1   **Classification:** Biological Sciences: Microbiology

2   **Title:** Altered Stool Microbiota of Infants with Cystic Fibrosis Shows Reduction in Genera

3   Associated with Immune Programming

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27 **Key words:** cystic fibrosis, microbiota, stool, intestine, exacerbation, immune

28   **Abstract.** Previous work from our group indicated a connection between the gastrointestinal
29   microbiota of infants and children with cystic fibrosis (CF) and airway disease in this population.
30   Here we examine the stool microbiota of infants with CF and from the general population who
31   did not have CF over the first year of life. CF children had reduced gastrointestinal *Bacteroides*
32   and *Bifidobacterium* beginning in infancy, even after adjusting for antibiotic treatment. We also
33   identify several metabolic pathways that are enriched or under represented among the
34   microbial communities in the stool of these young patients with CF as compared to children
35   without CF. In vitro studies demonstrated that exposure of the apical face of a polarized
36   Intestinal cell line to *Bacteroides thetaiotaomicron* significantly reduced production of IL-8
37   secreted from both the apical and basolateral face of these cells, suggesting a mechanism
38   whereby changes in the intestinal microflora could impact systemic inflammation. This work
39   further establishes a link between gastrointestinal microbiota, systemic inflammation and
40   airway disease, and presents the opportunity for therapeutic probiotic interventions.

41

42   **Significance statement.** There is a surprising link between gastrointestinal microbial
43   communities and airway disease progression in CF. Here we show that infants with CF ≤ 1 year
44   of age show a distinct stool microbiota compared with children of a comparable age from a
45   general population cohort. We detect associations between stool microbes and airway
46   exacerbation events in the cohort of infants with CF, and in vitro studies provide a possible
47   mechanism for this observation. These data argue that current therapeutics do not establish a
48   healthy-like gastrointestinal microbiota in young patients with CF, and we suggest that
49   interventions that direct the gastrointestinal microbiota closer to a healthy state may provide
50   benefit to these patients.

51

52   **Text**

53   Cystic fibrosis (CF) is an autosomal recessive disease that is known to significantly alter the
54   microenvironments in the airways, intestine and other organs, and is associated with systemic
55   complications such as chronic infections, pancreatic insufficiency, and ultimately premature
56   morbidity and mortality (1-6). While a great deal of attention has been focused on therapies to
57   treat the chronic airway infections that are often the proximal cause of mortality for these
58   patients, it is the gastrointestinal complications that are at the forefront for CF patients in early
59   life (7-10). Young patients with CF experience difficulty in absorbing nutrients secondary to
60   meconium ileus, pancreatic insufficiency and small bowel bacterial overgrowth (10-13). Poor
61   weight gain is common, resulting in the need for a high calorie diet starting early in life and
62   treatment with pancreatic enzyme replacement therapy (14).

63   Recent work has suggested an unexpected connection between the gastrointestinal tract and
64   airway function in young patients with CF. Our group has shown that select genera (e.g.,
65   *Enterococcus*, *Escherichia*) colonizing the airway of CF patients tend to first appear in the
66   intestines (15). Furthermore, breastfed infants showed a longer period of pulmonary health
67   and stability prior to their first pulmonary exacerbation, with 20% of breastfed children having
68   no exacerbation over the first 30 months of life, compared to exclusively formula-fed infants, all
69   of whom suffered exacerbations by 9 months (16). Given the impact of breastfeeding on
70   intestinal microbiota composition (17-19), one possible explanation for these findings in CF is
71   an effect of intestinal microbial communities on airway disease outcomes. Consistent with this
72   idea, an analysis of a small group of patients (n = 13, ages 0-34 months) showed that
73   community composition in the intestine, rather than the airway microbiota, was significantly
74   associated with pulmonary exacerbations prior to age 6 months (16).

75   Here we further probe the relationship between the intestinal microbiota utilizing the
76   Dartmouth Cystic Fibrosis Infant and Children's Cohort compared with a subset of infants from
77   a general population cohort -- the New Hampshire Birth Cohort Study. Using these two
78   cohorts, we characterize the differences between the intestinal microbiota of patients with CF
79   and the general population over the first year of life, both to understand the differences in the
80   microbiota associated with the intestinal tract in these two populations beginning in infancy, as
81   well as to determine if there is indeed a mechanistic link between intestinal microbiota and
82   airway disease outcomes through cell culture studies.

83

84   **Results**

85   **Patient population.** In this study, we analyzed 14 patients with CF and 20 non-CF infants at 4
86   months and 13 CF patients and 45 non-CF infants at 1 year. Of the subjects with CF, 8 had
87   paired samples analyzed at 4 months and 1 yr, and 6 samples were unique at the 2 time points.
88   The non-CF cohort were randomly selected from a larger cohort of children recruited through
89   the New Hampshire Birth Cohort Study for whom stool samples were collected at
90   approximately 4 months and one year of age (20-22). There was no overlap with the non-CF
91   subjects analyzed at 4 months and 1 year. DNA was extracted from stool samples and analyzed
92   for microbial communities as described in the Materials and Methods.

93   **The alpha diversity of stool communities is altered in infants with CF in the first year of life.**
94   To examine the impact of CF on community (alpha) diversity we calculated the Shannon
95   Diversity Index for infants with CF and without CF at 4 months and one year of age (Figure 1).
96   Two key differences could be identified. First, at 4 months, the alpha diversity of the microbial
97   community in the stool of infants with CF was significantly higher than that of those subjects
98   without CF (p = <0.001). No observable change in diversity occurred between 4 months and 1

99 year in CF infants (p = 0.87); in contrast, the α-diversity did increase during this period in non-
100 CF infants (p < 0.0001). Interestingly, the α-diversity of CF and non-CF children did not
101 significantly differ from each other at 1 year. These data indicate a significant difference in the
102 microbial diversity associated with the stool of infants with CF versus the comparison group of
103 non-CF children as early as 4 months of age.

104 **The gut microbial communities of infants with CF differ from non-CF controls subjects over**
105 **the first year of life.** We next analyzed the beta diversity (microbial community composition) of
106 the infants with CF in comparison to the non-CF comparison group. The beta diversity of
107 patients with CF and non-CF controls at 4 months is significantly difference (Fig. 2A;
108 PERMANOVA of generalized Unifrac distances; p = 0.002). Interestingly, despite the
109 observation that the alpha diversity of the subjects with CF and the comparison group is not
110 different at 1 yr, these stool samples show a significant difference in beta diversity (Fig. 2B;
111 PERMANOVA of generalized Unifrac distances; p = 0.001). Furthermore, the similar alpha
112 diversity of patients with CF at 4 months and non-CF children at 1 year is not reflected by a
113 similar microbial community, that is, the beta diversity of these samples is significantly different
114 (Fig S1; PERMANOVA of generalized Unifrac distances; p = 0.001).

115 Consistent with the significant difference in alpha diversity for non-CF children at 4 months and
116 1 year, there is a significant difference in beta diversity between these samples (Fig. 2C;
117 PERMANOVA of generalized Unifrac distances; p = 0.001). For children with CF, the similar
118 alpha diversity at 4 months and 1 year is also reflected by the clustering of communities, with
119 no significant difference in beta diversity (Fig. 2D; PERMANOVA of generalized Unifrac
120 distances; p = 0.396).

121 **Alterations in the beta diversity of stool of infants with CF are driven by modest changes in**
122 **microbes for patients at 4 months of age**. To further explore the basis of the differences in
123 beta diversity between subjects with CF and non-CF controls, we investigated differences in
124 individual OTUs, assigned at the genus level, when possible, or otherwise at the next lowest
125 taxonomic level. Selecting the top 25 OTUs in each data set resulted in a collection of microbes
126 that almost perfectly overlapped, and captured the top 20 OTUs for each cohort in terms of
127 relative abundance (Fig. 3). Using a Wilcoxon rank-sum test to compare the relative abundance
128 of genera present at 4 months, the abundance of *Bifidobacterium* is significantly higher in the
129 non-CF controls while *Streptococcus, Veillonella,* and *Clostridium* are significantly higher in
130 subjects with CF (Fig. 3). Three lower abundance groups (~1% relative abundance for most
131 samples) are also significantly different: *Faecalibacterium* and two unassigned genera in the
132 order Clostridiales. None of the other nineteen most abundant OTUs are significantly different
133 at 4 months between these cohorts, thus only 6/25 microbes differ at the 4 month time point.
134 By one year of life, there are more significant differences between the cohorts, with 11/25
135 showing a significant difference in relative abundance between these cohorts (Fig. S2). For

136 example, *Bacteroides* and *Faecalibacterium* are significantly higher in the non-CF comparison
137 group. There is no significant difference in *Bifidobacteria* levels between the cohorts at 1 year,
138 despite the significant difference at 4 months (Fig. 3). Thus, particularly early in life (i.e., at 4
139 months), we find that the shift in microbes is primarily driven by a small subset of genera.

140 **Microbes associated with immune programing are lower in patients with CF compared to**
141 **infants without CF.** Given that subjects with CF demonstrate a lower abundance of *Bacteroides*
142 and/or *Bifidobacterium* at 4 months and/or 1 year than the comparison group, a more indepth
143 analysis of these two keystone immune programming genera was undertaken. While both
144 cohorts have individual subjects with low abundance of *Bacteroides* or *Bifidobacterium*, a
145 comparison of the relative abundance of each microbe at each sampling time revealed that for
146 the CF cohort at both 4 months and 1 year, there were a higher percentage of patients with
147 <1% relative abundance of these microbes and a lower maximal relative abundance compared
148 to the non-CF comparison cohort (Figure S3).

149 Given the importance of *Bacteroides* and *Bifidobacterium* in immune programming (23, 24), it
150 was unexpected that even for the non-CF comparison cohort, some individuals had <1% of
151 these microbes. To investigate this observation further, the relative abundance of *Bacteroides*
152 versus *Bifidobacterium* on a subject-by-subject basis for both cohorts was plotted (Fig. S4). For
153 the non-CF cohort, many subjects with low *Bacteroides* showed a high relative abundance of
154 *Bifidobacterium*, and visa versus (Fig. S4A). There was also a group of non-CF patients with an
155 intermediate level of each microbe and ~50% of samples from the non-CF cohort had >40%
156 relative abundance of *Bacteroides* and/or *Bifidobacterium*. In contrast, for the CF cohort many
157 of the subjects clustered in a group with <20% of each microbe (Fig. S4B), and there is only 1
158 subject with >40% of either *Bacteroides* or *Bifidobacterium* (~5% of the samples analyzed).

159 **Subjects with CF and non-CF controls demonstrate differential enrichment of predicted**
160 **metabolic functions.** Relative to the patients with CF, the non-CF comparison infant's microbial
161 community was associated with increased in functions connected with β-lactam resistance,
162 polyketide sugar unit biosynthesis, and carbohydrate metabolism (Fig. S5). In contrast, subjects
163 with CF showed enrichment in processes that include restriction enzymes, streptomycin
164 biosynthesis, penicillin and cephalosporin biosynthesis, and galactose metabolism (Fig. S5). Our
165 analysis of the 4 month samples showed no significant enrichment of pathways for either
166 cohort, despite the significant differences in abundance of several OTUs (Fig. 3, S3).

167 **Apical application of *Bacteroides* to intestinal epithelial cells reduces basolateral release of IL-**
168 **8**. A recent report examining the impact of *Bacteroides* in intestinal epithelial provides a clue to
169 the role of *Bacteroides* in reducing systemic inflammation (25). Some *B. fragilis* strains produce
170 enterotoxins that can stimulate the production of IL-8. Cho and colleagues showed that
171 exposing the apical face of intestinal epithelial cells to such a toxin resulted in the increased
172 production of IL-8 from the basolateral face of the same cell monolayer. These data indicate

173   that impacts to the host cells in the lumen of the intestine can have systemic impacts on

174   cytokine production. Thus, we assessed whether apical application of *Bacteroides* spp. can

175   reduce the basolateral production of IL-8.

176   To investigate this mechanisms, we performed an experiment whereby Caco-2 cells were grown

177   on transwell filters as polarized monolayers, then inoculated on the apical face with ~3.5 x 10^7

178   CFU *Bacteroides thetaiotaomicron* (*B. theta*). *B. theta* was <u>not</u> introduced into the basolateral

179   compartment. The IL-8 produced in the apical and basolateral compartments of the transwells

180   were measured after 24 hrs of exposure to the bacteria. We found apically inoculated *B. theta*

181   significantly reduced both the apical and basolateral production of IL-8 (Fig. 4).

182

183   **Discussion.** In our study, children with CF had an intestinal microbial community that was

184   distinct from infants from the general population as early as 4 months, and despite a

185   convergence of alpha diversity by 1 year between these cohorts, the composition of the

186   communities remained distinct at 1 year of life. Furthermore, the altered microbiome of

187   infants with CF did not change markedly across the first year of life. At 4 months of age, only

188   5/25 genera identified differed between the cohorts, but among those genera depleted at 4

189   months are those microbes with key immune modulatory roles. By one year of age, while the

190   alpha diversity of the communities for the CF and the comparison cohort are not statistically

191   significantly different, nearly half of the genera differed in relative abundance, indicating that

192   individual taxa continue to be distinct between these two cohorts. This distinct microflora is

193   reflected by differences in several metabolic pathways at one year.

194   Importantly, our previous data (15, 16) and the work here indicate a link between intestinal

195   microbial communities and airway disease. Thus, we hypothesized that there must be a way for

196   local effects in the intestine (i.e., altered microbiota) to impact systemic levels of inflammation

197   and/or a gut-derived immune response, which likely contributes to airway exacerbation (26-

198   28). Our in vitro data show that *B. theta* applied to the apical face of an intestinal cell line can

199   reduce IL-8 secretion from both the *apical* and *basolateral* face of the cells, a finding consistent

200   with another report in the literature that examined the impact of a *Bacteroides*-derived toxin

201   (25). Therefore, beneficial strains of *Bacteroides* can alter systemic signals of inflammation, and

202   thereby may impact airway disease through this mechanism.

203   There are several important implications of our findings. First, while enzyme replacement

204   therapy has clearly had positive transformative impacts on CF patients (6, 29, 30), the

205   differences we observe in the intestinal microbiota of the CF cohort indicate that these subjects

206   still have underlying defects and suggests room for improvement in such therapeutic

207   interventions. Additionally, our data, in combination with prior work, suggest that probiotic

208   therapies that shift the intestinal microbiota towards a more healthful state may mitigate the

209   pulmonary exacerbations. Several systematic reviews of randomized controlled trials of
210   probiotics in groups with CF have shown a positive impact of probiotics on reducing frequency
211   of CF exacerbation and improving gastrointestinal inflammation (31-33), however mechanisms
212   behind benefits of shifting the intestinal microbiome in CF are not yet clear. Given that
213   pulmonary exacerbations are associated with the progressive loss of lung function in this
214   population, morbidity and ultimately mortality, reducing the frequency of such clinical episodes
215   beginning in early life would likely assist in maintaining lung function in patients with CF.

216

217   **Materials and Methods.**

218   **Patient population.** Institutional review board approval was obtained from the Center for the
219   Protection of Human Subjects at Dartmouth College with yearly renewal of the approval.
220   Additional detail can be found in the Supplemental Material. Eligibility criteria for the CF cohort
221   included: diagnosis with CF prenatally or postnatally based upon newborn screening results and
222   subsequent confirmation with sweat chloride and genetic testing. Subjects were eligible if their
223   care for CF would be at the Dartmouth-Hitchcock Medical Center in Lebanon, NH or
224   Manchester, NH affiliated clinics. Subjects were excluded if they had non-CF chromosomal
225   anomalies. For the non-CF comparison group, infants were randomly selected from the New
226   Hampshire Birth Cohort Study (NHBCS). For the NHBCS, pregnant women ages 18–45 were
227   recruited from prenatal clinics beginning at approximately 24 to 28 weeks gestation as
228   described: women were screened for eligibility at an initial prenatal care visit and were enrolled
229   at 24–28 weeks gestation if they reported using water from a private, unregulated well in their
230   home since their last menstrual period and were not planning a change in residence prior to
231   delivery. Only singleton, live births were included in the cohort.

232   **Analysis of microbial populations in stool.** Stool samples were analyzed as reported (16), with
233   minor modifications, as described in the Supplemental Material.

234   **Statistical analyses**. Differences in the microbial profiles of stool samples from 14 patients with
235   CF and 20 comparison infants at 4 months and 13 CF patients and 45 comparison infants at 1
236   year were analyzed using Generalized Unifrac Distances. Distances were visualized using PCoA
237   plots and the significance of clustering was determined through PERMANOVA. Within sample
238   alpha-diversity was determined using the Shannon diversity index as calculated for each sample
239   in the phyloseq R package function estimate_richness. Significant differences in the relative
240   abundance of individual taxa were determined with a Wilcoxon rank-sum test and verified with
241   linear models. Bonferroni correction was used to adjust for multiple testing.

242   **PICRUSt analysis.** Open reference OTUs were removed from the OTU table using the QIIME
243   function filter_otus_from_otu_table for use with Phylogenetic Investigation of Communities by

244   Reconstruction of Unobserved States (PICRUSt), as reported (34). Additional detail can be found

245   in the Supplemental Material.

246   **Cell line studies**. Caco-2 lines were grown on transwell filters and inoculated with bacteria in

247   the apical compartment. After 24 hours of exposure, the bacterial numbers were quantified

248   and IL-8 measured in the apical and basolateral compartments. Additional detail can be found

249   in the Supplemental Material.

250

260

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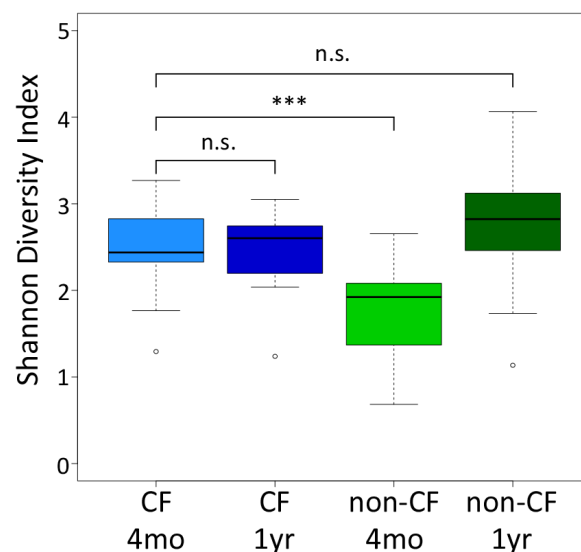348   **Figures**

349

350   **Figure 1. Alpha diversity varies between subjects with CF and infants without CF.** Using the
351   Shannon diversity of samples from subjects with CF at 4 months of age as the reference group
352   in a generalized linear model, samples from CF subjects at 1 year of age are not significantly
353   different from the same cohort at 4 months (p = 0.87). Samples collected for the comparison
354   group (non-CF) at 4 months of age have significantly lower alpha diversity than CF subjects at
355   the same age (p = 0.00025). While there is a trend toward increased diversity for the non-CF
356   comparison group at 1 year of age compared to CF subjects, this trend did not reach
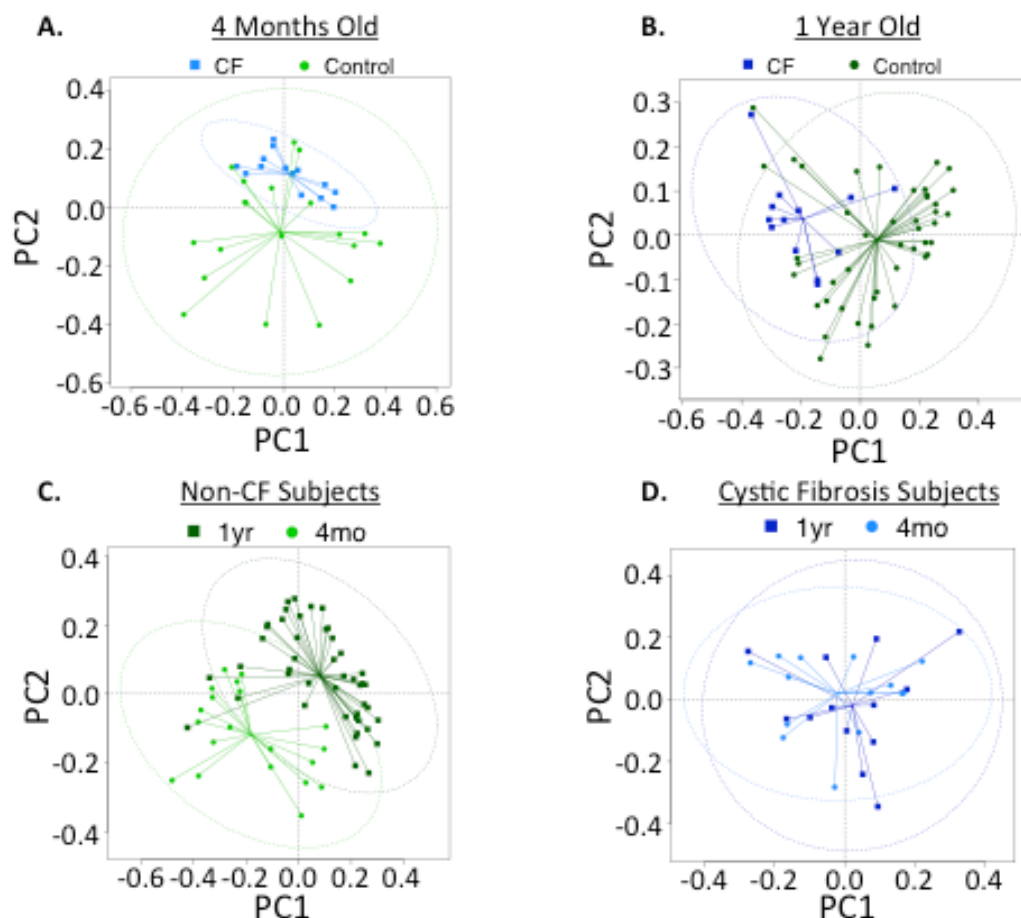357   significance (p = 0.091).

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Figure 2. Stool microbial profile remains stable but differ between the CF and the non-CF comparison group over the first year of life. PCoA plots generated from Generalized UniFrac distances between samples. **A**. At 4 months of age samples from CF subjects cluster separately from the non-CF comparison group (PERMANOVA p = 0.002). **B**. At 1 year samples from CF subjects continue to cluster together and separately from samples provided by the non-CF comparison group (PERMANOVA p = 0.001). **C**. Samples from the non-CF comparison group at 4 month cluster separately from samples provided at 1 year (PERMANOVA p = 0.001). **D**. Samples provided by CF subjects at 4 months and 1 year do not cluster separately from each other (PERMANOVA p = 0.396).
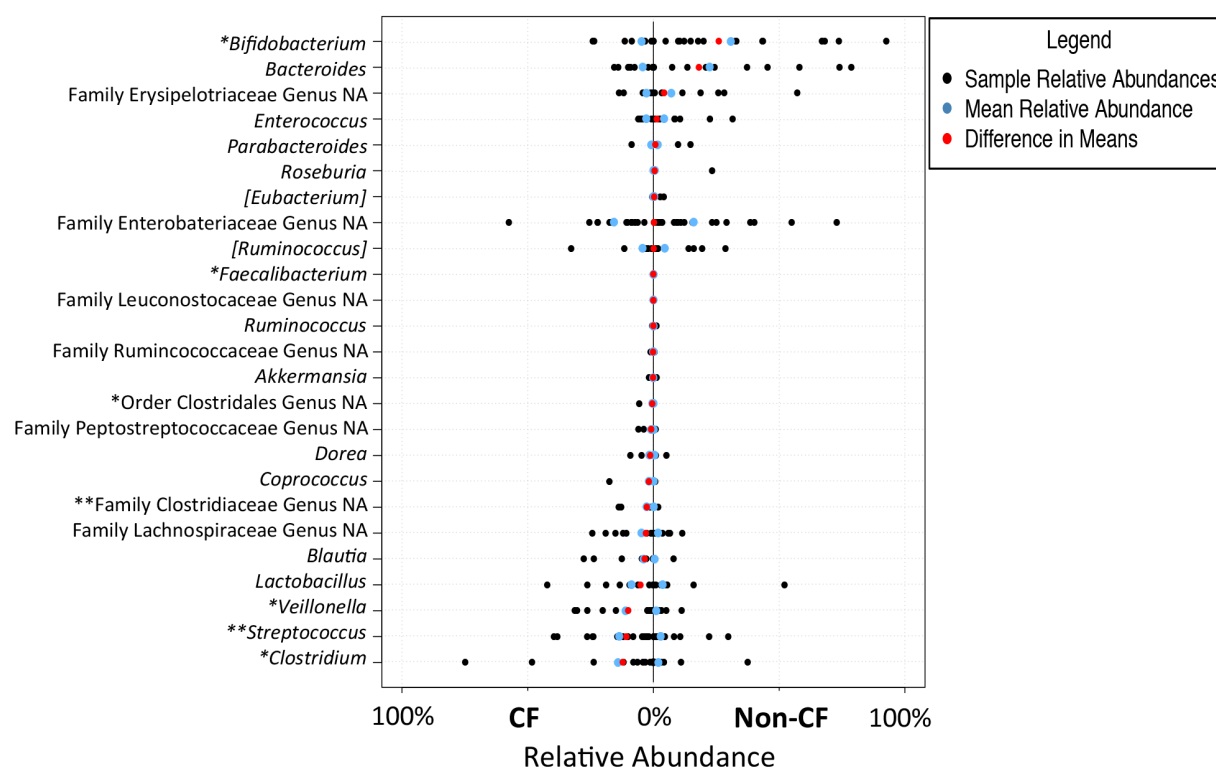
Figure 3. Comparison of relative abundances of genera for subjects with CF and the non-CF comparison group at 4 months. Relative abundance of the top 25 taxa present in stool samples. At 4 months there were 14 samples from CF subjects and 20 samples from the non-CF comparison group. *, **, and ***, indicate significantly different taxa by the Wilcoxon rank-sum test after Bonferroni correction at the p<0.05, p<0.01, and p<0.001 levels respectively. Genera in brackets are the taxonomic assignments suggested by the Greengenes database based on whole genome phylogeny. Abbreviations: NA, not assigned.

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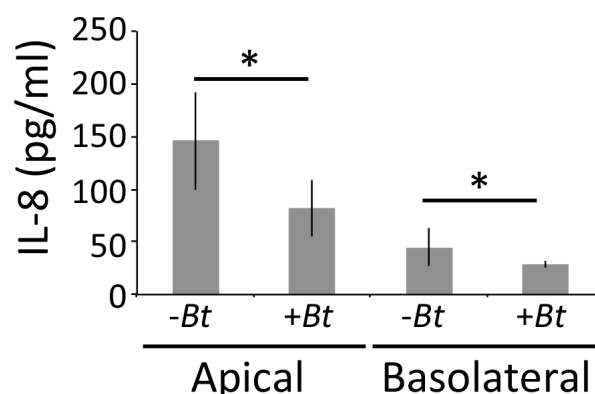
379 **Figure 4. *B. theta* reduces IL-8 production by intestinal epithelial cells.** Levels of IL-8,
380 determined by ELISA, and indicated for Caco-2 cells grown on transwell filters for ~4 weeks.
381 After addition of *B. theta* VPI 5482 (*Bt*) to the apical compartment of the transwell, the filters
382 were incubated anaerobically for 3 hrs, and then the added suspension of bacteria was
383 removed and replaced with fresh medium. Filters were returned to anaerobic incubation
384 overnight. Twenty four hours after application of *B. theta*, supernatants were collected and IL-8
385 levels measured via ELISA assay (PromoKine). To determine CFU, filters were scraped and
386 plated (n=6); at the end of the experiment there were ~2 x $10^9 \pm 4$ x 10^8 CFU *B. theta*/well
387 associated with the apical face of the monolayer. *p < 0.05 by students t-test compared to the
388 condition with no *B. theta* added (*-Bt*).