) controlled by MetaMorph software (Nashville, TN). Quantification was only performed when the hybridization signals were strong and could clearly distinguish intact bacteria morphologically by either 2-color (universal and bacterial-specific FISH probe) or 3-color (FISH probes and DAPI stain) identification. A minimum of 2 optimally oriented, paraffin-embedded proximal colonic tissue blocks per mouse were chosen for FISH analysis. Grossly, these tissue block cross-sections extended 360 degrees full-circle and contained luminal contents. A total of 7 serial 3-μm-thick mucosal cross-sections were cut from a single paraffin-embedded tissue block for hybridization using the 6 FISH probe array. Bacterial quantification was performed in 10 nonoverlapping and evenly displaced mucosal fields/section in most mice at a final observed magnification of ×600. The number of total bacteria counted in the 10 fields of each mouse was summed and that value averaged across all the mice in that treatment group. In some instances, tissue-processing artifacts required the use of additional serial sections of colonic tissue obtained from the same paraffin block. This technique resulted in bacterial quantification across the full circumference of the murine colonic mucosa for an individual probe. The 10 mucosal fields for in situ quantification included bacteria found within 3 well-defined mucosal compartments: (1) bacteria contained within the mucosa, (2) bacteria attached to the surface epithelium, and (3) bacteria localized within free mucus/interlaced area (Fig. 1).

Statistical Analysis

Tabular data were organized by mucosal region and infection status of the mice. Mean, median, minimum, and maximum values were calculated from the bacterial counts. Median values were compared among groups using the Wilcoxon rank–sum test. Associations between bacterial numbers and histopathologic score were assessed using linear mixed models with mouse as random effect. Histopathologic scores were compared between mouse groups over time using a 1-way analysis of variance and Student's *t* test. Association among variables was assessed using Spearman's rank correlation and tested for significance. The level of significance was set at $P < 0.05$.

TABLE 1. Probe Sequences Used for Fluorescence In Situ Hybridization of Colonic Tissues

Probe	Target	ASF Species	16S rRNA Sequence
Eub338	All bacteria	All	5′-GCT GCC TCC CGT AGG AGT-3'
Erec482	*Eubacterium plexicaudatum*	ASF492	5′-GCT TCT TAG TCA RGT ACC G-3′
	Pseudoflavonifractor spp.	ASF500	
	Clostridium spp.	ASF356/502	
Lab158	*Lactobacillus murinus*	ASF361	5′-GGT ATT AGC ATC TGT TTC CA-3′
	Lactobacillus intestinalis	ASF360	
Bac303	*Parabacteroides goldsteinii*	ASF519	5′- CCA ATG TGG GGG ACC TT -3′
457	*Mucispirillum schaedleri*	ASF457	5′-GGG ACG CGA GTC CAT CTT T-3′
Hel717	*Helicobacter* spp.	None	5′-AGG TCG CCT TCG CAA TGA GTA-3′

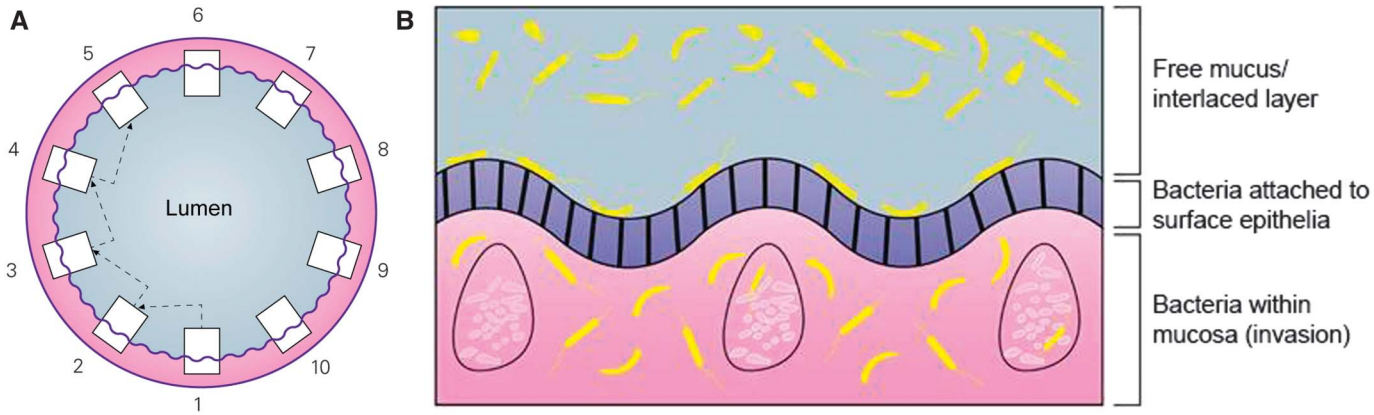

FIGURE 1. Methods for performing FISH bacterial counts. A, Bacterial quantification was performed in 10 microscopic regions covering the complete colonic mucosa of each mouse. B, Within a representative mucosal region, bacteria were found within the free mucus/interlaced layer, attached to surface epithelia, and/or located within the mucosa were enumerated.

RESULTS

Histopathologic Analysis

Defined microbiota (ASF) mice colonized with *H. bilis* developed mild-to-moderate microscopic colitis that peaked sometime between 0 and 6 weeks after bacterial colonization. Significant mucosal inflammation was observed in 3- and 6-week *H. bilis*–infected mice as compared with healthy controls, $P = 0.015$ and $P = 0.007$, respectively (Fig. 2). Histopathologic lesions were characterized by increased mononuclear cellular infiltrate into the lamina propria, crypt hyperplasia, lymphoid hyperplasia, and submucosal edema (Fig. 3).

16S rRNA Microbial Profiling

A total of 1,390,757 sequences passed quality filtering with 27,269.75 ± 5182.50 sequences per sample. Because of the limited composition of the ASF gut community, we use weighted Unifrac to assess the variation between samples (beta diversity). As shown in Figure 4, the control and *H. bilis*–infected groups clustered discretely. Adonis tests resulted in a *P*-value of 0.001 and an R^2 value of 0.3034, indicating that 30.34% of the variation can be attributed to the clustering. Analysis of similarity tests resulted in a *P*-value of 0.001 and a test statistic of 0.188. To assess the contribution of *H. bilis* to the beta diversity, 16s rRNA gene sequences specific to this species were removed and the beta diversity was reanalyzed. The resulting plot showed a much tighter clustering of the data points indicating that the addition of *H. bilis* was the major determinant of variation between the groups (data not shown).

Over the 12-week period, the ASF community within the cecum remained stable. ASF519 was the dominant organism, followed by ASF457, ASF492, and ASF361 (Fig. 5). Because ASF360 primarily colonizes the upper GI tract, it was not detected in the cecal contents.[21] Wilcoxon rank–sum tests were used to compare the control and *H. bilis*–infected mice at each time point. At week 3, ASF457 decreased in the *H. bilis*–infected group ($P = 0.03896$). At week 6, none of the ASF was significantly different. At the end of the 12-week period, ASF356 was enriched in the *H. bilis*–infected group ($P = 0.0153$).

In Situ Hybridization and Bacterial Quantification Using FISH

The mucosal microbiota in the proximal colon of healthy mice differed from bacterial populations observed at one or more time points in *H. bilis*–colonized mice. Subpopulations of bacteria

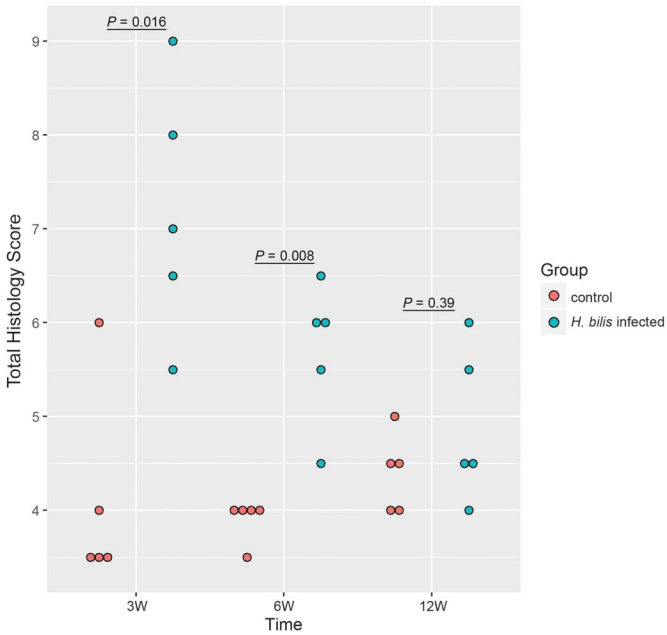

FIGURE 2. Dot plots showing total histopathologic scores of colonic tissues obtained from control and *Helicobacter bilis*–colonized mice during the trial. *P*-values show differences between murine groups at 3-, 6-, and 12-weeks post–*H. bilis* colonization. Data are representative of duplicate experiments with 5 mice/murine group at each of the 3 time points.

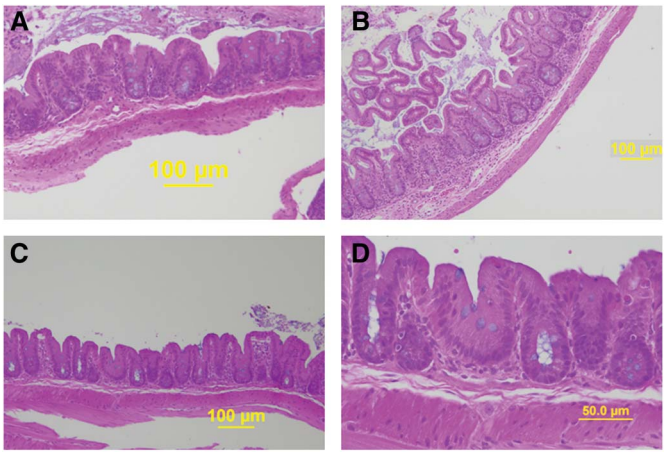

FIGURE 3. Histopathologic lesions observed in *Helicobacter bilis*–colonized defined microbiota mice vary temporally. A, Mild inflammatory changes (colitis) with infiltration of low numbers of lymphocytes and plasma cells into the lamina propria at 5 weeks post–*H. bilis* colonization. B, Colitis of moderate severity (3 weeks post–*H. bilis* colonization) showing inflammatory changes and moderate infiltration of lymphocytes and plasma cells into the lamina propria and submucosa. There is also mild edema of the submucosa. C and D, Colonic tissues obtained from control mice show no inflammation. Images (A–C) at ×10 magnification; image (D) at ×40 magnification.

hybridized with probes directed against all bacteria (Eub338), ASF519 (Bac303), ASF356/492/500/502 (Erec482), ASF360/361 (Lab158), and probe 457 (ASF457) but not *Helicobacter* spp. (Hel717) (Table 2 and Fig. 6). The proportions of total bacteria (i.e., the number of hybridized bacteria summed across all 3 mucosal compartments) hybridizing to probes Erec482 and 457 were greatest, with bacteria hybridizing to probes Bac303 (ASF519) and Lab158 (ASF360/361) representing only 17% of the Eub-positive total mucosal microbiota in healthy mice.

In *H. bilis*–colonized mice, the total number of colonic bacteria hybridizing to probes Eub338, Bac303, and Hel717 were increased ($P < 0.05$), whereas Lab158-positive bacteria were decreased ($P < 0.05$) when compared with control mice (Table 2). These differences were greatest early in the disease course (i.e., 3 and 6 week *H. bilis*–infected mice) for Eub338-, Lab158-, and Hel717-positive bacteria, with $P < 0.05$ for each probe. Total bacteria hybridizing against probes Bac303 and Hel717 were positively correlated ($P = 0.03$, $r = 0.39$ and $P < 0.0001$, $r = 0.73$, respectively) to total histopathologic score, whereas total bacteria hybridizing against probe Lab158 were negatively correlated ($P = 0.003$, $r = -0.53$) to total histopathologic score in *H. bilis*–infected mice.

The spatial distribution of mucosal bacteria in colitic mice was significantly ($P < 0.05$) different from healthy mice, with increased numbers of Eub338-, Bac303-, and Hel717-positive bacteria observed in the free mucus/interlaced layer versus other mucosal compartments (Table 3). Adherent films of bacteria observed on colonic tissues of colitic mice were composed of increased ($P < 0.05$) numbers of bacteria hybridizing against probes Eub338, 457, and Hel717 (Table 3 and Fig. 7). Bacterial attachment to the surface epithelia (versus those bacteria localized within the adherent mucus in close proximity to the surface epithelia) was variably observed in all mice with colitis. In these instances, bacteria lined the mucosal surface in a continuous or discontinuous pattern and were located below the mucus/interlaced layer. In some instances, the same bacteria attached to the surface epithelia were invasive within the mucosa, and this was especially true for bacteria that hybridized with Eub338 and Hel717 probes ($P < 0.05$ for both probes). There was a positive correlation between the number of Eub338- and Hel717-positive adherent/invasive bacteria and histopathologic score in *H. bilis*–infected mice ($P = 0.04$, $r = 0.38$ and $P < 0.0001$, $r = 0.73$, respectively).

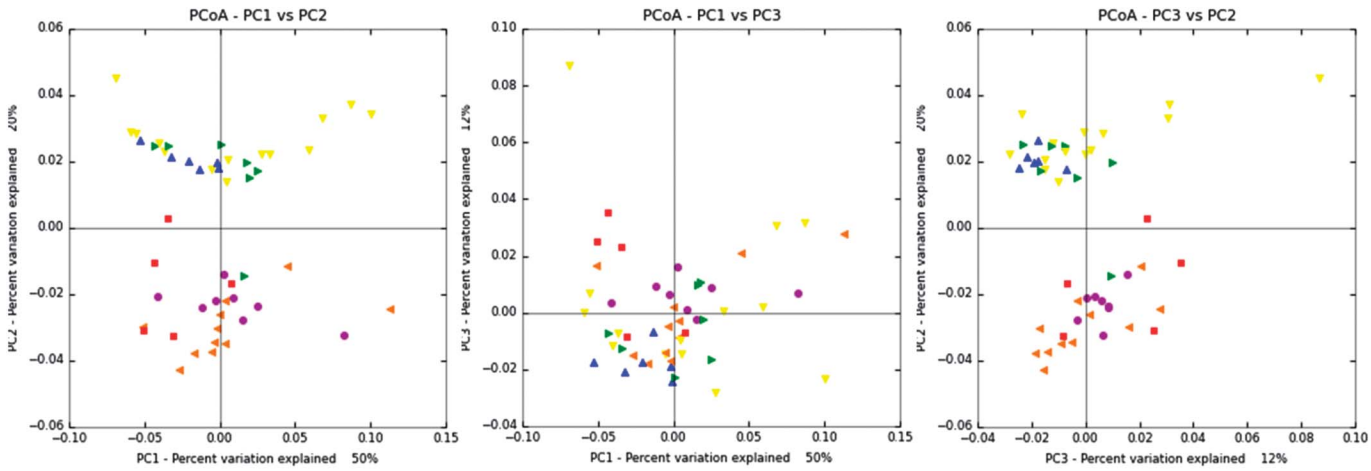

FIGURE 4. Principal Coordinates Analysis of weighted UniFrac distances of 16S rRNA genes. The clustering indicates differences (analysis of similarity: $P = 0.001$, test stat = 0.188; Adonis: $P = 0.001$, $R^2 = 0.30$) in microbiota composition between controls and *Helicobacter bilis*–infected mice. Control mice (3w = blue triangle, 6w = green triangle, 12w = yellow triangle) separate from *H. bilis*–infected mice (3w = red square, 6w = brown arrow, 12w = purple circle) at all weeks indicating differences in microbiota ecology.

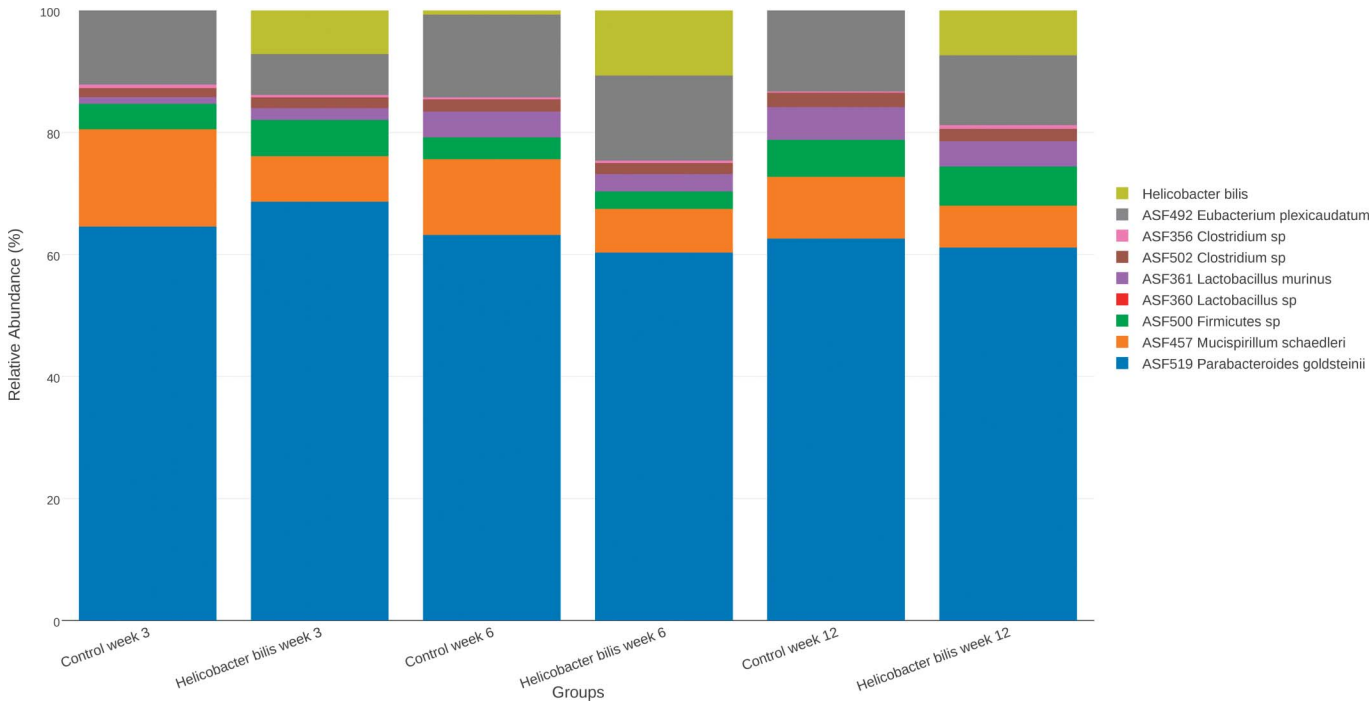

FIGURE 5. Comparison of murine disease states using 16S rRNA gene sequencing of cecal contents. Microbial abundance of *Helicobacter bilis* and individual ASF community members remained relatively stable over the experimental period. Exceptions to this trend included ASF492 was decreased in 3w *H. bilis*–infected mice versus 3w controls ($P = 0.038$), and ASF356 increased in 12w *H. bilis*–infected mice versus 12w controls ($P = 0.015$).

DISCUSSION

In our study, we examined the extent to which intestinal provocation with a bacterium effects disrupts the structure of a stable defined microbiota to instigate intestinal disease. When analyzed by FISH, our results show that oral inoculation with *H. bilis* caused major changes (i.e., spatial redistribution) in the colonic mucosal-associated microbiota of ASF-colonized C3H mice compared with ASF-colonized mice in the absence of *H. bilis*. Although bacterial inoculation affected both the structure and composition of mucosal-associated bacteria in localized regions of the colitic mice, it had minimal effects on the luminal cecal microbiota. The relative abundance of luminal ASF members in the cecum remained relatively stable throughout the experimental period with few changes, as measured directly from total cecal contents. Conversely, using FISH techniques that allow abundance measurements to be made with more precision at specific locations, we identified specific changes in the composition and spatial redistribution of mucosal-associated microbiota that were correlated with severity of histopathologic inflammation in *H. bilis*–inoculated ASF-colonized mice.

An association between *Helicobacter* spp. colonization and intestinal inflammation has been observed in a number of different mouse models. *Helicobacter hepaticus* was first isolated from either colonic or cecal mucosal scrapings of mice having chronic, active hepatitis.[22] Subsequently, *H. hepaticus* was isolated from the bile, liver, and intestines of aged A/JCr mice with hepatocellular tumors.[23] Experimental infection with either *H. bilis* or *H. hepaticus* induced typhlocolitis in mice lacking B and T lymphocytes (i.e., SCID),[24–26] in mice deficient in interleukin-10 (IL-10),[26] and in mice with abnormal T-cell receptor alpha (TCR$\alpha^{-/-}$) gene function.[27] Moreover, specific *Helicobacter* spp. may reduce (*H. hepaticus*) or enhance (*H. bilis*) colitis susceptibility in mdr1a-/- mice,[28] whereas co-infection with both *H. bilis* and *H. hepaticus* results in high-grade dysplasia and neoplasia.[29] Similarly, *H. bilis* infection or co-infection with *H. hepaticus* leads to both colitis and colorectal tumor development in Smad3-[30] or Smad3/Rag2-deficient mice[31] as shown in separate studies.

We have previously demonstrated that *H. bilis* colonization could induce host immune responses to the resident microbiota associated with DSS-induced colitis in gnotobiotic C3H/HeN mice harboring the ASF.[12–14] In subsequent experiments, we reported that temporal alterations in host responses preceded the onset of gross and histopathologic inflammation and that bacterial antigen-specific antibody and T-cell responses against the resident ASF bacteria were manifested differentially over time with respect to the development of typhlocolitic lesions.[15] However, these earlier data did not evaluate whether changes in mucosal microbial composition and/or spatial distribution might contribute to immune activation and tissue injury.

It is clear from our investigation that the introduction of an exogenous, phlogistic bacterium causes changes in the localized microbial ecosystem that are associated with intestinal inflammation in defined (ASF-colonized) microbiota mice. Our results indicate that significant shifts in spatial organization occurred for

TABLE 2. Number of Total Colonic Bacteria Determined by FISH in Control and *Helicobacter bilis*–Infected Mice

Probe	Hb3	Hb6	Hb12	Control
Eub338				
Mean	1292[a,b]	772[b]	663[b]	857
Median	1307	618	614	772
Range	594–2056	331–1117	173–1615	262–1818
Bac303				
Mean	128	184[a]	152[a]	103
Median	122	167	146	104
Range	35–265	103–311	45–400	21–180
Erec482				
Mean	887[b]	400[b]	628	741
Median	830	390	608	569
Range	199–1987	173–606	122–1440	191–1711
Lab158				
Mean	26	19[a]	32	41
Median	22	20	15	36
Range	5–78	7–35	2–108	5–140
457				
Mean	386	516[b]	133[a,b]	297
Median	178	308	133	275
Range	25–1907	102–1360	35–294	89–660
Hel717				
Mean	85[a]	55[a]	72[a]	0
Median	63	47	45	0
Range	18–306	20–117	9–481	0

Hb3, 3 weeks post-*H. bilis* infection; Hb6, 6 weeks post-*H. bilis* infection; Hb12, 12 weeks post-*H. bilis* infection.
Data expressed as mean and range.
[a]Significant ($P < 0.05$) difference between control versus *H. bilis*–infected mice.
[b]Significant ($P < 0.05$) difference between *H. bilis* infection groups. Data are representative of duplicate experiments with 5 mice/murine group at each of the 3 time points.

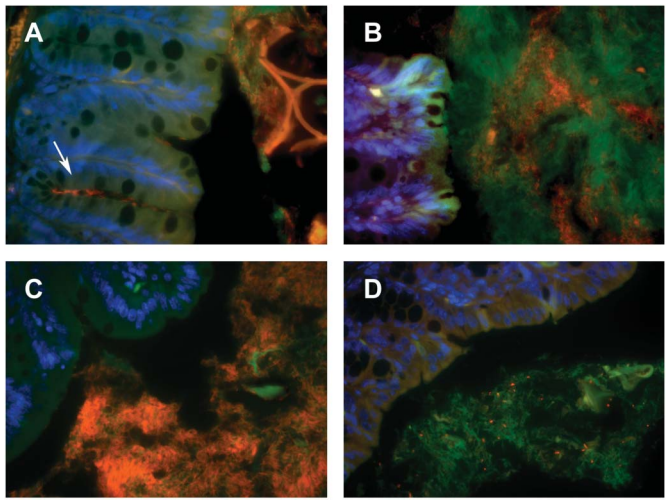

FIGURE 6. Triple-color FISH identifies bacteria within different mucosal compartments of healthy ASF mice. Panel A = colon section hybridized with Cy3-457. Sparse ASF457 bacteria are observed within one colonic gland (orange, ×600 magnification, arrow); Panel B = colon section hybridized with Cy3-457. Numerous ASF457 bacteria are observed within the free mucus/interlaced bacterial layer and positioned away from the mucosal surface (orange, ×600 magnification); Panel C = colon section hybridized with Cy3-Erec482. Erec482-labeled bacteria are numerous within the adherent mucus and interlaced compartments close to the surface epithelia (orange, ×600 magnification); Panel D = colon section hybridized with Cy3-Lab158. Lab158-positive bacteria are sparsely observed within the intestinal lumen (orange, ×600 magnification). All other bacteria in panels A–D that hybridize exclusively with the universal probe (Eub338-FITC) are shown in green. DAPI-stained colonic mucosa with goblet cells are shown in blue.

most of the ASF members after *H. bilis* colonization, including the total number of mucosal Eub338-, Bac303-, 457-, Hel717-, and Lab158-positive bacteria, and that adherent and infiltrative bacteria are observed in inflamed colon tissues, with *M. schaedleri* (ASF457) and *Helicobacter* spp. predominating. Although not statistically significant, numerous bacteria hybridizing against probe Erec482 (*Clostridiales*) were routinely visualized near the mucosa of *H. bilis*–infected mice as compared with control mice. These data documenting the changes in the spatial distribution of the ASF lend support to earlier observations from our laboratory[14,15] regarding the induction of adaptive responses directed against the resident bacteria after *H. bilis* infection; namely, that upregulated antibody and proinflammatory cytokine production occurs against select ASF members, including ASF457, ASF356, ASF492, ASF500, and ASF502. Moreover, *H. bilis* colonization of ASF mice has been shown to significantly increase the expression of mucosal genes associated with lymphocyte activation and inflammatory cell infiltration into the colonic mucosa.[13] It is also possible that host responses to ASF457 and *H. bilis* relate to their mobility (i.e., flagellated bacteria) and their tendency to make their way into the mucosa where they may more easily interact with various components (e.g., dendritic cells) of the mucosal immune system.

Different models have provided valuable insight on the putative role of the resident microbiota in mice infected with *Helicobacter* species. The structure of the intestinal microbiota was shown to be essential for the development of typhlocolitis in *H. hepaticus*–infected IL-10–deficient mice; in addition, where disease could be initiated, severity of histopathologic lesions varied significantly in the presence of different microbial communities.[32] Although *H. hepaticus* drives immune responses in this model, the specific structure of the microbiota was believed to modulate host gene expression pathways that lead to chronic intestinal inflammation. Similarly, others showed that IL-10–deficient mice housed under SPF conditions in 2 different institutions displayed significant differences in their susceptibility to *H. hepaticus*–induced colitis that could be linked to differences in the resident microbiota within the 2 facilities.[33] Finally, Wiskott–Aldrich syndrome protein–deficient mice failed to

TABLE 3. Spatial Distribution of the Number of Colonic Bacteria by Mucosal Compartment

Probe	Target	ASF	Group	FM/I	SE	WM
Eub338	Eubacteria	All	Control	853 (262–1813)	4 (0–25)	0 0
			Hb3	1277[a,b] (587–2044)	15 (0–76)	1 (0–6)
			Hb6	710[b] (317–1112)	10 (0–60)	1[a] (0–7)
			Hb12	643[b] (168–1500)	17[a] (0–100)	3[a] (0–15)
Bac303	*Parabacteroides goldsteinii*	ASF519	Control	103 (21–180)	0 (0–3)	0 0
			Hb3	128 (35–265)	0	0
			Hb6	183[a] (103–309)	1 (0–10)	0 (0–3)
			Hb12	152 (45–400)	0	0
Erec482	*Eubacterium plexicaudatum*	ASF492	Control	734 (189–1700)	6 (0–45)	1 (0–13)
	Pseudoflavonifractor spp.	ASF500	Hb3	873[b] (198–1968)	11 (0–77)	3 (0–45)
	Clostridium spp.	ASF356/502	Hb6	393[b] (160–600)	7 (0–25)	0
			Hb12	611 (122–1400)	16 (0–100)	0 (0–3)
Lab158	*Lactobacillus murinus*	ASF361	Control	41 (5–140)	0	0
			Hb3	25 (5–78)	0	0
	Lactobacillus intestinalis	ASF360	Hb6	19[a] (7–35)	0	0
			Hb12	32 (2–108)	0	0
457	*Mucispirillum schaedleri*	ASF457	Control	295 (89–660)	3 (0–25)	0 (0–4)
			Hb3	382 (25–1900)	3 (0–25)	0 (0–5)
			Hb6	509 (100–1352)	6[b] (0–35)	1 (0–8)
			Hb12	132[a] (35–294)	0[a,b] (0–7)	0 (0–14)
Hel717	*Helicobacter* spp.	None	Control	0	0	0
			Hb3	75[a] (8–259)	5 (0–45)	5[a] (0–26)
			Hb6	45[a] (10–103)	4[a] (0–15)	6[a] (0–20)
			Hb12	62[a]	5	5[a]

Data expressed as mean and range.
[a]Significant ($P < 0.05$) difference between control versus *Helicobacter bilis*–infected mice.
[b]Significant ($P < 0.05$) difference between *H. bilis* infection groups.
FM/I, free mucus/interlaced bacterial layer; SE, attached to surface epithelia; WM, within mucosa. Data are representative of duplicate experiments with 5 mice/murine group at each of the 3 time points.

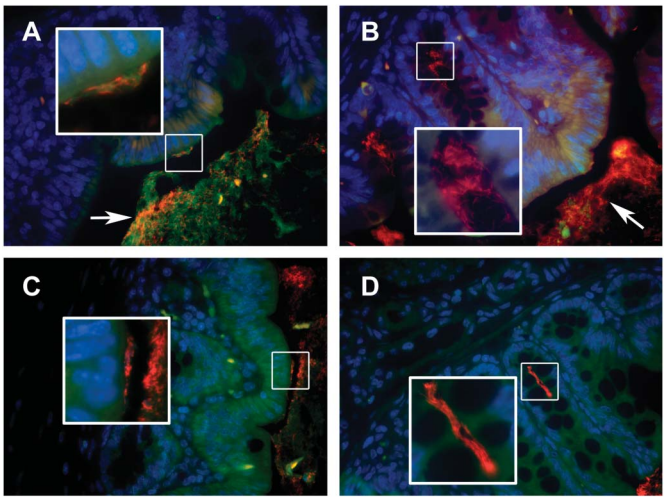

FIGURE 7. Triple-color FISH identifies bacteria within different mucosal compartments of *Helicobacter bilis*–infected ASF mice. Panel A = colon section hybridized with Cy3-457. The interlaced layer contains abundant ASF457 bacteria near the epithelium (orange, ×600 magnification at arrow) and adherent to the surface epithelia (orange, ×900 magnification insert); Panel B = colon section hybridized with Cy3-457. Numerous ASF457 bacteria are observed within adherent mucus (orange, ×600 magnification at arrow) and translocated in multiple colonic glands (orange, ×900 magnification insert); Panel C = colon section hybridized with Cy3-Hel717. *Helicobacter bilis* bacteria are numerous within adherent mucus (closest to the epithelium) and attached to the surface epithelia (orange, ×600 and ×900 magnification insert); Panel D = colon section hybridized with Cy3-Hel717. *Helicobacter bilis* bacteria are clustered within a colonic gland (orange, ×600 and ×900 magnification insert) and attached to the surface epithelia (orange, lower right corner of image). All other bacteria in panels A–D that hybridize exclusively with the universal probe (Eub338-FITC) are shown in green. DAPI-stained colonic mucosa with goblet cells are shown in blue. Panels A and B show the proximal colonic tissues of 6 weeks *H. bilis*–infected mice. Panels C and D show the proximal colonic tissues of 3 weeks *H. bilis*–infected mice.

develop colitis after rederivation in *Helicobacter* spp.–free SPF settings; however, colitis developed once again in these mice after reintroduction of *H. bilis*.[34] Collectively, these data demonstrate a strong association between *Helicobacter* spp. infection, the intestinal microbiota, typhlocolitis, and/or the development of colon cancer in different murine models. The results of our investigation provide new information on how an intestinal provocateur, such as *H. bilis*, perturbs the mucosal microbiota and may provoke host responses to resident bacteria contributing to the development of colitis.[15]

Strong evidence exists for an important role of the resident gut microbiota in IBD pathogenesis because changes in microbial composition are frequently reported in Crohn's disease and ulcerative colitis patients[1,2,4,5] and in companion animals.[19,35,36] Although arguably mucosal bacteria are more critically involved in IBD versus luminal populations, which is consistent with the results presented here, feces is still often used to characterize the inflammation-associated changes in the intestinal microbiota.[9,37] This is despite the fact that mucosal bacterial populations in healthy humans may differ significantly from those in feces.[38] Reports investigating changes in the spatial distribution of the mucosal microbiota composition in the gut are relatively sparse. Previous studies using different patient populations, sampling sites, and/or FISH techniques have reported mucosal invasion,[39–41] a lower proportion of *Faecalibacterium prausnitzii,*[37] increased colonization by Enterobacteriaceae[41] or the gamma subdivision of Proteobacteria, and *Bacteroides*/*Prevotella*[39] or a dense noninvasive *Bacteroides* biofilm[40] on mucosal biopsy specimens of IBD patients. Similarly, the altered spatial structure and differential response of specific bacterial groups to mucosal inflammation in IL-10–deficient mice with colitis have been reported. The potential for localized repair of wounded or inflamed tissues holds promise for new therapeutic approaches.[42]

There are some potential limitations in this study. Regarding the effect of formalin fixation on integrity of the intestinal mucus layer[40] (versus Carnoy's fixative), our experiences using FISH in multiple species suggest that this is not a major problem and that the mucus layer remains largely intact even with routine tissue processing. We have previously demonstrated the presence of a epithelial mucus layer (using Alcian blue stain) in formalin-fixed colonic specimens of C3H mice involved in other studies (data not shown). Moreover, other investigators have shown the utility of FISH used on formalin-fixed biopsy specimens obtained from the GI tract of companion animals[19,20,43] and humans.[37,44–47]

Another potential factor impacting quantification of mucosal bacteria might be mechanical artifacts associated with tissue processing (microtome cutting) and/or nonintended wash of biopsy specimens by formalin during transport to the pathology laboratory.[48] Our previous experiences have allowed us to readily identify these tissue artifacts (in companion animals and mice) and to avoid these areas, if present, when performing mucosal bacterial counts.

Lastly, differences in colonization dynamics of ASF bacteria may have impacted mucosal bacterial populations in mice of the present study. For example, the distribution and colonization of select ASF bacteria were shown to be influenced by differences in age, gender, and infection status with *H. hepaticus* in outbred Swiss Webster mice.[49] Whether these same factors influenced colonization levels of mucosal bacteria in the C3H mice of our study will require further investigation.

Given that each member of the ASF is now sequenced, use of defined microbiota C3H/HeN mice harboring the ASF as a resident microbial community will facilitate the ability to exploit mechanisms governing host–microbiota relationships associated with changes in diet, introduction of various pathobionts, and induction of inflammation. In addition, the ability to identify each member of a stable microbial consortium will allow us to interrogate the interactions between each member of the microbiota and assess functional dysbiosis using RNA-Seq. As has been pointed out previously,[50] the ASF is an excellent and relevant enteric model community based on the observations that mice

colonized with the ASF exhibit normal GI function, immune system development, and overall health. Furthermore, the ASF community is stably transmitted from dam to offspring as evidenced over 16 years of continuous breeding within our gnotobiotic facility.[16,21] Lastly, the use of the ASF murine model allows for the controlled population of murine GI tracts and simplifies the examination of the colonic microenvironment relevant to GI pathologies, such as idiopathic IBD.

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