GUT DYSBIOSIS IN CYSTIC FIBROSIS

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In people with CF, intestinal exocrine malfunction, antibiotic usage (1) and swallowing of infected respiratory mucus (2) likely perturb the normal community of commensal bacteria in the gut. People with CF report various intestinal problems which may be alleviated by probiotic administration (3). There is also evidence that probiotic bacteria can help people with CF fight respiratory infection (4,5). However, CF-related gut dysbiosis has only recently been subjected to detailed investigation. Using DGGE and culture-based methods, Duytschaever and colleagues (6) showed that children with CF have a quantitatively and qualitatively different fecal microbiota from their healthy siblings. We conducted a pilot study using culture-independent stool microbiome profiling and found evidence consistent with these results, strengthening the case for more targeted exploration of the gut microbiota in CF.

We obtained stool samples from four people with CF and four unrelated, healthy, age- and sex-matched controls. Total DNA was extracted and the 16S rRNA gene amplified. We analysed bacterial community composition using a high-density phylogenetic microarray (PhyloChip) that has been used extensively in microbial ecology surveys, including analyses of the human gut (7) and CF airways (8). Full methods are available as Supplementary Content.

In 3 of 4 pairs the CF patient exhibited lower taxonomic richness, evenness and diversity than the healthy control (Fig 1). Further, non-metric multidimensional scaling (9) revealed greater inter-individual variation in gut microbiota in the CF group than in the healthy controls. Given the range of factors that produce variation in the human gut microbiome it is impossible within this small cohort to speculate whether this is simply a function of disease severity and/or antibiotic use, or of other variables such as host genetics, diet, environment or age. Finally, consistent with Duyschaever *et al*.'s data, we found decreased relative abundance of *Bifidobacterium* species in the CF group; these are widely used in probiotic products and one of the species with decreased abundance in our cohort, *B. breve*, may mediate intestinal homeostasis (10). A full list of taxa with altered relative abundance in the CF group are provided in the Supplementary Content.

While our pilot data set is too small to draw firm conclusions, our data support the hypothesis that people with CF have intestinal microbial dysbiosis. This, together with the prophylactic potential of probiotics for preventing respiratory infections, highlights a need to investigate the intestinal microbiota of CF in a larger cohort. In the future this may inform studies that investigate the prophylactic potential of probiotics for people with CF.

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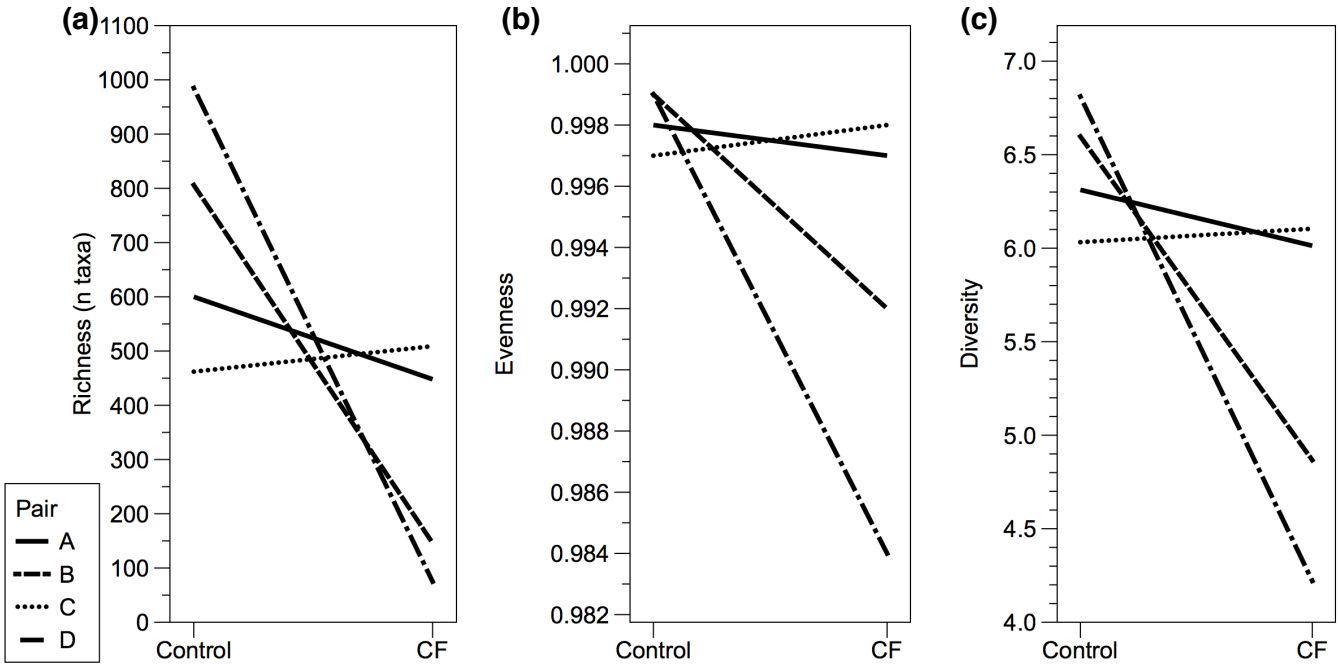

Figure 1. In three of four participant pairs, the microbial community isolated from the individual with CF showed lower species richness, evenness and diversity than that isolated from the healthy control individual. Evenness measures the relative distribution of community members and diversity is a metric that takes both richness and evenness into account.

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Supplementary Content

(1) Methods and participants

Participant recruitment

We recruited four people with cystic fibrosis (Table 1) via CF support groups and advertisments placed in public places. Three of the four participants carried the most common, severe CF mutation (ΔF508). Participant D did not know his genotype, but given his age it seems likely that he carried a milder variant of CF. All participants were pancreatic insufficient; participants A-C were using the pancreatic enzyme replacement supplement Creon. All participants were taking antibiotics at the time of sampling and all reported an acute respiratory episode or lung infection 0-3 months prior to the sample date. Participant C reported a positive culture test for respiratory *P. aeruginosa* infection. Each of these participants was paired with an age- and sex-matched healthy control participant. None of the control participants used probiotics or had recently used antibiotics. None of the CF or control participants reported any family history of gastrointestinal disorders and all reported regular bowel movements. Participants were asked to complete a short questionnaire about their health and diet and to provide a faecal sample. The two participants who were over the age of 18 provided informed consent and a parent provided informed consent for the other two participants. The study received ethical approval from the University of Oxford's Interdivisonal Research Ethics Committee for Medical Sciences (ref: MSD/IDREC/C1/2010/49).

Table 1. Participant information (CF group).

Participant	Sex	Age (years)	Genotype	Pancreatic sufficient	*P. aeruginosa*	Antibiotic usage	Pro/prebiotic usage
A	M	3	ΔF508 / Q493X	No, Creon	Negative	Colomycin (colistimethate sodium, nebulised); flucloxacillin (oral)	Live yoghurt
B	F	18	ΔF508 / 1898+1G>T	No, Creon	Negative	Augmentin (amoxicillin + clavulanate, oral)	Lactofermented whey
C	M	42	ΔF508	No, Creon	Positive	Colomycin (colistimethate sodium, nebulised); azithromycin (oral)	Lactulose
D	M	72	Not known	No	Negative	Flucloxacillin; promixin (colistimethate sodium)	None

Sample processing

Faecal samples were obtained from each individual and stored at –80°C until required for extraction of bacterial DNA. Samples used were thawed on ice and DNA was extracted using the Qiagen QIAamp MiniStool kit (Qiagen, Hilden, Germany) according to manufacturer's instructions for pathogen isolation, with an initial bead-beating step of 30 s at 5,000 rpm following by lysis at 95ºC. Partial 16S rRNA genes were amplified using an MJ Research PTC-200 Thermal Cycler and the PCR primers designed by Lane (1) 27f (5' AGA GTT TGA TCM TGG CTC AG) and 1492r (5' GGT TAC CTT GTT ACG ACT T). To minimise any potential PCR bias, each sample was amplified five times on two separate occasions and the reactions were pooled and purified into a single volume using a Qiagen QIAquick Gel Extraction kit. Each PCR mixture (50 µl final volume) contained 1 X Buffer (20 mM Tris pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.25 U of *Taq* polymerase (Invitrogen, UK), and 10 pmol of each primer. Appropriately diluted gDNA (1 -10 ng) was added to the final PCR. The PCR conditions were: 95ºC for 5 min initial denaturation, followed by 20 cycles of amplification at 95ºC denaturation for 30 s, 55°C for 40 s and extension of 72ºC for 1 min, with a final extension of 72ºC for 5 min. PhyloChip analyses were performed as described by Brodie et al. (2), with the exception that 500 ng of 16S rRNA amplicon per sample was hybridized to each array.

Data analysis

Relative distances among all microbiota compositions were calculated based on the Bray-Curtis metric using log-transformed PhyloChip fluorescence data. Distance matrices were analyzed by non-metric multidimensional scaling (NMDS) and hierarchical clustering with the *vegan* package in the R statistical environment. Gut microbiota richness (number of bacterial taxa present), evenness (relative distribution of community members: (3)) and diversity (metric based on both richness and evenness indices: (4)), were also calculated using the *vegan* package in the R statistical environment. Taxon presence or absence was determined based on probe set positive-fraction (pf) value of ≥0.9 (1).

The NMDS and non-hierarchical clustering analyses shwoed that the gut microbiota of healthy individuals clustered together, relative to the CF samples. One exception was the CF individual in pair C, who clustered with the healthy individuals, suggesting that this participant shared microbiota community membership with the healthy participants in this cohort. This is consistent with the observation that this participant's sample had slightly higher community richness, evenness and diversity than that of his matched control (see Fig. 1). This result is interesting as this participant was the only member of the CF group to have been colonised by *P. aeruginosa* and had CF-related diabetes, which would have led us to expect him to be the least healthy of the group.

T-tests were employed to examine the difference in relative abundance of detected taxa between CF patients and healthy individuals. A total of 59 taxa were detected at $p < 0.05$ and these are listed in part 2 below, though it should be noted that, likely due to the small number of samples in this study and the wide variation in community composition exhibited by CF patients, none of the findings passed our threshold for false discovery ($q < 0.05$).

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(2) Comparison of taxonomic relative abundance (fluorescence intensity) changes across CF and healthy groups.

i. Increased relative abundance in CF patients

p-value	q-value	Abundance change	Representative species	Phylum
0.047	0.611	3360	*Aeromonas sp.* PAR2A	Proteobacteria
0.028	0.611	2641	Antarctic pack ice Lasarev Sea Southern Ocean clone ANTXI/4_14-62 sea	Proteobacteria
0.038	0.611	2276	*Ferribacter thermoautotrophicus str.* JW/JH-Fiji-2	Firmicutes
0.032	0.611	1699	anoxic marine sediment clone LD1-PB1	Verrucomicrobia
0.016	0.611	1678	a member of Alphaproteobacteria	Proteobacteria
0.046	0.611	1675	soil clone RB24	Acidobacteria
0.021	0.611	1666	hydrothermal vent polychaete mucous clone P. palm C 85	Verrucomicrobia
0.031	0.611	1666	anoxic marine sediment clone LD1-PB20	Verrucomicrobia
0.016	0.611	1078	a member of Alphaproteobacteria	Proteobacteria
0.049	0.611	1002	trichloroethene-contaminated site clone FTL276 bacterium	Chloroflexi
0.016	0.611	1000	*Mannheimia sp.* R19.2 str. R19.2; CCUG 38463 R19.2	Proteobacteria
0.033	0.611	989	termite gut homogenate clone Rs-J10 bacterium	Actinobacteria
0.041	0.611	609	Guaymas Basin hydrothermal vent sediments clone B01R005	OP9/JS1

ii. Decreased relative abundance in CF patients

p-value	q-value	Abundance change	Representative species	Phylum
0.043	0.611	-385	*Lyrodus pedicellatus* symbiont	Proteobacteria
0.020	0.611	-430	*Nitrosomonas eutropha str.* Nm57	Proteobacteria
0.043	0.611	-511	*Calyptogena magnifica* symbiont	Proteobacteria
0.012	0.611	-541	a memberof Lachnospiraceae	Firmicutes
0.007	0.611	-748	*Fucophilus fucoidanolyticus str.* SI-1234	Verrucomicrobia
0.023	0.611	-770	*Dietzia maris*	Actinobacteria
0.045	0.611	-817	forested wetland clone RCP1-33	Actinobacteria

0.048	0.611	-854	*Clostridium acetobutylicum str.* ATCC 824 (T)	Firmicutes
0.009	0.611	-864	*Bifidobacterium breve str.* KB 92	Actinobacteria
0.042	0.611	-924	human colonic clone HuCA22	Firmicutes
0.049	0.611	-929	*Thermosipho sp. str.* MV1063	Thermotogae
0.014	0.611	-966	a member of Clostridiales	Firmicutes
0.050	0.611	-978	termite gut clone Rs-050	Firmicutes
0.013	0.611	-989	*Thiomicrospira crunogena str.* XCL-2	Proteobacteria
0.040	0.611	-999	anaerobic bioreactor clone SHD-238	Chloroflexi
0.036	0.611	-1003	travertine hot spring clone SM2B11	Cyanobacteria
0.024	0.611	-1040	*Sphingobacterium sp. str.* HC-6155	Bacteroidetes
0.006	0.611	-1066	*Hymenobacter sp.* str. NS/50	Bacteroidetes
0.011	0.611	-1134	a member of Clostridiaceae	Firmicutes
0.025	0.611	-1147	a member of Lachnospiraceae	Firmicutes
0.028	0.611	-1166	*Nodularia sphaerocarpa str.* UTEX B 2093	Cyanobacteria
0.036	0.611	-1219	*Bifidobacterium thermacidophilum porcinum subsp. suis str.* P3-14 subsp.	Actinobacteria
0.024	0.611	-1232	human gut clone adhufec382	Firmicutes
0.020	0.611	-1298	termite gut homogenate clone Rs-G04 bacterium	Firmicutes
0.038	0.611	-1339	*Bifidobacterium adolescentis str.* E-981074T	Actinobacteria
0.011	0.611	-1413	hypersaline pond clone LA7-B27N	Deinococcus-Thermus
0.044	0.611	-1523	faecal clone adhufec35.25	Firmicutes
0.029	0.611	-1542	soil isolate Ellin301	Actinobacteria
0.010	0.611	-1594	anaerobic bioreactor clone SHD-235	Chloroflexi
0.005	0.611	-1621	swine intestine clone p-2013-s959-5	Firmicutes
0.042	0.611	-1634	*Mitrastema yamamotoi*	Cyanobacteria
0.038	0.611	-1691	human colonic clone HuCA19	Firmicutes
0.004	0.611	-1698	a member of TM7	TM7
0.049	0.611	-1793	termite gut homogenate clone Rs-K21 bacterium	Firmicutes
0.040	0.611	-1850	sphagnum peat bog clone K-5b5	Proteobacteria
0.040	0.611	-1925	swine intestine clone p-953-s962-5	Firmicutes
0.034	0.611	-1942	*Symbiobacterium toebii str.* SC-1	Firmicutes
0.044	0.611	-2003	a member of Clostridiales	Firmicutes
0.004	0.611	-2037	ckncm326-B4-13 clone	Firmicutes
0.039	0.611	-2039	*Sphingopyxis flavimaris str.* SW-151	Proteobacteria
0.004	0.611	-2053	swine intestine clone p-5263-4Wb5	Firmicutes
0.041	0.611	-2065	termite gut homogenate clone Rs-J39 bacterium	Firmicutes
0.041	0.611	-2124	*Corynebacterium jeikeium str.* ATCC 43734	Actinobacteria
0.001	0.488	-2190	a member of TM7	TM7
0.045	0.611	-3052	uranium mining waste pile clone JG37-AG-81 sp.	Acidobacteria
0.049	0.611	-3414	termite gut homogenate clone Rs-F27 bacterium	Firmicutes