Disordered Gut Microbiota in Children Who Have Chronic Pancreatitis and Different Functional Gene Mutations

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OBJECTIVES: Chronic pancreatitis (CP) is a serious condition whose pathogenic mechanism is unclear. Interactions of host genetic factors with gut microbiota have a role, but little is known, especially in children with CP (CCP), in which the external factors are less important. Our objective was to identify the main gut microbiota genera in CCP and to characterize the functional mutations of these patients.

METHODS: We used 16S rRNA sequencing to compare the gut microbiota of healthy controls with patients who had CCP and different functional gene mutations.

RESULTS: CCP is characterized by gut microbiota with remarkably reduced alpha diversity. Receiver operating characteristic curve analyses indicated that the abundances of 6 genera—*Faecalibacterium*, *Subdoligranulum*, *Phascolarctobacterium*, *Bifidobacterium*, *Eubacerium*, and *Collinsella*—were significantly decreased in CCP, with an area under curve (AUC) of 0.92 when considering all 6 genera together. Functional analysis of gut microbiota in CCP indicated reduced ribosomal activity, porphyrin and chlorophyll metabolism, starch and sucrose metabolism, and aminoacyl-tRNA biosynthesis, but an enrichment of phosphotransferase system pathways. The abundance of *Butyricicoccus* was significantly decreased in CCP in the presence of *CFTR* mutations when combined with mutations in *CASR*, *CTSB*, *SPINK1*, and/or *PRSS1*. The abundance of *Ruminococcaceae* was significantly increased in CCP when there were mutations in *CASR, CTSB, SPINK1*, and/or *PRSS1*. Patients with CCP but no gene mutations had greater abundances of *Veillonella* and reduced abundances of *Phascolarctobacterium*.

DISCUSSION: CCP is associated with a depletion of probiotic gut microbiota, and CCP patients with different functional gene mutations have different gut microbiota.

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A229

INTRODUCTION

Chronic pancreatitis (CP) is a persistent fibroinflammatory process of the pancreas, which is associated with genetic, environmental, and other pathogenic factors, eventually leads to irreversible pancreas injury, and often causing pain and/or permanent loss of function. CP patients also have an increased risk of pancreatic cancer and impaired mental health (1,2). CP is especially harmful to children because malnutrition and malabsorption of food could potentially lead to a delay in their physical growth and development (3).

There is no effective treatment for CP and no simple and reliable diagnostic test for early CP (4). The annual incidence of CP ranges from 5 to 12 per 100,000, and the prevalence is about 50 per 100,000 (5). A survey in China reported that the prevalence of CP was 13.52 per 100,000 (6). On the other hand, based on the previous findings that most CP patients were 15–20 years old and a recent epidemiological study in France, which found an annual incidence of 7.8 per 100,000, the annual prevalence of CP may be as high as 120 to 143 per 100,000. The incidence and prevalence of CP are thus greatly underestimated, especially in China, and this is further compounded by the difficulty in diagnosing CP (4).

Clarification of the pathogenesis of CP is important for defining and treating this disease (1). Although there are well-established internal factors (genetic predispositions) and external factors (smoking, drinking alcohol) that increase the risk for CP, it remains a poorly understood disease (1,2,7,8).

Many researchers have examined the relationship of genomic variations of human hosts with the composition of the gut

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microbiome. There is strong evidence that an interaction of host genetics and gut microbiota underlie the onset and clinical presentation of many gastrointestinal diseases and that host genetics influence gut microbiota (9). Similarly, there is evidence that patients with CP and type 3c (pancreatogenic) diabetes mellitus (T3cDM) have higher levels of *Bacteroidetes* and lower levels of *Faecalibacterium* than patients with CP alone and that patients with T3cDM and PEI have lower levels of *Bifidobacterium* than those with T3cDM alone (10). Other studies that used gas liquid chromatography reported lower levels of *Bifidobacterium* and *Lactobacillus* and higher levels of *Enterobacter*, *Proteus*, *Klebsiella*, and *Morganella* in patients with CP than in healthy controls (HCs) (11,12). A study of oral microbiota reported that patients with CP had lower levels of *Granulicatella adiacens* than that of those with pancreatic ductal carcinoma (13). An analysis of the colonic microenvironment reported that patients with CP had lower levels of *Lactobacillus* and higher levels of *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus faecium* than that of HCs, but these 2 groups had no difference in the levels of *Bifidobacterium* (14). However, no studies have yet examined the link between gut microbiota and children with CP (CCP).

External pathogenic factors, such as tobacco and alcohol consumption, are generally irrelevant in studies of many children. Thus, the studies of children who had no smoking or drinking habits may simplify the identification of pathogenic factors associated with CP because there is no confounding by these factors. In this study, we used 16S rRNA sequencing and bioinformatics analysis to characterize the gut microbiota associated with CCP, the gut microbiota of hosts with different functional mutations, and the functional traits of hosts and metabolic pathways associated with the pathogenesis of CP.

MATERIAL AND METHODS
Inclusion and exclusion criteria
The diagnosis of CP was based on the criteria of the INternational Study Group of Pediatric Pancreatitis: In Search for a CuRE (INSPPIRE), as previously described (7). All cases and HCs were 4–18 years old and were examined from 2014 to 2017. Individuals who used an antibiotic or an oral probiotic drink within the previous 3 months were excluded.

Collection of samples
Samples were collected at Ruijin Hospital (Shanghai, China), which is affiliated with the Shanghai Jiao Tong University School of Medicine. All samples were stored at −80 °C within 2 hours after collection.

Isolation of fecal bacterial DNA
Fresh fecal specimens were collected in sterile containers and immediately subjected to DNA extraction using the OMEGA-soil DNA Kit (Omega Bio-Tek, Norcross, GA) following the instructions of the manufacturer. DNA concentration and purification were determined by using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE). DNA quality was confirmed using 1% agarose gel electrophoresis, and isolated DNA was stored at −80 °C before sequencing.

16s rRNA amplicon sequencing of bacteria
PCR (GeneAmp 9700; Applied Biosystems, Foster City, CA) was used to amplify the V3-V4 hypervariable regions of the 16S rRNA gene with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′)

and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR reactions (3 minutes of denaturation at 95 °C; 27 cycles of 30 seconds at 95 °C, 30 seconds for annealing at 55 °C, and 45 seconds for elongation at 72 °C; final extension at 72 °C for 10 minutes) were performed in triplicate in 20 μL mixtures (4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer [5 μM], 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA). DNA was extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA). Quantification was performed using QuantiFluor-ST (Promega, Madison, WI).

Illumina MiSeq sequencing
Purified amplicons were pooled in equimolar amounts and sent to Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). Paired-end sequencing (2 × 300) was performed on an Illumina MiSeq platform (Illumina, San Diego, CA) using standard protocols.

Processing of sequencing data
Raw fastq files were subjected to read-quality filtering using Trimmomatic and merged by FLASH. First, reads were truncated at any site with an average quality score below 20 over a 50 bp sliding window. Second, sequences with overlap longer than 10 bp were merged so that the overlap with mismatch was no more than 2 bp. Third, sequences were separated by barcodes (exact matching) and primers (allowance for mismatch of 2 nucleotides); reads containing ambiguous bases were removed.

UPARSE (version 7.1, http://drive5.com/uparse/) was used to cluster the operational taxonomic units. A 97% similarity cutoff was implemented with a novel "greedy" algorithm that simultaneously removes chimeras and clusters operational taxonomic units. The identification of each 16S rRNA gene sequence was determined using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) and a 16S rRNA database (Silva, SSU release 123), with a confidence threshold of 70%.

The α-diversity (within-sample diversity) was calculated from the gene profiles of each sample using the Shannon index, as described previously (15). The β diversity (between-sample diversity) was calculated by the principal coordinate analysis and nonmetric multidimensional scaling. The functional traits of each sample were assigned according to the known genomes and prediction using the KEGG orthology (KO) (16). The functional differences between groups were determined using Linear Discriminant Analysis Effect Size (LEfSe).

The abundances of different gut genera in CP patients and HCs were compared by AUROC curve analysis to determine their performance in the diagnosis of CP.

Genetic analysis and grouping
Genomic DNA was extracted from peripheral blood leukocytes of patients using the FlexiGene DNA Extraction Kit (Qiagen, Hilden, Germany). *PRSS1*, *SPINK1*, *CFTR*, *CTRC*, *CASR*, *CTSB*, *KRT8*, *CLDN2*, *CPA1*, and *ATP8B1* were sequenced and analyzed, as previously described (6). The patients were classified into 3 groups, according to the sequencing results, and the abundance of gut bacteria in these groups were compared. Patients in group 1 had heterozygous or homozygous mutations in *CFTR* that were responsible for abnormal secretions in the pancreatic duct (17,18) and also had or had no heterozygous or homozygous mutations in *CASR*, *CTSB*, *SPINK1*, and/or *PRSS1* that were responsible for the abnormal activation of trypsinogen

Table 1. Basic clinical data of children with chronic pancreatitis

Patient ID	Sex	Age (yrs)	Dur (mo)	Clinical symptoms[a]	ERCP findings	Group and Patient ID
CP-1	Female	9	12	Repeated abdominal pain	Pancreatic duct stones, pancreatic duct stricture and dilation	Group 1-1
CP-2	Male	12	1	Repeated abdominal pain, nausea, vomiting	Pancreatic duct stricture	Group 1-2
CP-3	Male	13	12	Repeated abdominal pain or pancreatitis	Pancreatic duct dilation	Group 1-3
CP-4	Female	7	3	Recurrent pancreatitis	Pancreatic duct stricture and dilation	Group 1-4
CP-5	Male	4	24	Recurrent pancreatitis	Pancreatic duct stones, pancreatic duct dilation	Group 1-5
CP-6	Female	4	1	Repeated abdominal pain	Pancreatic duct stones, anomalous junction of pancreaticobiliary duct	Group 1-6
CP-7	Male	13	24	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct dilation	Group 1-7
CP-8	Male	4	18	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct stricture and dilation	Group 2-1
CP-9	Female	16	96	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct dilation	Group 2-2
CP-10	Female	6	8	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct dilation	Group 2-3
CP-11	Female	15	48	Chronic abdominal pain	Pancreatic duct stones, pancreatic duct stricture and dilation	Group 2-4
CP-12	Female	7	48	Chronic abdominal pain	Pancreatic duct stones, pancreatic duct stricture and dilation	Group 2-5
CP-13	Male	12	6	Recurrent pancreatitis	Pancreatic duct stones, pancreatic duct dilation	Group 2-6
CP-14	Male	10	12	Repeated abdominal pain	Pancreatic duct stones, pancreatic duct dilation	Group 3-1
CP-15	Female	13	1	Chronic abdominal pain	Anomalous junction of pancreaticobiliary duct, pancreatic duct stricture	Group 3-2
CP-16	Male	6	0.3	Repeated abdominal pain, nausea, diarrhoea	Pancreatic duct stones, pancreatic duct stricture and dilation	Group 3-3
CP-17	Female	3	12	Recurrent pancreatitis	Pancreatic duct stones, pancreas divisum	Group 3-4
CP-18	Male	8	24	Repeated abdominal pain	Pancreatic duct stones, pancreas divisum	Group 3-5
CP-19	Male	6	12	Repeated abdominal pain or pancreatitis	Pancreatic duct dilation	Group 3-6
CP-20	Female	8	60	Repeated abdominal pain	Pancreatic duct stones, pancreatic duct dilation	Group 3-7
CP-21	Male	6	9	Repeated abdominal pain or pancreatitis	Anomalous junction of pancreaticobiliary duct, pancreatic duct stricture	Group 3-8
CP-22	Male	4	12	Repeated abdominal pain or pancreatitis	Pancreatic duct stricture and dilation	—
CP-23	Female	15	24	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct stricture and dilation	—
CP-24	Female	6	4	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct dilation	—
CP-25	Male	9	24	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct stricture and dilation	—
CP-26	Female	4	6	Repeated abdominal pain or pancreatitis	Pancreatic duct stones, pancreatic duct stricture and dilation	—
CP-27	Male	11	96	Repeated abdominal pain or pancreatitis	Pancreatic duct dilation	—
CP-28	Female	4	0.7	Acute pancreatitis	Pancreatic duct stones, pancreatic duct dilation	—

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Table 1. (*continued*)

Patient ID	Sex	Age (yrs)	Dur (mo)	Clinical symptoms[a]	ERCP findings	Group and Patient ID
CP-29	Female	7	0.3	Acute pancreatitis	Pancreatic duct dilation and tortuous	—
CP-30	Female	6	1	Acute pancreatitis	Pancreatic duct stones, anomalous junction of pancreaticobiliary duct	—

CP, chronic pancreatitis; CT, computed tomography; Dur (mo), duration of symptoms (months); ERCP, endoscopic retrograde cholangiopancreatography.
[a]For diagnosis, a patient with abdominal pain and a blood amylase level 3 times higher than the normal reference value or a CT diagnosis of acute pancreatitis was classified as having pancreatitis; a patient not meeting these criteria was classified as having abdominal pain; a patient who had abdominal pain but no record of blood amylase level or CT examination was classified as having abdominal pain (4). Patients were stratified into group 1, 2, or 3 as described in the Methods and Table 2. In the "Group and Patient ID" column, the first number indicates the group (based on sequencing data) and the second number indicates patient ID.

(19). Patients in group 2 only had mutations in *CASR*, *CTSB*, *SPINK1*, and/or *PRSS1*. Patients in group 3 had no mutations in any of these genes.

Clinical trial registry

This study was approved by the Institutional Review Board (IRB) of Shanghai Jiao Tong University, and the protocols were approved by the Committee of Human Subjects Protection of Ruijin Hospital. Informed consent was obtained from the parents of all recruited children. The clinical trial registry number is NCT03809247 (https://register.clinicaltrials.gov/).

Statistical analysis

Quantitative data are presented as means ± SDs and analyzed using the *t*-test (for normal distributions), the Wilcoxon signed-rank test (for non-normal distributions), or one-way analysis of variance (for multiple samples). Categorical data are presented as frequencies and analyzed by the χ^2 tests (with or without the Yates correction for continuity) or Fisher exact tests, where appropriate. SPSS 17.7 software for Windows (SPSS, Chicago, IL) was used for statistical analyses. A *P* value below 0.05 was considered significant.

AUROC curve calculations were performed using R software, as previously described (20). In these calculations, the relative

Table 2. Mutations present in the different groups

Patient ID	Mutations associated with abnormal secretions in the pancreatic duct				Mutations associated with abnormal activation of trypsinogen			
	CFTR	*CPA1*	*KRT8*	*CTRC*	*CASR*	*CTSB*	*SPINK1*	*PRSS1*
Group 1-1	I556V				R990G	L26V	IVS 3+2 T>C	
Group 1-2	E217G							G208A
Group 1-3	I556V/Q1352H				R990G	L26V		N29I(N29T)
Group 1-4	I556V							
Group 1-5	I556V				A986S	L26V		N29I(N29T)
Group 1-6	I556V				R990G	L26V		
Group 1-7	M469V				R990G	L26V	UTR3, G>A	
Group 2-1						Q334P		G208A
Group 2-2					A986S			
Group 2-3					R990G	L26V	IVS 3+2 T>C/UTR3, G>A	G208A
Group 2-4						L26V	IVS 3+2 T>C	
Group 2-5					R990G	L26V	IVS 3+2 T>C	G208A
Group 2-6					R990G	L26V	IVS 3+2 T>C	
Group 3-1								
Group 3-2								
Group 3-3								
Group 3-4								
Group 3-5								
Group 3-6								
Group 3-7								
Group 3-8								

Figure 1. Alterations of the gut microbiota diversity in CCP. (**a** and **b**), Alpha diversity in patients and controls was calculated using the Shannon index and the Simpson index, and a two-tailed Wilcoxon signed-rank test was used for comparisons. **$P < 0.01$. (**c** and **d**), Beta diversity in patients and controls was calculated using PCoA and NMDS. (**e**), KEGG orthology (KO), showing differences in the functional pathways of gut microbiota in patients and controls. LEFSe was used for comparison. The predetermined threshold on the logarithmic linear discriminant analysis (LDA) score for discriminative features was set at >3.0; the predetermined α-value for the factorial Kruskal-Wallis test was set at >0.01. CCP, children with chronic pancreatitis; NMDS, nonmetric multidimensional scaling; PCoA, principal coordinate analysis.

abundance of each genus was subjected to arcsine square-root transformation before the analysis. To evaluate the diagnostic performance of the fitted logistic regression models, the area under the curve (AUC) values were calculated. The significance of differences between different AUCs was determined using the DeLong method.

RESULTS

Basic clinical data
We compared the gut microbiota of 30 CCP patients with 35 HCs (Table 1, see Table 1, Supplementary Digital Content 1, http://links.lww.com/CTG/A229). These 2 groups had no

significant differences in age (7.2 ± 0.5 vs 8.3 ± 0.7 years, $P = 0.224$) or sex ratio (53.3% vs 65.7% men, $P = 0.122$).

We collected the peripheral blood samples from 21 CCP patients (Tables 1–3). The 3 groups of CCP patients had no statistically significant differences in age (8.9 ± 4.0 vs 10.0 ± 5.0 vs 7.5 ± 3.0 years, $P = 0.515$) or sex ratio (57.1% vs 33.3% vs 62.5% men, $P = 0.646$).

Alterations of gut microbial diversity in CCP
CCP was associated with a significantly reduced alpha diversity of gut microbiota ($P < 0.01$) (Figure 1a,b). Analysis of beta diversity showed that the abundance of gut microbial genera had distinct clustering in CCP patients and in HCs (Figure 1c,d). In particular, the KEGG

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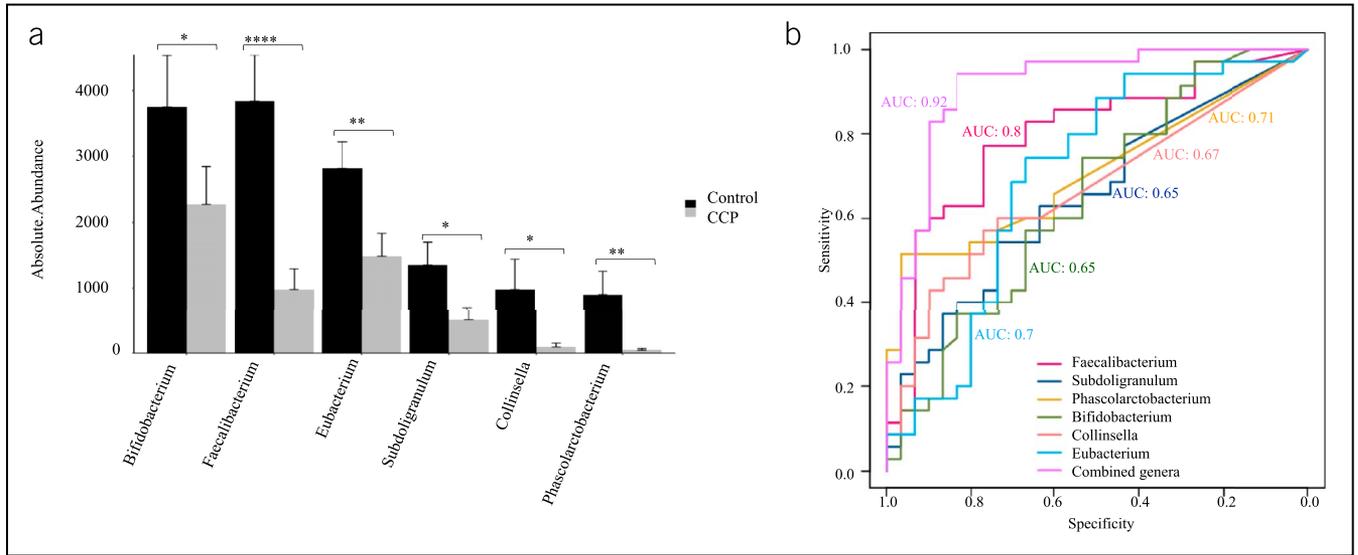
Figure 2. Alterations in the abundances of gut microbial genera in CCP. (**a**), Six genera whose abundances had the greatest reductions in CCP patients relative to HCs, with comparisons by a two-tailed Wilcoxon signed-rank test (sum of the mean <2.3, difference of the mean >2.5, see Table 2, Supplementary Digital Content 1, http://links.lww.com/CTG/A229). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. (**b**), The AUROC curve analysis of the performance of 6 genera in the diagnosis of CCP. *Faecalibacterium*: AUC = 0.80 (0.68–0.80, $P < 0.001$), *Subdoligranulum*: AUC = 0.65 (0.52–0.65, $P < 0.05$), *Phascolarctobacterium*: AUC = 0.71 (0.59–0.71, $P < 0.01$), *Bifidobacterium*: AUC = 0.65 (0.51–0.65, $P = 0.07$), Collinsella: AUC = 0.67 (0.54–0.67, $P < 0.67$) Eubacterium: AUC = 0.70 (0.56–0.70, $P < 0.01$). The use of each of the 6 individual arcsine square root transformed abundance values, together with the coefficients from multivariate logistic regression: AUC = 0.92 (0.84–0.92, $P < 0.001$). AUC, area under curve; CCP, children with chronic pancreatitis; HC, healthy control.

analysis indicated that CCP patients had gut microbiota that were enriched in the phosphotransferase system and depleted in ribosomal activity, porphyrin and chlorophyll metabolism, starch and sucrose metabolism, and aminoacyl-tRNA biosynthesis (Figure 1e).

Identification of disordered genera

We identified 28 genera with different abundances in the CCP and HC groups. Relative to the HCs, CP patients had 19 genera with decreased abundance and 9 genera with increased abundance (see Table 2, Supplementary Digital Content 1, http://links.lww.com/CTG/A229). *Faecalibacterium, Subdoligranulum, Phascolarctobacterium, Bifidobacterium, Collinsella,* and *Eubacterium* were the 6 genera with the greatest decreases in absolute abundance in CCP patients (Figure 2a).

The AUROC analysis showed that when each genus was considered individually, the diagnostic performance of *Faecalibacterium* (AUC = 0.80, 95% confidence interval [CI]: 0.68–0.80, $P < 0.001$), *Phascolarctobacterium* (AUC = 0.71, 95% CI: 0.59–0.71, $P < 0.01$), and *Eubacterium* (AUC = 0.70, 95% CI: 0.56–0.70, $P < 0.01$) were "fair"; and the diagnostic performance of *Subdoligranulum* (AUC = 0.65, 95% CI: 0.52–0.65, $P < 0.01$), *Bifidobacterium* (AUC = 0.65, 95% CI: 0.51–0.65, $P = 0.07$), and *Collinsella* (AUC = 0.67, 95% CI: 0.54–0.67, $P < 0.05$) were "poor." The use of each individual arcsine square root transformed abundance, together with the coefficients from multivariate logistic regression, led to "excellent" diagnostic performance (AUC = 0.92, 95% CI: 0.84–0.92, $P < 0.001$) (Figure 2b).

Relationship of host functional mutations with gut microbiota

Relative to the HCs, the abundance of *Butyricicoccus* was significantly lower in group 1, but not significantly different in group 2 and group 3 (Figure 3a). The abundance of *Ruminococcaceae* was

significantly greater in group 2, but not significantly different in group 1 and group 3 (Figure 3b). The abundance of *Veillonella* was greater and the abundance of *Phascolarctobacterium* was lower in group 3, but not significantly different in group 1 and group 2 (Figure 3c,d).

DISCUSSION

This is the first study of the gut microbiota of CCP patients who have mutations in different specific functional genes. There were several major findings. First, CCP was associated with a significantly reduced gut microbial alpha diversity, and the subsequent KEGG analysis indicated that the disordered gut microbiota probably contributed to the pathogenesis of CCP. Second, our comparison of the gut microbiota of CCP patients and HCs using the AUROC curve analysis indicated the presence of 4 new genera (*Subdoligranulum, Phascolarctobacterium, Collinsella,* and *Eubacterium*) and 2 known genera (*Faecalibacterium* and *Bifidobacterium*) (10,21). The use of all 6 genera together led to excellent diagnostic performance (AUC = 0.92, 95% CI: 0.84–0.92, $P < 0.001$). Thus, determination of the abundances of these 6 genera might be useful as a diagnostic marker of CCP, and these genera may also be considered as therapeutic targets for treatment of CCP. Third, *Butyricicoccus, Ruminococcaceae, Veillonella,* and *Phascolarctobacterium* are associated with different functional gene mutations in the host that are linked to CCP, thus confirming that specific functional gene mutations in the host might affect the composition of gut microbiota. This further supports the hypothesis that the presence of disordered gut microbiota is related to the pathogenesis of CCP. Further in-depth studies of gut microbiota are thus necessary to investigate the pathogenic mechanisms of CCP and to develop new treatments for CCP.
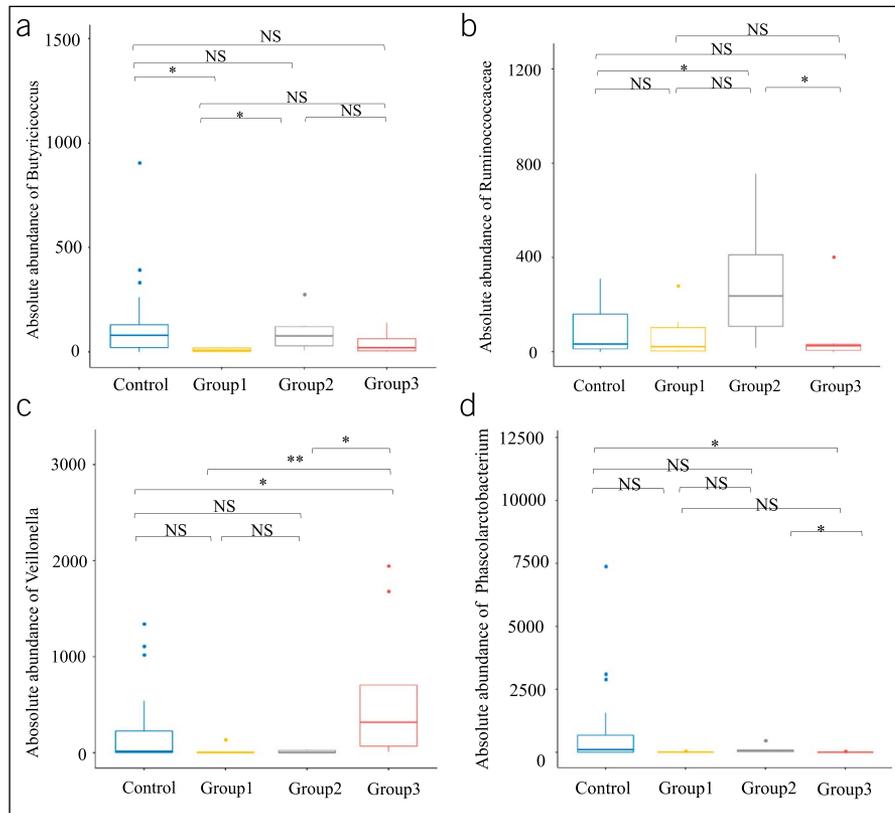
Figure 3. Relationship of gut microbial genera with functional mutations in hosts who have CCP. (**a**), *Butyricicoccus* was significantly less abundant in group 1 (*CFTR* mutations and mutations in *CASR, CTSB, SPINK1*, and/or *PRSS1*). (**b**), *Ruminococcaceae* was significantly more abundant in group 2 (mutations only in *CASR, CTSB, SPINK1*, and/or *PRSS1*). (**c** and **d**). *Veillonella* was significantly more abundant and *Phascolarctobacterium* was significantly less abundant in group 3 (no mutations of the tested genes). Box plots were drawn using R software, and a two-tailed Wilcoxon singed-rank test was used for comparisons. $*P < 0.05$, $**P < 0.01$. CCP, children with chronic pancreatitis.

Among the genera of gut microbiota that had significantly decreased abundance in CCP, *Faecalibacterium* (10) and *Bifidobacterium* (21) were also less abundant in adults with CP. *Faecalibacterium prausnitzii* can produce butyrate (22) and salicylic acid (23), participate in the inflammatory process by blocking NF-kB activation and IL-8 production (24), and negatively correlate with the plasma endotoxin and glycemic status (10). A reduced level of *Faecalibacterium* is also associated with Crohn's disease (24), colitis (25), and colorectal cancer (26). A decreased abundance of *Faecalibacterium* might promote CP by weakening the inflammatory process. *Bifidobacterium* is a well-known probiotic that can improve the nutritional status and enhance the immune capacity of patients with severe acute pancreatitis (27). Therefore, these 2 groups of gut microbiota should be considered as novel therapeutic targets in future applications that seek to prevent or treat CCP.

No previous studies of CP have examined 4 other genera of gut microbiota that we examined, *Subdoligranulum*, *Phascolarctobacterium*, *Eubacterium*, and *Collinsella*. However, as potentially beneficial bacteria, previous research reported that a reduction of *Subdoligranulum* was related to glycemic control, inflammatory processes (28), and neoplasms of the stomach, colon, and rectum (29). In addition, the abundance of *Subdoligranulum* increases after the administration of vitamin D (30) and is associated with a healthy eating index (31). These results suggest that CP patients

might benefit from vitamin D supplements and healthy eating. *Phascolarctobacterium* is a genus of proinflammatory bacteria that is associated with the onset and development of inflammation in metabolic diseases and mental illnesses (32,33). In addition, patients with nephrolithiasis have a greater abundance of *Phascolarctobacterium* than HCs, and the abundance of these bacteria correlates with the concentration of trace elements in blood, including potassium, sodium, calcium, and chlorinum (34). *Eubacterium*, a potential probiotic, is a butyrate-producing bacterium whose level is decreased in Crohn's disease (35) but increased in primary biliary cholangitis (36). The abundance of *Collinsella* is closely related to high levels of alpha aminoadipate and asparagine and the production of the proinflammatory cytokine IL-17A, and animal studies indicated it has a role in altering gut permeability and disease severity in arthritis (37). On the other hand, an increased abundance of *Collinsella* is associated with symptomatic atherosclerosis (38).

To our knowledge, no previous studies have examined the relationship of gut microbiota with functional gene mutations in CP patients. Thus, one of our important findings is that 4 genera of gut microbiota appear to be related to different functional mutations in their hosts. We found that the abundance of *Butyricicoccus* was significantly decreased patients with mutations in *CFTR*, which manifests as abnormal secretions of the pancreatic duct. *Butyricicoccus* is a butyrate-producing clostridial

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Table 3. Molecular characteristics of mutated genes in 13 patients

Gene	db SNP	HGMD	Nucleotide changes	Amino acid changes	Cases (n)
CFTR	rs121909046	CM972939	c.A650G	p.E217G	1
CFTR	—	CM034785	c.A1405G	p.M469V	1
CFTR	rs75789129	CM920985	c.A1666G	p.I556V	5
CFTR	rs113857788	—	c.G4056C	p.Q1352H	1
CASR	—	—	c.A2968G	p.R990G	7
CASR	rs1801725	CM012741	c.G2956T	p.A986S	2
CTSB	—	—	c.C76G	p.L26V	9
CTSB	rs117613666	—	c.A1001C	p.Q334P	1
SPINK1	rs148954387	CM001461	IVS 3,+2,T>C	Splicing	5
SPINK1	rs11319	—	UTR3,CAGGCCTCGC[G] GTGACCTGAT/G-A	UTR3, G>A	2
PRSS1	rs111033566	CM971250	c.A86T	p.N29I(N29T)	2
PRSS1	rs117497341	CM066596	c.C410T	p.T137M	2
PRSS1	rs189270875	CM066598	c.G623C	p.G208A	4
PRSS1	rs6666	—	c.T486C	p.D162D	1

cluster IV genus (39). Previous research showed that its abundance was reduced in the stools of patients with ulcerative colitis. In addition, *Butyricicoccus*-conditioned medium can prevent the cytokine-induced increase of epithelial permeability *in vitro*, and a specific strain of *Butyricicoccus pullicaecorum* can reduce intestinal inflammation in a rat model of colitis (40). Butyrate and *B. pullicaecorum*-conditioned medium can reduce the *CLDN1* expression while preserving the epithelial integrity (41). These results suggest that *Butyricicoccus* may affect gene expression in its host, thereby altering physiological function and inducing disease, thus indirectly confirming our hypothesis that the presence of disordered gut microbiota is related to the pathogenesis of CCP.

The abundance of *Ruminococcaceae* was significantly increased CP patients who had mutations in *CASR*, *CTSB*, *SPINK1*, and/or *PRSS1*, each of which is associated with abnormal activation of trypsinogen. A reduced abundance of these bacteria is associated with increased innate immune responses and the levels of proinflammatory cytokines (IL-6 and TNFα) (42). A reduced abundance of *Ruminococcaceae* appears to be implicated in food sensitization and to precede the development of atopic eczema (43). Han et al. showed that a decreased abundance of *Ruminococcaceae* had a positive correlation with the Treg/Th17 ratio, thus suggesting that intestinal microbiota might induce acquired graft-vs-host disease by altering the Treg/Th17 balance (44). Several other studies demonstrated that host heredity affects the abundance of gut *Ruminococcaceae* (7,8).

The abundance of *Veillonella* was significantly increased and that of *Phascolarctobacterium* was significantly decreased in CCP patients who had no mutations in any of the tested genes. *Veillonella* is a genus of anaerobic Gram-negative cocci and considered as part of the normal flora of the animal and human mouth, gastrointestinal tract, and genitourinary tract (45). Matera et al. demonstrated that *Veillonella parvula* lipopolysaccharides (LPS) are recognized by toll-like receptors (TLR4) and that activation of

MAP kinases plays an important role in *V. parvula* LPS-stimulated signaling of human peripheral blood mononuclear cells (46). Disorders of these cells are associated with many inflammatory diseases, such as chronic anaerobic pneumonitis (47), acute pyelonephritis, and secondary bacteraemia during pregnancy (48).

Our results showed that 8 groups of gut microbiota are potential probiotics because of their anti-inflammatory potential. Disruption of these microbes is associated with many different inflammatory diseases. The first human intervention trial that used a butyrate-producing *Clostridium* cluster IV isolate demonstrated that *B. pullicaecorum* 25-3[T] administration was safe and well tolerated by healthy participants (49). This safety study paved the way for the further development of this strain as a next-generation probiotic. We found no association between these genera and the CP phenotype in the GMrepo database, which continues to annotate the human gut macrogenome (50). Thus, our findings are a useful complement to the GMrepo database.

There were some limitations in our study. We examined only a small number of patients, and we therefore need to verify the relationships of the different functional mutations of hosts with different genera of gut microbes by examining more patients. The sequencing and comparison of fecal samples from CCP patients who improve or do not improve during treatment and follow-up would also be useful. Deep sequencing, including metagenomic sequencing, is also necessary. The anti-inflammatory and therapeutic effects of probiotics still need to be verified *in vivo* and *in vitro*.

Our major findings are that the presence of disordered gut microbiota occurs in CCP and that specific functional gene mutations in the host appear to influence the gut microbiota composition. These findings may be helpful for further investigations into the pathogenic mechanisms of CP and provide several promising targets for the diagnosis and treatment of CP.

CONFLICTS OF INTEREST

Study Highlights

WHAT IS KNOWN

✓ CP is a serious condition, particularly in children because it reduces dietary intake and delays growth and development. However, the pathogenesis of CP is still unclear. Interactions of host genetics with gut microbiota underlie many diseases, but little is known about these interactions in the pathogenesis of CP, especially in CCP, in whom external pathogenic factors relative are less important.

WHAT IS NEW HERE

✓ This is the first study of gut microbiota in CCP to analyze the impact of different functional gene mutations in the host. CCP is characterized by gut microbiota with remarkably reduced alpha diversity compared to age-matched HCs. Receiver operating characteristic analysis indicated that 6 genera, including 4 newly described genera, were significantly less abundant in CCP. Patients with CCP who had different functional gene mutations had different gut microbiota.

TRANSLATIONAL IMPACT

✓ These findings may help to identify new biomarkers or therapeutic targets for CP.

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