

## Original Research

# Fecal Bacterial Microbiota of Healthy Free-Ranging, Healthy Corralled, and Chronic Diarrheic Corralled Rhesus Macaques (*Macaca mulatta*)

Nicole R Compo,<sup>1,2</sup> Luis Mieles-Rodriguez,<sup>1</sup> and Diego E Gomez<sup>3,4</sup>

A clinical challenge to nearly every primate facility in North America is chronic idiopathic diarrhea (CID), the pathogenesis of which has yet to be fully elucidated. However, wild macaques appear resistant to CID, a trend that we observed in the free-ranging population of the Caribbean Primate Research Center. The gastrointestinal microbiota has been shown to have a significant role in the pathogenesis of disease and in maintaining normal health and development of the gut. In humans, chronic diarrhea is associated with alteration of the gut microbiota, which has lower bacterial diversity than does the microbiota of healthy humans. The current study was designed to describe and compare the fecal bacterial microbiota of healthy corralled, CID corralled, and healthy, free-ranging macaques. Fresh fecal samples were collected from healthy corralled (HC;  $n = 30$ ) and CID ( $n = 27$ ) rhesus macaques and from healthy macaques from our free-ranging colony (HF;  $n = 43$ ). We excluded macaques that had received antibiotics during the preceding 60 d (90 d for healthy animals). Bacterial DNA was extracted, and the V4 region of the 16S rRNA gene was sequenced and compared with known databases. The relative abundance of Proteobacteria was higher in CID animals than HC animals, but otherwise few differences were found between these 2 groups. HF macaques were differentially enriched with Christensenellaceae and *Helicobacter*, which are highly associated with a 'healthy' gut in humans, as compared to corralled animals, whereas CID animals were enriched with Proteobacteria, which are associated with dysbiosis in other species. These results indicate that environment has a greater influence than health status on the gut microbiota. Furthermore, the current data provided targets for future studies on potential clinical interventions, such as probiotics and fecal transplants.

**Abbreviations:** CID, chronic idiopathic diarrhea; HC, healthy corralled; HF, healthy free-ranging; IBD, inflammatory bowel disease; PCoA, principal coordinate analysis; SSFS, Sabana Seca Field Station

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Chronic idiopathic diarrhea (CID; also called idiopathic chronic diarrhea and chronic enterocolitis) is a clinical challenge that plagues nearly every large primate facility in North America. For example, the Oregon National Primate Center reports that CID comprises nearly 30% of their clinical caseload.<sup>20</sup> At the Caribbean Primate Research Center, a review of the medical records database at the Sabana Seca Field Station (SSFS), where animals are housed in large, outdoor corrals, indicates that treatment for diarrhea comprises nearly 50% of the clinical caseload.

Information on CID in wild macaques is sparse, and an exact cause for CID in research macaques has not been identified, despite extensive study. Fecal bacterial culture has yielded mixed results, with no specific pathogen consistently isolated from animals with CID. An increased prevalence of *Campylobacter*,

*Shigella*, and *Yersinia* species in animals with chronic diarrhea compared with healthy animals has been reported.<sup>59</sup> However, the overall prevalence in diarrheic animals was around 25% for *Campylobacter* and well below 25% for *Shigella* and *Yersinia*.<sup>59</sup> Similarly, one study reported that approximately 30% of chronic diarrheic animals had at least one historic bout of diarrhea that was culture positive and 40% culture positive for *Campylobacter* at the time of necropsy.<sup>38</sup> Others have reported that fecal cultures are regularly negative for these and other common gastrointestinal pathogens,<sup>28,38</sup> which is consistent with our experience.

The collective, interacting genomes of the symbiotic microorganisms in the gastrointestinal tract are referred to as the gastrointestinal microbiome.<sup>34</sup> The microbiome has a significant role in the pathogenesis of disease and contributes to normal health and development of the gut.<sup>19,67</sup> In humans, chronic diarrhea due to *Clostridium difficile* infection is associated with alteration of the gut microbiota (also known as dysbiosis), which has lower bacterial diversity than does the microbiota of healthy humans. This finding led to the successful use of fecal bacterial transplantation to restore the flora to normal.<sup>17,39</sup> Similarly, our group identified significant differences in the bacterial microbiota and

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<sup>1</sup>Caribbean Primate Research Center, Unit of Comparative Medicine, University of Puerto Rico, Sabana Seca, Puerto Rico; <sup>2</sup>SoBran Bioscience, Norfolk, Virginia; <sup>3</sup>Department of Large Animal Clinical Studies, College of Veterinary Medicine, University of Florida, Gainesville, Florida; and <sup>4</sup>Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

\*Corresponding author. Email: ncompo@sobran-inc.com

enrichment of Proteobacteria (a phylum associated with dysbiosis) in diarrheic calves and horses as compared with healthy ones.<sup>3,23</sup> We also reported that diarrheic calves had lower relative abundance of genes responsible for metabolism of various nutrients, indicating that nutrient availability can be altered in diarrheic states.<sup>21</sup> A better understanding of the organisms present in the gut of healthy and diarrheic macaques may offer new insights into the pathogenesis of this condition, and lead to new approaches to prevent and treat CID in NHP.

The current study was designed to describe and compare the fecal bacterial microbiota of healthy free-ranging, semiwild rhesus macaques (HF group), healthy macaques living in large, outdoor corrals (HC group), and corralled macaques with CID. The composition of the fecal bacterial microbiota from these 3 groups was compared to determine whether differences in bacterial composition are present among the groups. Identification of such changes may provide feasible starting points for studying the role of the intestinal microbiota in the pathophysiology of CID and possible treatment and preventive measures.

## Materials and Methods

**Animal housing.** The study was conducted at 2 AAALAC-accredited field sites using Indian-origin rhesus macaques (*Macaca mulatta*). At the SSFS, macaques are socially housed in outdoor corrals. At the Cayo Santiago Field Station, animals are free ranging on a 15.2-ha (37.5 acres) island near Puerto Rico and live in distinct social groups. At both stations, animals are fed Teklad NIB Primate Diet (Envigo, Madison WI) and provided filtered water, although both populations regularly consume standing water that results from the ample rain of Puerto Rico. At SSFS, animals receive additional food enrichment, such as fresh produce, seeds, and popcorn. At Cayo Santiago, free-ranging animals can forage on native foliage found on the island.

Housing and care for all animals are provided in accordance with the standards set forth in the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act.<sup>1,29</sup> All procedures for this study were approved by the Caribbean Primate Research Center IACUC (protocol no. 338300).

**Sample size calculation.** The sample size was determined by using a Dirichlet multinomial distribution model.<sup>37</sup> With an expected number of 20,000 sequence reads (per animal) available for comparison and an  $\alpha$  of 1%, 25 subjects per group were required for a power of 90%.

**Animal selection.** HC macaques were selected according to the schedule for upcoming routine semiannual exams. A list of male and female macaques, 2 to 10 y old, from a variety of social groups was created. Medical records were reviewed, and animals were excluded for any of the following criteria: previous episode of diarrhea, recent use (<90 d from sample collection) of antibiotics for any reason, and other clinical comorbidities. Once animals were determined to be eligible for inclusion, samples were collected directly from the rectum during routine annual exams performed under sedation with ketamine (10 to 15 mg/kg IM; Dechra Veterinary Products, Overland Park, KS).

CID macaques were all housed in corrals. Colony animals were screened using our internal database system for a diagnosis of 'diarrhea, chronic' and age 2 to 10 y. From the selection of animals, individual medical records were reviewed. Animals diagnosed with CID were considered for inclusion in the study when at least one of the following was met: documented as having diarrhea for  $\geq 30$  d during the preceding 90 d; multiple episodes of diarrhea unresponsive to nonantimicrobial treatment; permanent removal from the social group due to ongoing need for treatment; or removal from social group at least 3 times for

diarrhea treatment within preceding 1 y.<sup>20</sup> Once this list of eligible animals was created, it was cross-referenced whenever an animal presented to the clinic for diarrhea, as determined by animal health technician during daily observations. The clinical veterinarian (NC) then conducted a secondary review of the individual medical record to determine final eligibility for inclusion. Animals with CID were excluded from the study if any antimicrobial had been administered within the 60 d period preceding sample collection or if any concurrent health problem was diagnosed by the veterinarian. Different criteria for recent antibiotic use were used for CID and HC groups because it was prohibitively difficult to identify CID animals that had not received antibiotics within 90 d of sample collection. Approximately 10 g of fresh fecal samples were taken from the collection pan of animal's cage immediately after observation of a bowel movement, labeled, and stored in a  $-80^\circ$  freezer until processing. All samples were collected during May through July 2018.

Samples from HF animals were collected during the annual trapping event during October through December 2018. As part of the ongoing colony management to address overpopulation on HF, a single social group is removed from the island each year. Animals were trapped in feeding corrals, sedated with ketamine (10 to 15 mg/kg IM), and placed in a transport cage overnight with food and water. The next morning, animals were transported to SSFS. Animals were sedated with ketamine (10 to 15 mg/kg IM) and xylazine (100 mg/mL, 0.1 mL/animal IM; Bayer, Shawnee Mission, KS), various morphometric data were collected, and animals were euthanized by intravenous overdose of sodium pentobarbital (390 mg/mL, 1 mL/5 kg; Med-PharmEx, Pomona, CA). Macaques approximately 2 to 10 y old were selected for sample collection when they appeared generally healthy, were in good body condition, and had formed stool. Fecal samples were collected directly from the rectum, labeled, and stored in a  $-80^\circ$  freezer until processing.

**DNA extraction.** Frozen samples were softened (typically approximately 2 h) until they could be reasonably and gently stirred to homogenize in a biosafety cabinet. A commercial DNA extraction kit (E.Z.N.A. Stool DNA Kit, Omega Bio-Tek, Norcross, GA) was used to extract and isolate bacterial DNA from the stool sample according to the manufacturer's protocol. Briefly, 0.2 g of stool was placed in a collection tube, and samples were lysed in a formulated detergent-containing buffer. After a heat-freeze step, proteins, polysaccharides, and cellular debris were precipitated by using a buffer in the kit. A provided reagent and buffer were used to bind DNA and remove contaminants after centrifugation. The supernatant was transferred to a HiBind DNA Mini Column, the column was washed to remove trace contaminants, and the purified DNA was eluted with elution buffer. The buffered DNA was stored in a  $-20^\circ$  freezer until sequencing.

**Targeted library preparation.** The samples were processed and analyzed by using the ZymoBIOMIH Targeted Sequencing Service for Microbiome Analysis (Zymo Research, Irvine, CA). Bacterial 16S ribosomal RNA gene targeted sequencing was performed by using the Quick-16S NGS Library Prep Kit (Zymo Research). The bacterial 16S primers amplified the V4 region of the 16S rRNA gene. The sequencing library was prepared by using a library preparation process in which PCR reactions were performed in real-time PCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together to achieve equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean and Concentrator (Zymo Research) and then quantified by using TapeStation

**Table 1.** Demographics of rhesus macaques from which fecal samples were collected and used in the final analysis. Healthy HF = healthy free-ranging, Healthy SSFS = healthy corralled, CID SSFS = corralled animals with chronic idiopathic diarrhea.

Group	Median age (y; range)	Weight (kg; mean $\pm$ 1 SD)	no. of males	no. of females	Total
Healthy free-ranging	5.5 (0.5–14.5)*	5.0 $\pm$ 2.6	17	26	43
Healthy corralled	4.2 (2.1–9.5)	5.9 $\pm$ 2.5	15	15	30
CID	4.1 (1.8–10.3)	5.7 $\pm$ 2.6	9	18	27

\*Rounded to nearest half-year because exact birthdates for the free-ranging population are unknown

(Agilent Technologies, Santa Clara, CA) and Qubit (Thermo Fisher Scientific, Waltham, WA).

**Control samples.** The ZymoBIOMIHF Microbial Community DNA Standard (Zymo Research) was used as a positive control for each targeted library preparation. Negative controls (i.e., blank extraction control, blank library preparation control) were included to assess the bioburden level due to the wet-lab process.

**Sequencing.** The final library was sequenced on Illumina MiSeq with a V4 reagent kit (600 cycles; Zymo Research). The sequencing was performed by spiking with at least 10%  $\phi$ X DNA.

**Bioinformatic and statistical analysis.** Unique amplicon sequences were inferred from raw reads, and chimeric sequences were removed by using the DADA2 pipeline.<sup>9</sup> Taxonomy assignment was performed by using Uclust from QIIME version 2.0 and the Zymo Research Database, a 16S RNA database that is internally designed and curated, as a reference. Composition visualization,  $\alpha$  diversity,  $\beta$  diversity analyses, Bray–Curtis dissimilarity matrices, and weighted and unweighted UniFrac distances were performed by using QIIME version 2.0.<sup>10</sup> Vegan function ADONIS was used to perform PERMANOVA to assess differences in  $\beta$  diversity. Taxonomic groups that had significant enrichment among different groups were identified by linear discriminant analysis for effect size by using default settings.<sup>58</sup> Those default settings were  $\alpha$  parameters for pairwise tests set to 0.05 for both class normality and subclass tests, and the threshold on the logarithmic score of linear discriminate analysis was set to 2.0. Analysis of heatmaps and principal coordinate analysis plots were performed with internal scripts.

Animal weights and ages were checked for normal distribution by using the Shapiro–Wilk goodness-of-fit test within JMP (version 15.1, SAS, Cary, NC). Differences between groups in weight and age were assessed by using the Student *t*-test and Kruskal–Wallis rank–sum test, respectively. Differences in the relative abundance of taxa and  $\alpha$ -diversity parameters between groups were determined by using the Steel–Dwass method for nonparametric comparisons for all pairs (version 15.1, SAS). *P* values were adjusted for multiple comparisons according to the Benjamini–Hochberg false discovery rate.<sup>5</sup>

## Results

**Animal demographics.** A total of 100 samples were collected and sequenced from rhesus macaques at 2 fields sites. Animal weights were normally distributed, whereas animal ages were not. Neither weight nor age differed between groups (Table 1). Significantly more female than male macaques were included in the HF and the CID groups as compared with HC group, but no differences between male and female animals for any parameter, and thus data from both sexes were considered together throughout (data not shown).

**Rhesus macaque fecal microbiota: overall assessment.** A total of 15,765,498 raw sequences were obtained, with a range of 57,330 to 327,494 and median of 157,655 sequences per sample.

A subsample of 20,000 reads per sample was used to normalize sequence numbers across samples and was considered adequate, as evidenced by greater than 99% coverage for all samples and plateau of rarefaction curves (data not shown).

A total of 17 bacterial phyla were identified; however, only 6 had greater than 1% of the total sequences identified, and Firmicutes and Bacteroidetes together accounted for over 90% of the total sequences (Table 2). Bacteria were identified from 209 genera; however only 11 included more than 1% of the total sequences identified and could be classified to a known genus (Table 3). A total of 30 orders, 18 classes, and 81 families were identified (Tables 4, Table 5 and 6).

**$\alpha$  and  $\beta$  diversity.** HC and CID animals showed no differences ( $P > 0.05$ ) in the richness, evenness, or diversity of organisms identified (Chao 1, Shannon, and inverse Simpson analyses, respectively). These groups were therefore combined into a single group for comparison with HF animals. Samples from the combined HC+CID group were more rich, even, and diverse in bacterial organisms than were samples from HF animals ( $P = 0.034, 0.001, \text{ and } 0.008$ , respectively; Figure 1).

Principal coordinate analysis of the Bray–Curtis distance showed significant differences in the fecal microbiota of the macaques according to housing site but not health status (PERMANOVA  $P < 0.05$ ), separating along principal component 1 and explaining about 20% of the total variation in the data (Figure 2). Similarly, principal coordinate analysis of unweighted UniFrac distances show clustering of fecal samples by housing site (PERMANOVA  $P < 0.05$ ), but not by disease status. The samples separate along principal component 1, which explains approximately 26% of the total variation in  $\beta$  diversity (Figure 2).

**Linear discriminant analysis for effect size.** When comparing HC macaques, corralled macaques with CID, and HF macaques, a total of 127, 221, and 236 bacterial taxa (excluding species) were enriched, respectively, among 355 total taxa identified. Of the 3 groups, feces from HF animals had a higher number of bacterial taxa enriched from phylum Firmicutes (173, compared with 68 from HC macaques and 52 from animals with CID). In contrast, macaques with CID had more taxa enriched from phylum Proteobacteria (43, compared with 13 from HF and 5 from HC macaques; Figure 3). Figure 4 shows those taxa found to be enriched in HC compared with HF macaques. Figure 5 highlights the higher number of members of the phylum Proteobacteria, including *Campylobacter*, in HC compared with CID animals. Those phyla, classes, orders, families, and genera with a linear discriminant analysis score greater than 3.5 among all 3 groups are summarized in Table 7, and selected taxa of clinical interest, *Helicobacter*, *Campylobacter*, and *Clostridium* are shown in Figures 6, Figure 7 and 8. The phylogenetic relationships among *Helicobacter* species and strains, the only Proteobacteria differentially enriched in HF macaques, are depicted in Figure 9.

**Table 2.** Relative abundance of predominant bacterial phyla (overall relative abundance >0.5%) present in the feces of rhesus macaques

Phylum	Healthy free-ranging (HF)	Healthy corralled (HC)	CID	HF compared with HC	HF compared with CID	HC compared with CID	HF compared with HC+CID
	Median (range)			P			
Firmicutes	73 (53–95)	65 (44–92)	61 (4.6–86)	<b>0.004</b>	<b>0.003</b>	0.511	<b>&lt;0.001</b>
Bacteroidetes	19 (3.0–35)	28 (5.0–50)	29 (0.7–46)	<b>&lt;0.001</b>	<b>0.009</b>	0.983	<b>&lt;0.001</b>
Proteobacteria	1.3 (0–11)	0.9 (0.1–5.4)	1.7 (0.1–76)	0.212	0.320	<b>0.007</b>	not applicable
Spirochaetae	0.8 (0.0–3.8)	0.7 (0.1–8.1)	1.3 (0.0–7.6)	0.937	0.074	0.308	0.138
Actinobacteria	0.5 (0.0–3.1)	0.5 (0.2–1.3)	0.4 (0.1–56)	0.558	0.308	0.558	0.136
Euryarchaeota	0.0 (0.0–15)	0.4 (0.0–3.3)	0.4 (0.0–5.0)	0.203	<b>0.039</b>	0.354	<b>0.049</b>
Tenericutes	1.0 (0.0–3.1)	0.4 (0.0–1.1)	0.4 (0.0–2.0)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.986	<b>&lt;0.001</b>

**Table 3.** Relative abundance of predominant bacterial genera (overall relative abundance >1.0%) present in the feces of rhesus macaques

Genus	Healthy free-ranging (HF)	Healthy corralled (HC)	CID	HF compared with HC	HF compared with CID	HC compared with CID	HF compared with HC+CID
	Median (range)			P			
<i>Lactobacillus</i>	22 (0.0–73)	20 (1.4–42)	12 (0.0–41)	0.302	<b>0.009</b>	0.088	not applicable
<i>Prevotella</i>	2.4 (0.4–7.1)	4.5 (0.2–12)	4.0 (0.0–8)	0.002	0.165	0.685	<b>0.008</b>
<i>Streptococcus</i>	0.2 (0.0–11)	3.4 (0.2–17)	1.3 (0.1–9.1)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.099	not applicable
<i>Phascolarctobacterium</i>	0.8 (0.0–5.5)	2.6 (0.3–5.3)	1.5 (0.0–8.8)	<b>0.001</b>	0.321	0.070	not applicable
<i>Alloprevotella</i>	0.4 (0.0–2.8)	2.3 (0.1–6.2)	3.0 (0.0–8.6)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.181	<b>&lt;0.001</b>
<i>Faecalibacterium</i>	0.6 (0.0–5.1)	1.8 (0.1–4.9)	2.4 (0.1–4.8)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.309	<b>&lt;0.001</b>
<i>Blautia</i>	0.9 (0.1–4.9)	1.1 (0.3–4.7)	1.0 (0.0–3.1)	0.274	0.622	0.654	0.209
<i>Subdoligranulum</i>	0.9 (0.1–2.6)	1.4 (0.1–4.1)	1.6 (0.1–7.7)	<b>0.045</b>	<b>0.016</b>	0.436	<b>0.002</b>
<i>Megasphaera</i>	0.0 (0.0–0.1)	0.7 (0.0–5.7)	2.3 (0.1–11)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.008</b>	<0.001
<i>Roseburia</i>	0.3 (0.0–3.0)	1.5 (0.2–2.7)	1.3 (0.0–3.2)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.715	<b>&lt;0.001</b>
<i>Ruminococcus</i>	1.2 (0.0–8.8)	0.8 (0.3–2.0)	0.3 (0.0–2.0)	<b>0.018</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	not applicable

## Discussion

A number of studies<sup>20,28,32,38,47,55,57,59</sup> have explored CID in captive macaques, but, to our knowledge, none have specifically characterized and compared the fecal microbiomes of healthy adult and diarrheic animals or of corralled and free-ranging animals by using 16S rRNA gene sequences. The 2 field sites of the Caribbean Primate Research Center offer a unique opportunity to compare macaques in a semiwild state with those in a more traditional large, outdoor-corral facility. Animals at both sites received the same standard monkey chow, had access to purified water, and lived in the same climate and thus offer the most directly comparable populations of free-ranging and corralled

NHP that we know of, with one population afflicted with CID and the other unaffected to any significant degree. The HF macaques undergo no veterinary intervention (except for annual health exams, which include tuberculosis testing and vaccination against tetanus) and therefore have never received antibiotics or other treatment that may alter the gut microbiota. In the current study, we showed that rhesus macaques from the same housing site, regardless of disease status, were more similar in fecal bacterial composition than animals of similar health status at different housing sites with different housing modalities. This finding is in agreement with studies in other mammals.<sup>15,23</sup>

**Table 4.** Relative abundance of predominant bacterial phyla (overall relative abundance >0.5% among 17 classifiable orders) present in the feces of rhesus macaques

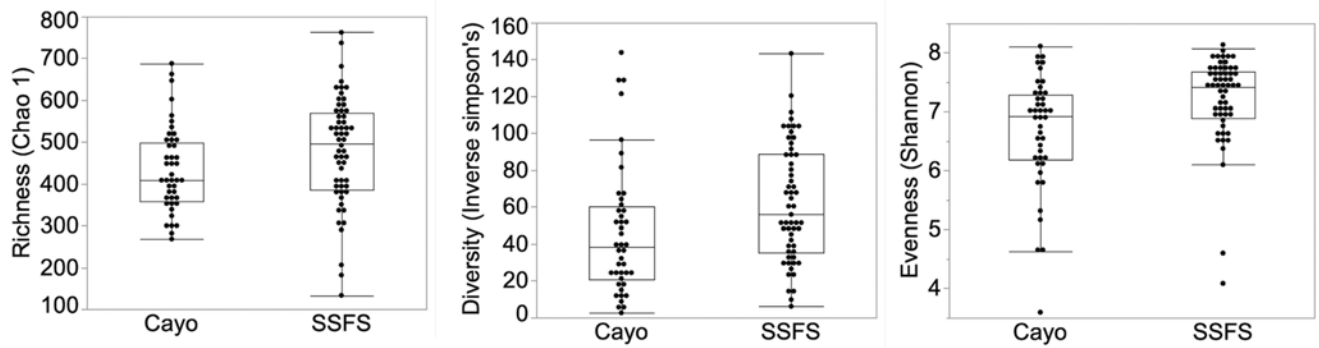
Class	Healthy free-ranging (HF)			Healthy corralled (HC)			CID			HF compared with HC	HC compared with CID	HF compared with CID	HF compared with HC+CID
	Min	Max	Median	Min	Max	Median	Min	Max	Median				
Clostridia	12	66.6	42.2	24.1	60.9	32.45	2.4	56.4	32.6	<0.001	0.017	0.799	<0.001
Bacteroidia	3	34.9	18.6	5	49.9	29.25	0.7	46	29	0.004	0.005	0.799	<0.001
Bacilli	0.2	72.9	22.9	2.4	45.7	26	0.3	41.8	16.3	0.799	0.064	0.040	n/a
Negativicutes	0	5.8	1.9	2.3	12.3	5.3	0.2	17.4	6.7	<0.001	<0.001	0.050	n/a
Erysipelotrichia	0.5	7.4	1.7	0.5	3.6	1.05	0.1	16	1.2	0.011	0.128	0.263	0.004
Spirochaetes	0	3.8	0.8	0.1	8.1	0.7	0	7.6	1.3	0.953	0.031	0.191	0.138
Methanobacteria	0	14.7	0	0	3.3	0.35	0	5	0.4	0.195	0.038	0.354	0.050
Epsilonproteobacteria	0	3.7	0.3	0	1.3	0.1	0	48.9	0.7	0.010	0.405	0.001	n/a
Mollicutes	0.1	6.4	0.8	0	3.7	0.65	0	3.1	0.8	<0.001	<0.001	0.802	<0.001
Actinobacteria	0	3.1	1	0	1.1	0.4	0	2	0.4	0.090	0.031	<0.001	n/a
Deltaproteobacteria	0	1.1	0	0	0.1	0	0	55.7	0	0.493	0.411	0.987	0.366

**Table 5.** Relative abundance of predominant bacterial phyla (overall relative abundance >0.5% among 27 classifiable orders) present in the feces of rhesus macaques

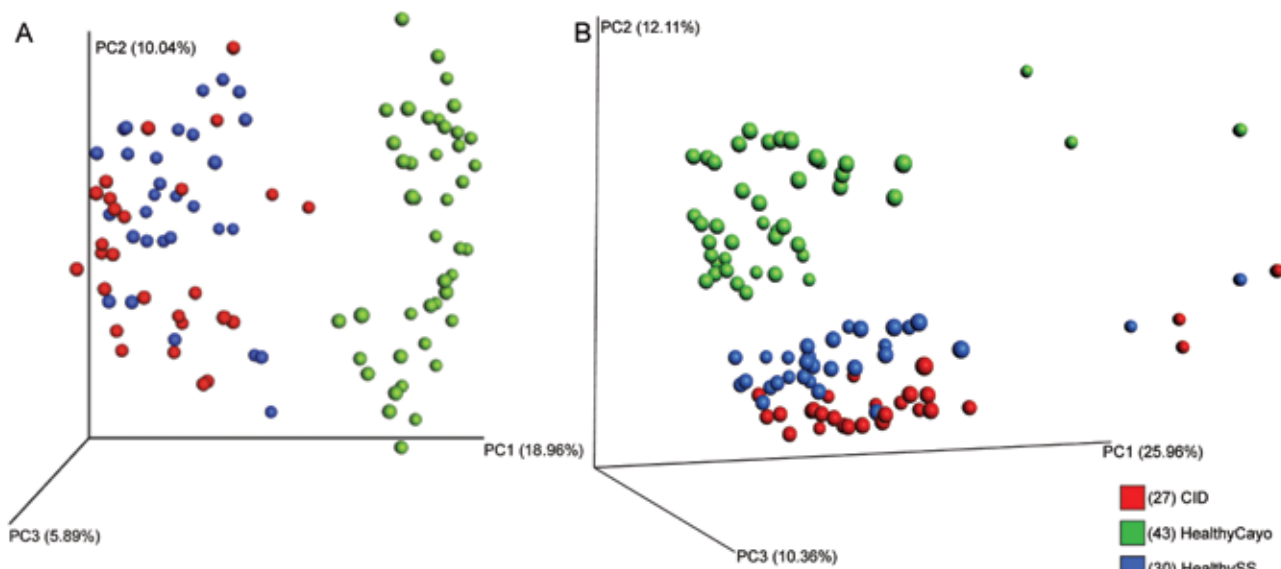
Order	Healthy free-ranging (HF)			Healthy corralled (HC)			CID			HF compared with HC	HF compared with CID	HC compared with CID	HF compared with HC+CID
	Min	Max	Median	Min	Max	Median	Min	Max	Median				
Bacteroidales	3	34.9	18.6	5	49.9	29.25	0.7	46	29	<0.001	0.020	0.998	<0.001
Methanobacteriales	0	14.7	0	0	3.3	0.35	0	5	0.4	0.482	0.277	0.634	0.047
Corynebacteriales	0	0	0	0	0	0	0	55.4	0	1.000	0.365	0.417	0.222
Coriobacteriales	0	2	0.4	0.2	1.3	0.5	0.1	2.3	0.5	0.582	0.993	0.993	0.357

**Table 6.** Relative abundance of predominant bacterial phyla (overall relative abundance >0.5% among 69 classifiable families) present in the feces of rhesus macaques

Family	Healthy free-ranging (HF)			Healthy corralled (HC)			CID			HF compared with HC	HF compared with CID	HC compared with CID	HF compared with HC+CID
	Min	Max	Median	Min	Max	Median	Min	Max	Median				
Lactobacillaceae	0	72.9	22.5	1.4	42	20.3	0	40.9	12	0.291	0.009	0.090	not applicable
Ruminococcaceae	5.2	47.6	24.1	10.6	41.6	15.65	1.5	28.2	14.6	<0.001	<0.001	0.523	<0.001
Prevotellaceae	1.2	26.6	9.5	4.3	43.6	25.3	0.7	36.1	25.6	<0.001	<0.001	0.696	<0.001
Lachnospiraceae	4.6	24.8	12	7.1	25.4	13.35	0.4	27.8	15.3	0.352	0.149	0.131	0.088
Unclassified Bacteroidales	0.1	14.6	3.9	0.3	12.8	2.6	0	26.7	2.3	0.040	0.026	0.808	0.003
Veillonellaceae	0	2.1	0.2	0.1	10.9	2.5	0.2	14.4	5.3	<0.001	<0.001	0.016	not applicable
Streptococcaceae	0	11.2	0.2	0.2	16.7	3.4	0.1	9.2	1.3	<0.001	<0.001	0.094	not applicable
Acidaminococcaceae	0	5.5	0.8	0.3	5.3	2.55	0	9.1	1.7	<0.001	0.146	0.161	0.001
Rikenellaceae	0.1	8.6	1.6	0	10.7	0.85	0	5.3	1.1	0.017	0.006	0.630	0.004
Christensenellaceae	0.1	7.4	2.9	0.1	3	0.65	0	1.9	0.5	<0.001	<0.001	0.132	<0.001
Erysipelotrichaceae	0.5	7.4	1.7	0.5	3.6	1.05	0.1	16	1.2	0.010	0.139	0.262	0.004
Spirochaetaceae	0	3.8	0.7	0.1	8.1	0.7	0	5.7	1.3	0.874	0.024	0.189	0.098
Family XIII	0.2	3.2	1	0.5	3.2	1	0	4	1.2	0.294	0.072	0.319	0.041
Unclassified Clostridiales	0.1	5.9	1.2	0.2	3.5	0.95	0	2.1	0.5	0.467	0.001	0.001	not applicable
Campylobacteraceae	0	1.5	0	0	1	0	0	42.5	0.7	0.610	<0.001	<0.001	not applicable
Unclassified Mollicutes	0	3.1	1	0	1.1	0.4	0	1.9	0.3	<0.001	<0.001	0.729	<0.001
Corynebacteriaceae	0	0	0	0	0	0	0	55.4	0	<0.001	0.141	0.228	0.222



**Figure 1.**  $\alpha$  diversity indices of bacteria observed in the feces of rhesus macaques. (A) Richness. (B) Evenness. (C) diversity.

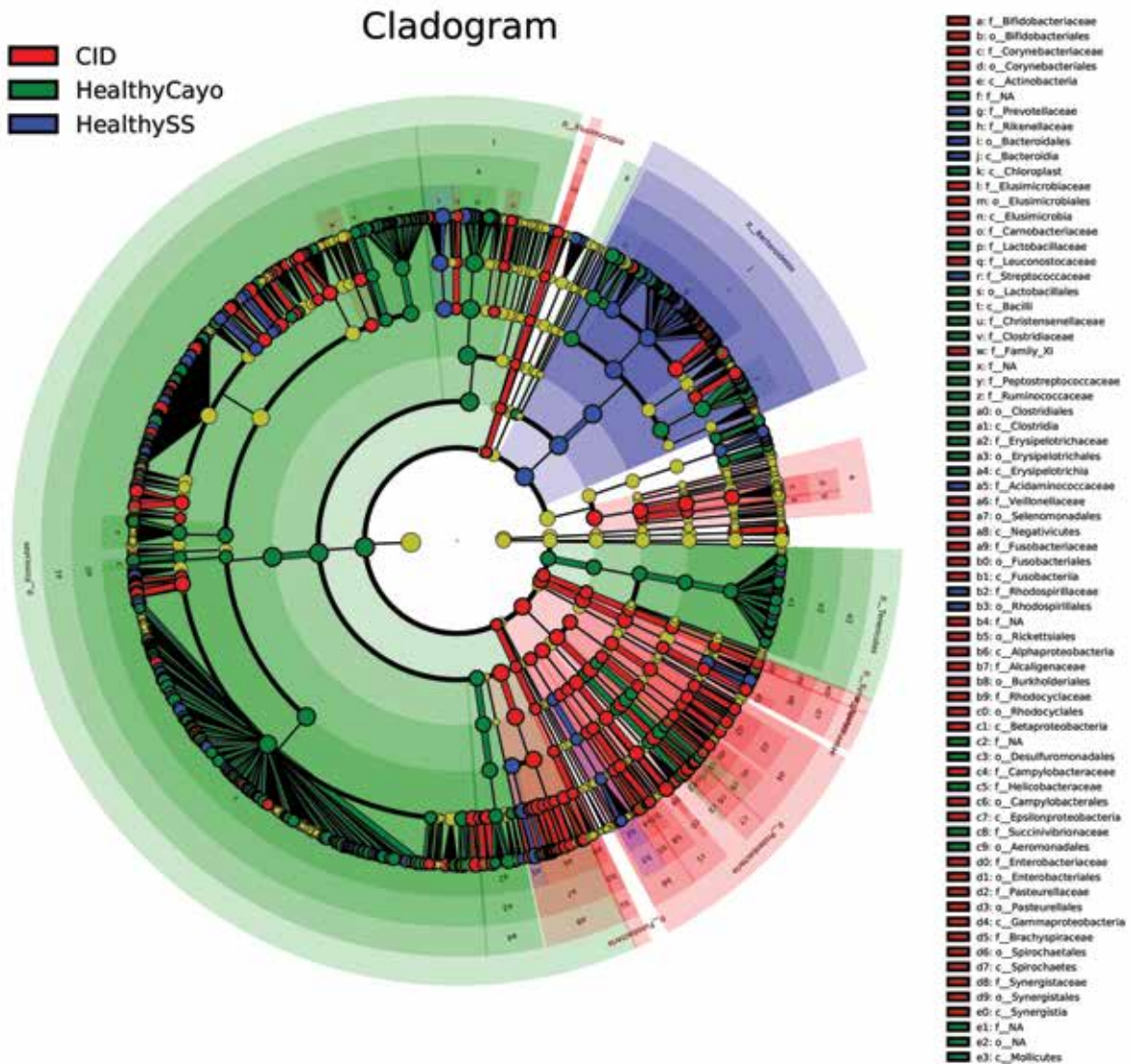


**Figure 2.** Principal coordinate analysis plot with (A) Bray–Curtis and (B) UniFrac unweighted distances, showing clustering of fecal bacterial composition by (A) abundance and (B) abundance and phylogenetic relatedness, respectively. Samples clustered significantly according to housing site but not health status.

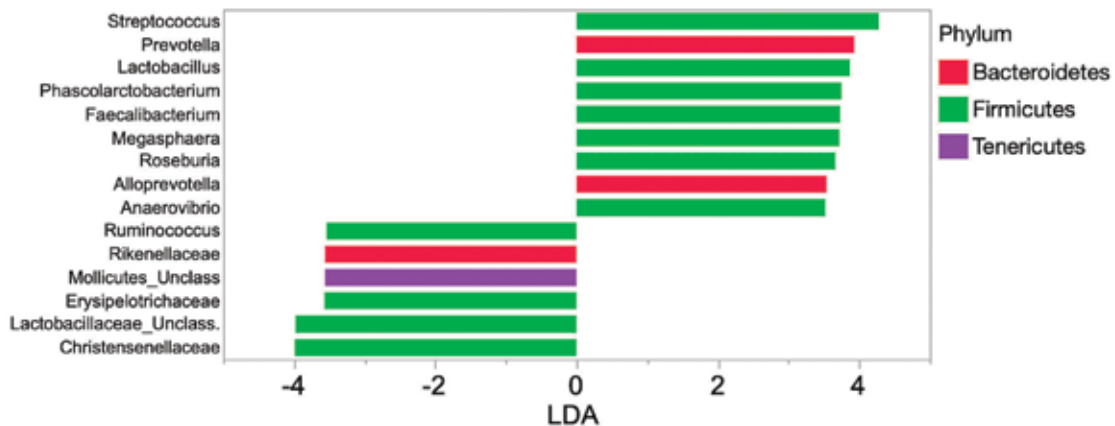
The 2 predominant bacterial phyla regardless of health status or housing modality were Firmicutes and Bacteroidetes, a finding that is consistent across species, including other NHP, humans, mink, rabbits, cows, and mice.<sup>15,18,23,36,57,61,68</sup> However, an overall lower relative abundance of Bacteroidetes was found in the HF macaques as compared with corralled animals and as compared with previously published reports in macaques.<sup>55,61,72</sup> In addition, taxa from the phylum Bacteroidetes were the most differentially enriched taxa of the HC animals, and were the only nonfirmicute taxa represented. Several studies in mice and humans have shown a negative correlation between obesity and the relative abundance of Bacteroidetes, with leaner subjects showing lower proportions of Bacteroidetes.<sup>31,40,41,64</sup> Although we did not account for body condition score and group ages and weights were similar, a cursory evaluation of the relationship between age and weight suggests that the free-ranging animals were leaner than their corralled counterparts. Free-ranging macaques had the highest mean age but the lowest mean weight, whereas HC animals had a lower mean age and highest mean weight. Given the frequent use of rhesus macaques as a model for human obesity and metabolic syndrome,<sup>4</sup> additional studies on the relationship between the gut microbiome and body

condition in this species are warranted. Reasons for these differences are unclear, but body condition, diet, or water sources may explain, at least in part, the disparities in the abundance of Bacteroidetes between groups.

Proteobacteria were differentially enriched in diarrheic animals. This phylum was the only one that differed in the relative abundance among HC and CID animals. This pattern is expected, given that higher proportions of Proteobacteria are regularly identified in diarrheic animals across species and are considered a hallmark of dysbiosis.<sup>21,23,33,44,51,60</sup> However, an unexpected finding was the lack of difference in the relative abundance of Proteobacteria between diarrheic and HF animals. In all other regards, the free-ranging animals would be considered to have a ‘normal’ or ‘healthy’ fecal microbiota, but the relative abundance of Proteobacteria in the HF group was even higher than that of the HC animals. This finding may challenge the hypothesis that, overall, enrichment of Proteobacteria is an indicator of dysbiosis or altered gut microbiota. Rather, it highlights the importance of taking into account gastrointestinal location when making generalizations regarding what determines a dysbiotic state. Certain bacteria may have a commensal or protective role in one



**Figure 3.** Cladogram created by using linear discriminant analysis for effect size showing differentially bacterial abundant taxa in fecal samples from healthy corralled (blue), chronic diarrheic corralled (red), and healthy semiwild (green) rhesus macaques.

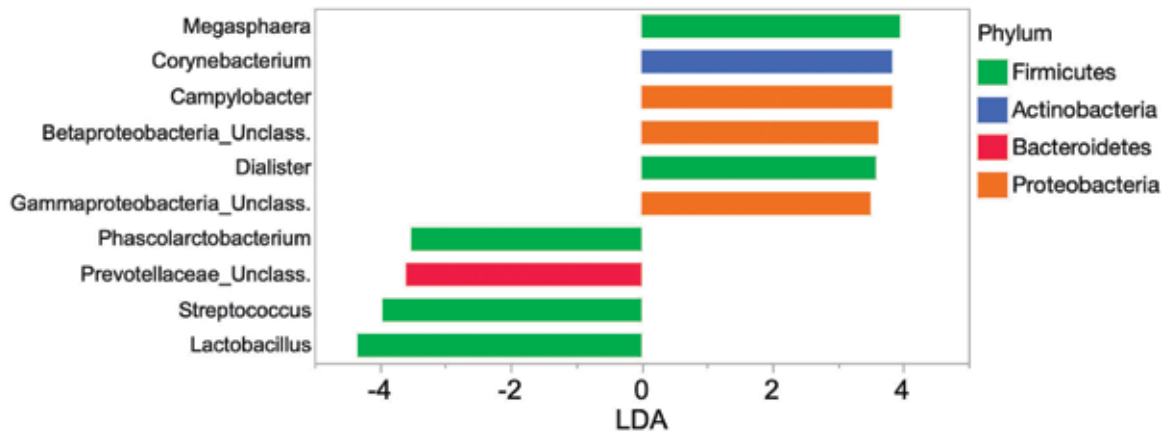


**Figure 4.** Linear discriminate analysis scores of differentially enriched taxa in fecal samples from healthy, semiwild (left panel) and healthy, corralled (right panel) rhesus macaques.

region of the gut but cause pathology if unchecked in other regions.

The unexpectedly high proportion of Proteobacteria in the free-ranging group is accounted for by the differential

enrichment of the genus *Helicobacter* and its corresponding family, Helicobacteraceae, the only bacteria from this phylum that was enriched in this group. One study reported that the colon from healthy control rhesus macaques had a superficial mucosa



**Figure 5.** Linear discriminant analysis scores of differentially enriched in fecal samples from healthy (left panel) and chronic diarrheic (right panel) corralled rhesus macaques.

densely populated with epithelium-adherent bacteria, identified as 100% *Helicobacter macacae*, whereas in animals with CID, these organisms were largely absent.<sup>38</sup> Similarly, healthy 8-mo-old rhesus macaques were differentially enriched with and had a significantly higher relative abundance of *H. macacae* in feces than did their diarrheic counterparts.<sup>55</sup> Studies in human diarrheal cases have reported an inverse relationship between *H. pylori* infection and diarrheal illnesses in children and lower risk of diarrhea of unknown origin and shigellosis in *H. pylori*-positive adults.<sup>14,56</sup> The literature on the relationship between *H. pylori* infection and inflammatory bowel disease (IBD) in humans, similar in nature to CID of macaques, is diverse. A meta-analysis found a higher prevalence of *H. pylori* infection in healthy people compared with those with IBD,<sup>45</sup> although the authors acknowledged that differing methodologies and possible publication bias may limit the certainty of their findings. Similar results were reported in a systematic review investigating the mechanisms underlying the potential link between *H. pylori* and IBD.<sup>52</sup> Moreover, a systematic review and meta-analysis found that *H. pylori* infection was indeed negatively associated with IBD, regardless of ethnicity, age, or detection methods.<sup>11</sup> Our findings, together with other studies in rhesus macaques<sup>39,56</sup> and extrapolation from the human literature, suggest that *H. macacae* may be important in maintaining homeostasis of the gut or exert a protective role in preventing CID.<sup>15,39,56,57</sup>

*Campylobacter* spp. are an important cause of diarrhea in humans and NHP worldwide, with the Center of Disease Control estimating that *Campylobacter* is the number-one cause of bacterial diarrheal illness in the United States.<sup>13</sup> Indeed, multiple studies have identified a higher prevalence of *Campylobacter* species in bacterial cultures of diarrheic as compared with asymptomatic rhesus macaques.<sup>38,59</sup> Similarly, using next-generation sequencing techniques, one study reported that identification of *Campylobacter* (*C. jejuni* and *C. coli*) was strongly associated with diarrhea, and 2 other studies each found that *Campylobacter* were differentially enriched in relative abundance and gene expression, respectively, in symptomatic as compared with asymptomatic counterparts.<sup>47,55,71</sup> However, identification of *Campylobacter* in as many as 42% of healthy subjects brings into question whether its presence is as a primary or an opportunistic pathogen.<sup>32,38,59</sup> *Campylobacter* spp. in diarrheic subjects overall had a much higher level of virulent gene expression than did *Campylobacter* spp. of healthy controls, and *Campylobacter*-specific transcriptomes suggested a closer association with the mucosa in chronic diarrhea than

in controls.<sup>71</sup> Taken together, differing expression of virulence factors, resulting in a more virulent phenotype, may explain why some animals positive for *Campylobacter* develop diarrhea whereas others do not.

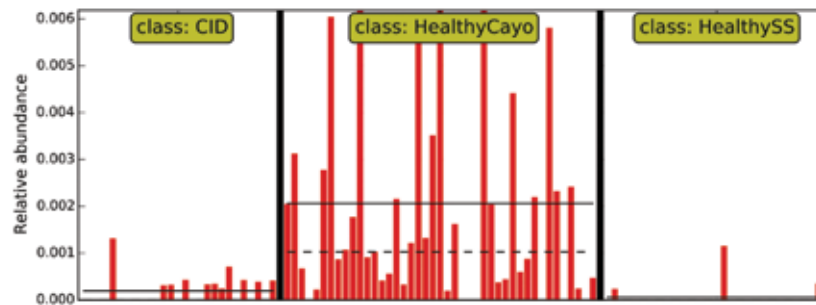
An alternative explanation for the presence of *Campylobacter* in diarrheic animals is rooted in its close phylogenetic relationship to *Helicobacter*, both of which are members of the order Campylobacterales (*Helicobacter* were previously classified as *Campylobacter* organisms). As previously discussed, *H. macacae* may have a role in prevention of CID. We showed that healthy macaques were differentially enriched with *Helicobacter* and diarrheic animals were differentially enriched with *Campylobacter*, mirroring results in healthy and diarrheic rhesus infants and in humans with and without IBD.<sup>11,55</sup> The inverse relationship between these closely related genera may represent a niche displacement event, with some inciting factor leading to displacement of *Helicobacter*, either prior to or concurrent with, increased expression of virulence factors by *Campylobacter*, ultimately leading to overgrowth and adherence of *Campylobacter*. The importance of this relationship is likely only recently being explored because of the inherent limitations of bacterial culture: *Helicobacter* is fastidious and requires specific, narrow culture conditions that are unlikely to be achieved unless specifically seeking to do so.<sup>6</sup> The use of next-generation sequencing allows the identification of organisms that have been historically difficult to culture. Although *Helicobacter* is known to be associated with gastric disease and ulcers in humans, macaques, and other mammalian species,<sup>6,14,56</sup> it is not generally considered to be an important cause of diarrhea; consequently, previous studies would have been unlikely to have specifically evaluated *Helicobacter*, such that its prevalence and thus its importance would not be recognized.

Bacteria of the class Clostridia are a diverse group of obligate anaerobes that we found to be enriched in HF rhesus macaques, as was its largest order, Clostridiales. This enrichment was due to a corresponding enrichment of 2 families: Ruminococcaceae and Christensenellaceae. Clostridia, including species of the infamous genus *Clostridium*, many of which produce highly pathogenic toxins and are associated with serious diarrheal disease in humans,<sup>7,12,35,49</sup> are known to digest cellulose and hemicellulose.<sup>65,73</sup> The free-ranging animals on the island of Cayo Santiago receive standard monkey chow daily but also are able to forage freely on the island fauna, whereas corralled animals at the SSFS have access to fresh produce (primarily fruits and seeds) only in limited

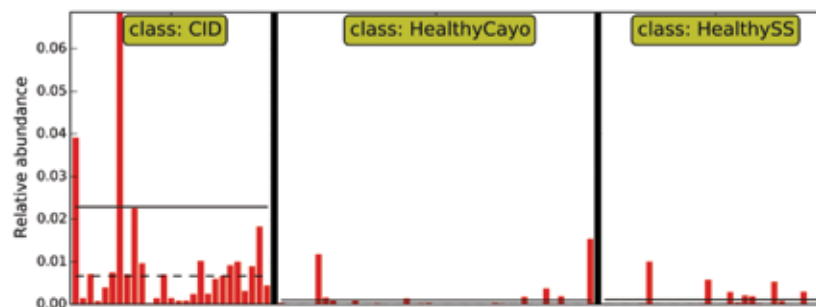


**Table 7.** Differentially enriched taxa with a linear discriminate analysis score (LDA) of  $\geq 3.5$  when comparing the feces of healthy free-ranging, healthy corralled, and chronic diarrheic rhesus macaques

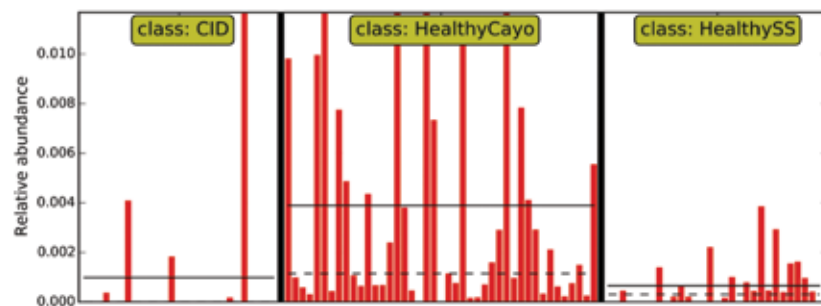
Group	Phylum	Taxonomic level	Taxon	LDA	
Healthy free-ranging	Firmicutes			4.8	
		Class	Bacilli	4.7	
		Class	Clostridia	4.6	
		Class	Erysipelotrichia	3.6	
		Order	Lactobacillales	4.7	
		Order	Clostridiales	4.6	
		Order	Erysipelotrichales	3.6	
		Family	Lactobacillaceae	4.8	
		Family	Ruminococcaceae	4.6	
		Family	Christensenellaceae	4.1	
		Family	Erysipelotrichaceae	3.6	
		Genus	<i>Lactobacillus</i>	4.8	
		Genus	<i>Ruminococcus</i>	3.8	
		Proteobacteria	Family	Helicobacteraceae	3.5
			Genus	<i>Helicobacter</i>	3.5
		Bacteroidetes	Family	Rikenellaceae	3.9
		Tenericutes			3.6
Mollicutes			3.6		
CID	Firmicutes	Class	Negativicutes	4.5	
		Order	Selenomonadales	4.5	
		Family	Veillonellaceae	4.4	
		Genus	<i>Megasphaera</i>	4.2	
		Genus	<i>Faecalibacterium</i>	3.9	
		Genus	<i>Dialister</i>	3.8	
		Genus	<i>Roseburia</i>	3.8	
		Genus	<i>Eubacterium</i>	3.6	
		Genus	<i>Subdoligranulum</i>	3.5	
		Proteobacteria			4.1
		Class	Epsilonproteobacteria	3.9	
		Class	Betaproteobacteria	3.6	
		Order	Campylobacteriales	3.9	
		Family	Campylobacteraceae	3.9	
		Family	Rhodocyclaceae	3.5	
		Genus	<i>Campylobacter</i>	3.9	
	Bacteroidetes	Genus	<i>Alloprevotella</i>	4.1	
	Spirochaetes		Spirochaetae	3.7	
		Class	Spirochaetes	3.7	
		Order	Spirochaetales	3.7	
	Actinobacteria			3.9	
		Order	Corynebacteriales	3.8	
		Family	Corynebacteriaceae	3.8	
	Genus	<i>Corynebacterium</i>	3.8		
Healthy corralled	Bacteroidetes			4.7	
		Class	Bacteroidia	4.7	
		Order	Bacteroidales	4.7	
		Family	Prevotellaceae	4.8	
		Genus	<i>Prevotella</i>	4.0	
	Firmicutes				3.7
		Class	Acidaminococcaceae	3.7	
		Family	Streptococcaceae	4.3	
		Genus	<i>Streptococcus</i>	4.3	
		Genus	<i>Phascolarctobacterium</i>	3.7	
		Genus	<i>Anaerovibrio</i>	3.5	



**Figure 6.** Linear discriminant analysis for effect size indicates that fecal samples from healthy, free-ranging rhesus macaques are differentially enriched with bacteria of the genus *Helicobacter*.



**Figure 7.** Linear discriminant analysis for effect size indicates that fecal samples from corralled rhesus macaques with CID are differentially enriched with bacteria of the genus *Campylobacter*.

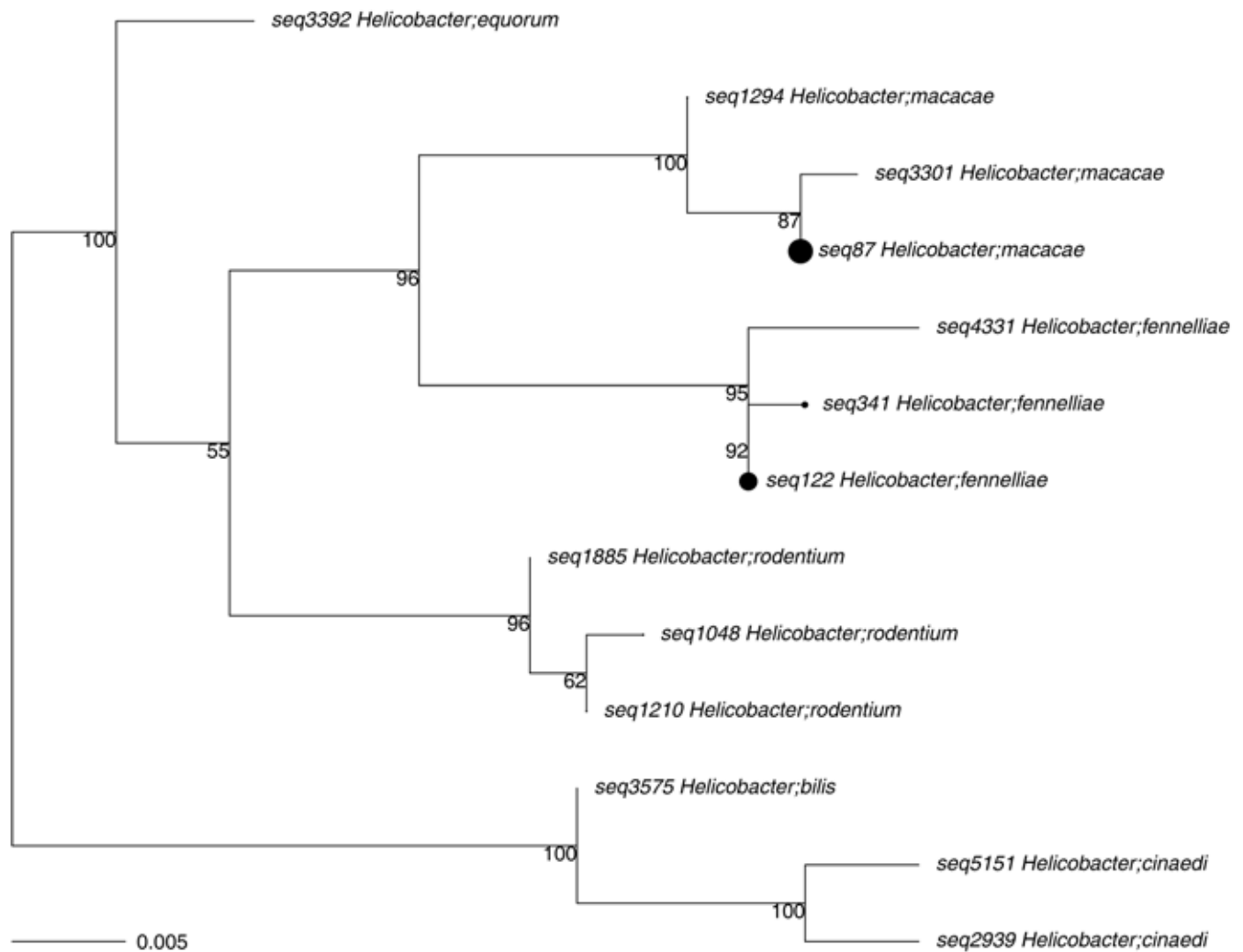


**Figure 8.** Linear discriminant analysis for effect size indicates that fecal samples from healthy, free-ranging rhesus macaques are differentially enriched with bacteria of the genus *Clostridium*.

supply as part of their enrichment program, suggesting the important role that diet plays in shaping the gut microbiome. Ruminococcaceae are depleted during chronic diarrhea in humans, horses, and piglets.<sup>2,16,26,62,66</sup> Christensenellaceae has recently been recognized as highly important to human health,<sup>69</sup> with several studies showing a highly negative correlation between relative abundance of Christensenellaceae and body mass index,<sup>8,43,54</sup> serum markers of metabolic disease,<sup>25,27,43</sup> and metabolic syndrome.<sup>25,42,50</sup> Moreover, Christensenellaceae is one of the most highly heritable and transmittable groups of bacteria in humans, with germ-free mice that receive feces amended with Christensenellaceae gain less weight than recipients of unamended feces.<sup>24</sup> Finally, Christensenellaceae was identified as a signature of a healthy gut in a meta-analysis of IBD<sup>46</sup> and is consistently depleted in individuals with IBD.<sup>22,30,51,53</sup> Taken all together, Ruminococcaceae and Christensenellaceae bacteria represent a reasonable starting point for experimental and interventional

studies aimed at using or optimizing the gut microbiome to combat CID, as well as obesity, in research macaques.

We recognize several limitations to the current study. Primarily, fecal samples are limited in their representation of all parts of the gut. In macaques, fecal samples were highly correlated with the microbiota of the large intestinal lumen and mucosa but less correlated with small intestinal luminal and mucosal samples.<sup>73</sup> The cited study also found that about 95% of the operational taxonomic units within the large intestinal mucosa and lumen were proportionally similar, such that feces can serve as a proxy for colonic contents.<sup>72</sup> In addition, sample collection times differed between the free-ranging and corralled groups, because the trapping schedule for the free-ranging macaques occurred during the fall. In horses, season, supplementary feeding and ambient weather conditions (for example, changes in temperature) are associated with changes in fecal microbiota structure and therefore, the differences in collection times could have impacted our results. However, the community membership



**Figure 9.** Phylogenetic tree of *Helicobacter* species and strains identified in the feces of rhesus macaques. Dot size represents relative contribution to the number of sequences identified.

and structure of the fecal microbiota of CID and HC and HF macaques were similar. The specific changes we found in CID macaques were similar to those previously reported in macaques and other species.<sup>3,23,26,32,38,55</sup> Therefore, the influence of different collection times on the fecal microbiota probably was minimal. A final limitation of our study is that frozen samples were softened in a biosafety cabinet for approximately 2 h before processing. During this period, proteases in the stool could have damaged the DNA, thereby reducing the quality of the data. However, several studies, including by our group, have reported that storage of fecal samples at room temperature or refrigerated at 4 °C for less than 6 h has minimal effect on the results of the microbiota analysis.<sup>48,63,70</sup>

In summary, we found that, overall, the housing site of rhesus macaques appeared to have a greater effect on the composition of the gut microbiome than did their health status. Our results suggest a possible role for *Helicobacter macacae* in maintaining gut health, and a potentially important niche relationship between the closely related genera *Helicobacter* and *Campylobacter*. In addition, our findings provide a basis for future studies on potential clinical interventions, such as probiotics and fecal transplants, which, if effective, could have a profound effect on animal welfare in research settings.

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