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Research Article

RESEARCH ARTICLE

Impact of cystic fibrosis disease on archaea and bacteria composition of gut microbiota

Francesco Miragoli[1,†], Sara Federici[1,†‡], Susanna Ferrari[1], Andrea Minuti[2], Annalisa Rebecchi[1], Eugenia Bruzzese[3], Vittoria Buccigrossi[3], Alfredo Guarino[3] and Maria Luisa Callegari[1,*]

[1]Centro Ricerche Biotecnologiche, Università Cattolica del Sacro Cuore, Cremona 26100, Italy, [2]Istituto di Zootecnica, Università Cattolica del Sacro Cuore, Piacenza 29122, Italy and [3]Department of Translational Medical Sciences, Section of Pediatrics, University Federico II, Naples 80131, Italy

*Corresponding author: Istituto di Microbiologia, Universita Cattolica del Sacro Cuore, via Milano 24, Cremona 26100, Italy. Tel: +390372499141; Fax: +390372499193; E-mail: marialuisa.callegari@unicatt.it
†These authors contributed equally to this work.
‡Present address: Weizmann Institute of Science, Department of Immunology, Rehovot, Israel.
One sentence summary: A different balance of hydrogenotrophic bacteria in cystic fibrosis intestinal microbiota, compared with healthy subjects, could increase hydrogen accumulation in the colon.
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ABSTRACT

Cystic fibrosis is often associated with intestinal inflammation due to several factors, including altered gut microbiota composition. In this study, we analyzed the fecal microbiota among patients with cystic fibrosis of 10–22 years of age, and compared the findings with age-matched healthy subjects. The participating patients included 14 homozygotes and 14 heterozygotes with the delF508 mutation, and 2 heterozygotes presenting non-delF508 mutations. We used PCR-DGGE and qPCR to analyze the presence of bacteria, archaea and sulfate-reducing bacteria. Overall, our findings confirmed disruption of the cystic fibrosis gut microbiota. Principal component analysis of the qPCR data revealed no differences between homozygotes and heterozygotes, while both groups were distinct from healthy subjects who showed higher biodiversity. Archaea were under the detection limit in all homozygotes subjects, whereas methanogens were detected in 62% of both cystic fibrosis heterozygotes and healthy subjects. Our qPCR results revealed a low frequency of sulfate-reducing bacteria in the homozygote (13%) and heterozygote (13%) patients with cystic fibrosis compared with healthy subjects (87.5%). This is a pioneer study showing that patients with cystic fibrosis exhibit significant reduction of H_2-consuming microorganisms, which could increase hydrogen accumulation in the colon and the expulsion of this gas through non-microbial routes.

Keywords: cystic fibrosis; gut microbiota; methanogen archaea; sulfate-reducing bacteria, DGGE-PCR; qPCR

INTRODUCTION

Cystic fibrosis (CF) frequently involves the gastrointestinal tract, with one of the most common complications being chronic inflammation (Li and Somerset 2014). Decreased bacterial diversity is associated with overgrowth of particular groups of resident bacteria that can induce inflammatory responses, which could be a factor leading to intestinal inflammation in CF. Several mechanisms linking microbial dysbiosis to gut inflammation in inflammatory bowel diseases have been proposed. An altered composition of the microbial communities might cause

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an immune response in the gastrointestinal tract due to the inflammatory potential of some commensal bacteria such as *Escherichia coli* and *Enterococcus faecalis* (Kim *et al.* 2005). Functional changes in the gut microbiota, such as the increase of stress oxidative pathways (Morgan *et al.* 2012), the decrease of butyrate production (Takaishi *et al.* 2008) or an increase of sulfate-reducing bacteria (SRB) generating toxic H_2S, might also be implicated in intestinal inflammation (Pitcher 2000). However, for several gastrointestinal diseases, it is still not clear if the reduction of species richness is a cause or a downstream consequence of the inflammatory process (Mosca, Leclerc and Hugot 2016). Concerning CF, recent studies have examined the intestinal microbiota composition using both culture-dependent and culture-independent techniques (Duytschaever *et al.* 2011, 2013; Scanlan *et al.* 2012; Schippa *et al.* 2013; Bruzzese *et al.* 2014). Results of these studies commonly describe reductions of members of the *Bacteroides-Prevotella* group, *Bifidobacterium* spp. and *Clostridium* cluster XIVa. However, the literature includes some discrepancies, for example, the complexity of CF gut microbiota is sometimes described as reduced and sometimes as similar compared to that of healthy subjects. Such discrepancies are likely because the studies use different methodology and because the investigated patient cohorts are heterogeneous in terms of age, genotype, antibiotic treatments, inflammation status, etc. To the best of our knowledge, there are no studies showing a clear relationship between reduced microbiota complexity and inflammation in CF; in one study (Bruzzese *et al.* 2014), the authors attempted to correlate bacterial biodiversity with fecal calprotectin (CLP) values but no association was found.

CF disease is characterized by an altered intraluminal environment in which many factors can affect the gut microbiota composition and biodiversity. Among these, alterations in intestinal motility, pH, production of digestive enzymes and mucus are mostly responsible for CF-associated gastrointestinal complications (De Lisle and Borowitz 2013). The excessive mucus production, together with its high viscosity (Garcia, Yang and Quinton 2009), not only could create niches in which bacterial colonization is altered, but also contribute to the onset of some gastrointestinal symptoms such as obstruction and malabsorption (Freudenberg *et al.* 2008; Houwen *et al.* 2010). Malabsorption and inadequate nutrient intake represent the primary causes of malnutrition in patients with CF (Stephenson *et al.* 2013). In this context, the gut microbiota could be one of the factors affecting nutrient metabolism and energy balance (Li and Somerset 2014).

Gut microbiota is directly involved in the anaerobic fermentation of undigested dietary components that reach the colon, and of mucin glycoproteins that constitute the mucus gel covering the intestinal epithelium. These complex metabolic reactions produce a variety of final products, mainly short-chain fatty acids (SCFAs). The type and quantity of SCFAs depend on the metabolic functions of the resident bacterial communities and on the gut environmental conditions as determined by pH, nutrient availability, transit time, etc. Besides butyrate, acetate and propionate, which are the major end products of fermentation, a lot of less represented SCFAs can result from substrate fermentations and bacterial cross-feeding, including valerate, hexanoate and octanoate. Secondary products of several microbial fermentations are gases such as CO_2 and H_2. Since high hydrogen levels can inhibit normal fermentative reactions (Carbonero, Benefiel and Gaskins 2012), mechanisms involved in H_2 disposal are required for proper function of the intestinal ecosystem. A low H_2 partial pressure is maintained in the colon by both H_2 expulsion as flatus or breath and microbial hydrogenotrophic activity (Nakamura *et al.* 2010). Therefore, microorganisms able to use hydrogen such as methanogenic archaea, acetogenic bacteria and SRB may play an important role in nutrient digestion and absorption through cross-feeding interactions.

In this study, we sought to determine whether the gut microbiota disruption in CF may involve modification of the archaea and SRB populations in response to the altered intestinal environment. We used PCR-DGGE and qPCR to investigate the gut bacterial and archaeal communities in 30 subjects with CF as well as in age-matched healthy controls. In addition, we evaluated potential correlations between gut microbiota composition and key clinical parameters as host genetic background, gut inflammation and status of lung function.

MATERIALS AND METHODS

Study subjects

For this study, we enrolled 30 clinically stable young adults with CF, who did not require antibiotics for at least 2 weeks and who did not manifest acute intestinal or extraintestinal diseases. We registered the patients' sex, age, body mass index (BMI) centile, CF genotype and *Pseudomonas aeruginosa* colonization, based on sputum cultures. Host genotype was classified as either mild or severe depending on the occurrence of at least one mild mutation or two severe mutations, respectively, according to the class of mutations (Zielenski 2000) and their related functional consequences (Castellani *et al.* 2008). Antibiotic burden in the last 12 months for patients with *P. aeruginosa* colonization consisted in four cycles of intravenous antibiotic therapy. One or two additional cycles of therapy were necessary in case of pulmonary exacerbation. Patients showing no *P. aeruginosa* colonization received on average two cycles of intravenous antibiotic therapy for pulmonary exacerbation.

The forced expiratory volume in 1 s (FEV1) and CLP levels were measured in order to evaluate lung function and intestinal inflammation, respectively. Patients' mean age was 14.54 years (range, 10–22 years). To investigate intestinal microbiota composition and inflammation, fecal samples were collected from these subjects. We also collected fecal samples from eight healthy volunteers of the same age (mean age, 14.38 years). Participants older than 18 years provided their written informed consent to participate in this study. Moreover, we obtained written informed consent from the parents of each enrolled child. The study protocol was approved by the Ethical Committee of University Federico II of Naples.

Intestinal inflammation and lung function

To assess the level of intestinal inflammation, we measured fecal CLP production using an enzyme-linked immunosorbent assay (Calprest Eurospital SpA, Trieste, Italy). Normal inflammation was defined as a fecal CLP level of 0–50 μg/g of stool, intermediate inflammation corresponded to 50–100 μg CLP/g stool and severe intestinal inflammation was indicated by greater than 100 μg CLP/g stool (Fagerberg *et al.* 2003). CLP levels were not assessed in healthy volunteers. FEV1 was measured as described by Corey *et al.* (1997). At least three maximum expiratory maneuvers were performed during each test and the best result was recorded.

Bacterial DNA extraction and PCR-DGGE

We performed DNA extraction and PCR-DGGE of the dominant bacterial communities and the *Bacteroides-Prevotella* group as

Table 1. qPCR primers used in this study.

Species/group	Target gene	Reference
Dialister invisus	16S rRNA	Kumar *et al.* (2003)
Collinsella aerofaciens	16S rRNA	Kassinen *et al.* (2007)
Ruminococcaceae	16S rRNA	Garcia-Mazcorro *et al.* (2012)
Blautia spp.	16S rRNA	Suchodolski *et al.* (2012)
Clostridium coccoides group	16S rRNA	Rinttilä *et al.* (2004)
Ruminococcus gnavus	16S rRNA	Joossens *et al.* (2011)
Methanobrevibacter smithii	16S rRNA	Dridi *et al.* 2009)
Methanosphaera stadtmanae	16S rRNA	Dridi *et al.* (2009)
Methanobrevibacter	16S rRNA	Skillman *et al.* (2004)
Total archaea	16S rRNA	Yu *et al.* (2005)
SRB	*dsrA*	Kondo *et al.* (2004)
Acetogens	*acsB*	Gagen *et al.* (2010)
Methanogens	*mcrA*	Denman, Tomkins and McSweeney (2007)
Butyrate-producing bacteria	*BcoAT*	Louis and Flint (2007)

dsrA = dissimilatory (bi)sulfite reductase gene, α subunit; *acsB* = acetyl-CoA synthase gene, β subunit; *mcrA* = methyl-coenzyme M reductase gene, α subunit; *BcoAT* = butyryl-CoA: acetate CoA transferase gene.

previously described (Bruzzese *et al.* 2014). Our analysis of the *Clostridium coccoides* group utilized previously reported primers and thermal protocol (Maukonen *et al.* 2006). All DGGE gels were run in an INGENYphor 2×2 System (INGENYphor, Goes, the Netherlands) using 1× buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). DGGE gels were digitally processed via a multistep procedure using Fingerprinting II SW (Bio-Rad Laboratories, Hercules, CA, USA). After profile normalization, dendrograms were constructed using Pearson's correlation coefficient and the unweighted-pair group method. Discriminating bands, i.e. bands whose intensity varied significantly between patients with CF and healthy controls, were excised, re-amplified, and sequenced (BMR Genomics, Padova, Italy). Next, the blastn algorithm was used to compare the nucleotide sequences with those deposited in GenBank (http://www.ncbi.nlm.nih.gov/) and in the Ribosomal Database Project (Maidak *et al.* 1994). We calculated the Shannon Wiener Diversity (H) Index of the biodiversity using the previously reported formula (Deng *et al.* 2012). We also attempted to perform PCR-DGGE analysis of archaea and SRB communities by using group-specific primers as described (Geets *et al.* 2006; Federici *et al.* 2015). Unfortunately, PCR amplification of methanogenic 16S rRNA genes resulted in bands that were all identified as *Methanobrevibacter smithii*, while for SRB no amplicons were obtained from fecal DNA of patients with CF.

qPCR

We used qPCR to quantify *Escherichia coli*, *Bifidobacterium* spp., *Bifidobacterium adolescentis*, *B. catenulatum*, *Eubacterium rectale*, the *Bacteroides-Prevotella* group, *Bacteroides uniformis*, *Ba. vulgatus* and *Faecalibacterium prausnitzii* as previously described (Bruzzese *et al.* 2014). Table 1 lists the remaining qPCR primers. For the detection of hydrogenotrophic groups, primers targeting key functional genes, namely the methyl-coenzyme M reductase α-subunit (*mcrA*), dissimilatory (bi)sulfite reductase α-subunit (*dsrA*), butyryl-CoA: acetate CoA-transferase (*BcoAT*) and acetyl-CoA synthase β-subunit (*acsB*) were used to quantify methanogens, SRB, butyrate-producing bacteria and acetogens, respectively. All qPCR reactions were performed in a LightCycler® 480 Instrument II (Roche Life Science, Mannheim, Germany). Standard curves were generated using reference DNA, and thermal conditions were set up to achieve optimal amplification efficiency. Results were expressed as gene copy number per gram of wet fecal sample.

Statistical analysis

Principal component (PC) analysis was performed using the PRINCOMP procedure of SAS release 8.0 (SAS Inst. Inc., Cary, NC, USA). The CLP concentration and FEV1 data of patients with CF were analyzed by ANOVA using the GLM procedure of SAS. The applied statistical model included the fixed effects of genetic mutation (heterozygote or homozygote) and the random effect of the patient. Spearman's rank correlation was calculated by separately considering the two groups (healthy and CF) using the CORR procedure of SAS. To investigate the relationship between CLP level, FEV1data and bacterial population in patients with CF, we performed a stepwise regression analysis using the REG procedure of SAS. Statistical significance was established using a conventional P level of 0.05.

RESULTS AND DISCUSSION

Study population

Of the included patients, 14 (46.7%) were homozygous and 14 (46.7%) were heterozygous for the delF508 mutation, which is the most common polymorphism of the CF transmembrane conductance regulator gene. The remaining two subjects (6.6%) displayed a mild genotype, which was not correlated with a mild inflammation status since CLP value was greater than 100 μg CLP/g stool for both individuals. The BMI centile, ranging from 10th to 75th centile, indicated that all enrolled patients had a good nutritional status. In the patients with CF, we measured fecal CLP concentration, which is reported as a good marker of intestinal inflammation (Hradsky *et al.* 2014; Dhaliwal *et al.* 2015). Severe inflammation was found in 19 subjects (63%), intermediate inflammation in 7 subjects (23%) and mild inflammation in 4 subjects (13%). Analysis by unpaired t test revealed no correlation between genotype and intestinal inflammation:

mean of 213.9 μg/g ±145.2 μg/g in homozygous subjects versus 231.3 μg/g ± 299.2 μg/g among heterozygous subjects. FEV1 (% predicted) ranged from 32 to 99 (mean value, 75.71±13.82). No correlation was found between genotype and FEV1 data: mean (% predicted) of 73 ± 17.35 in homozygous subjects versus 78 ± 9.9 (% predicted) among heterozygous subjects. FEV1 values indicated a mild pulmonary involvement in 23 patients, a moderate involvement in 5 patients and a severe pulmonary involvement in 2 patients. FEV1 is widely used as marker of lung function throughout the course of CF lung disease and it is considered the best clinical predictor of poor outcome. *Pseudomonas aeruginosa* lung colonization was detected in 11 patients (36%).

PCR-DGGE

PCR-DGGE analysis of amplicons obtained using universal primers revealed a more complex profile in healthy subjects than in the majority of patients with CF (Fig. 1a). The main Shannon index values obtained for healthy subjects and patients with CF were 1.17 and 0.94, respectively ($P = 0.007$). This difference in biodiversity was lower than that previously reported among younger subjects (Bruzzese *et al.* 2014). Figure 1b presents the dendrogram produced using software analysis. Healthy profiles formed two distinct subclusters: one containing subjects aged 10–14 years and the other including subjects from 16–17 years of age. In contrast, the clusterization of patients with CF was apparently unrelated to age or CLP level, and we did not find distinctiveness related to homozygote and heterozygote (delF508 and non-delF508) profiles (Fig. 1b). Notably, the frequent use of antibiotic therapy to treat pulmonary exacerbation could make individuals cluster distinctly regardless of host age or genotype. The effects on gut microbiota composition due to antibiotic treatments can be, in some cases, persistent (Jernberg *et al.* 2005, 2007; Jakobsson *et al.* 2010). Moreover, reduced transit time, occasionally resulting in intestinal constipation, may exert a great impact on the relative proportions of specific bacterial populations.

Our results differ from previous findings that genetic background (homozygous-delF508, heterozygous-delF508 and non-delF508) was correlated with differences in gut microbiota composition (Schippa *et al.* 2013). These discrepancies could be related to the analysis of different regions of the 16S rRNA gene between studies. Indeed, the quality of PCR-DGGE data reportedly depends on the number and the resolution of the amplicons in the gel (Yu and Morrison 2004). We chose the V3 region for PCR-DGGE analysis since it is considered a reliable target for gut microbiome studies (Huys, Vanhoutte and Vandamme 2008) although longer fragments, as those corresponding to V6–V8

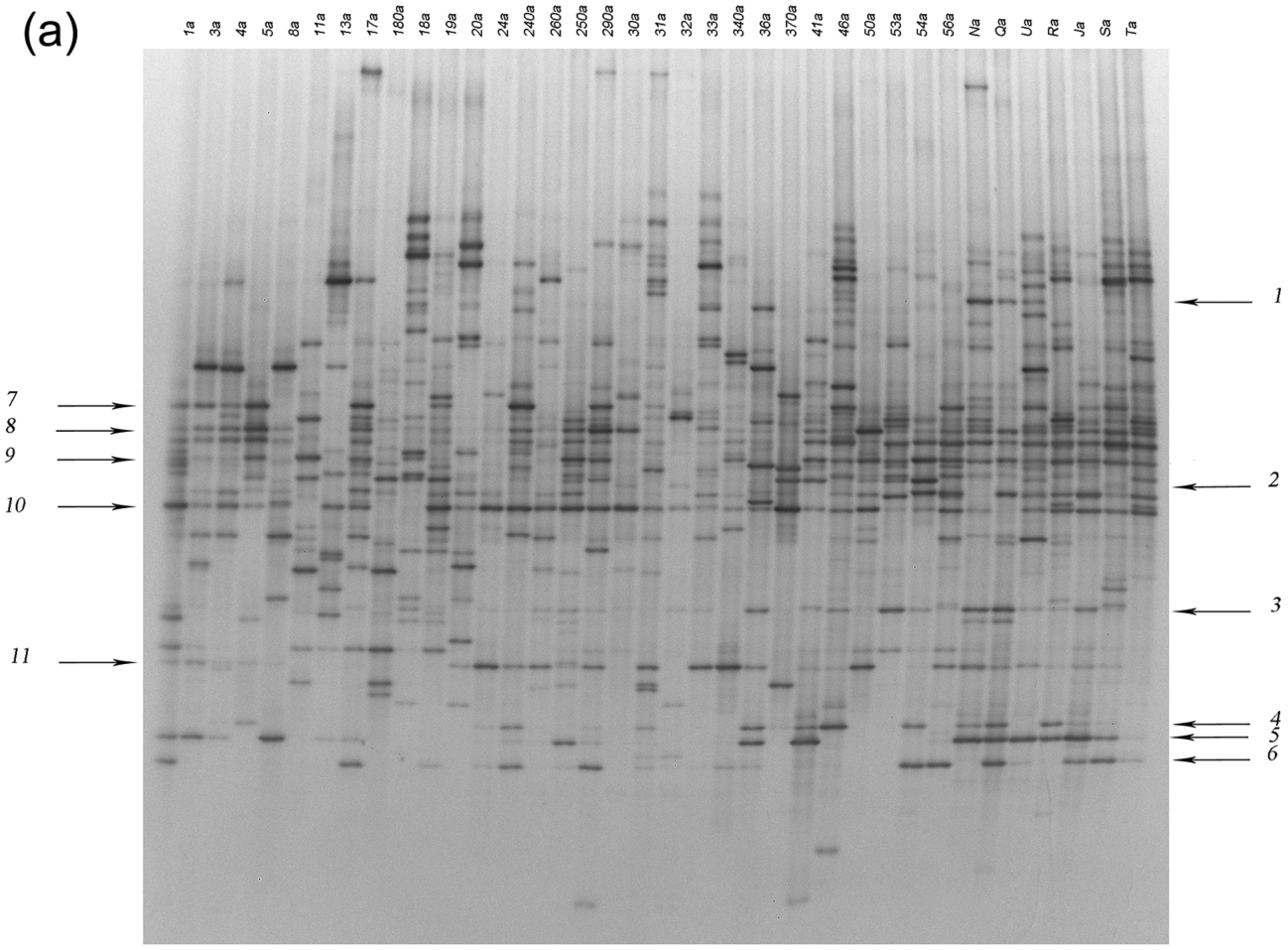

Figure 1. **(a)** DGGE profiles obtained using universal primers designed for the 16S rRNA gene. CF samples are indicated by a number, while samples from healthy subjects are indicated by capital letters. **(b)** Dendrogram constructed from analysis of DGGE gels using Pearson's correlation coefficient and the unweighted-pair group method. Red squares indicate homozygotes, green squares heterozygotes and light blue squares healthy subjects. Next to each patient's identification code, their age and CLP value are reported.

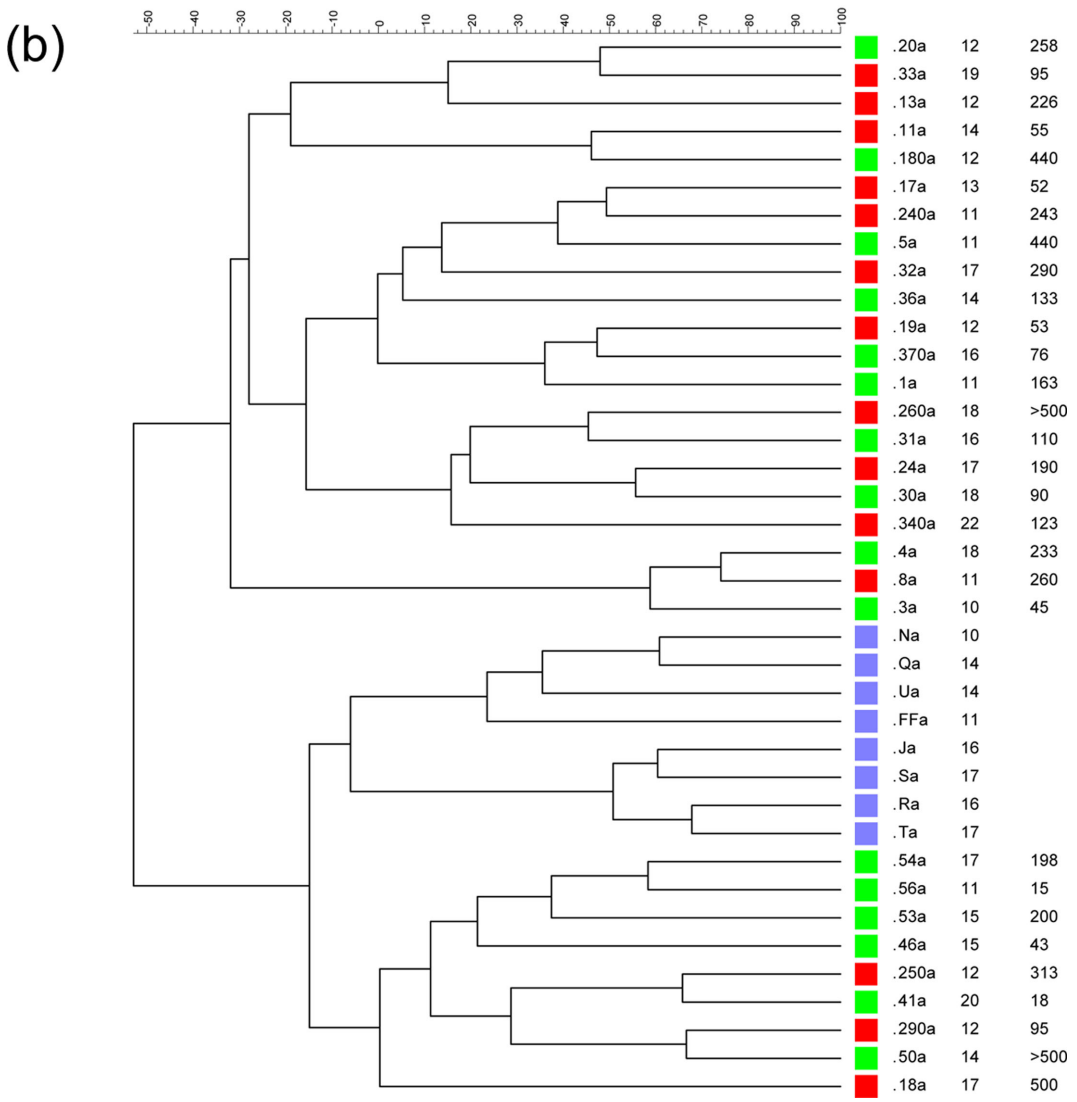

Figure 1. (*Continued*).

regions, can allow a more accurate taxonomic affiliation of DNA bands. Yu and Morrison (2004) recommended a different set of primers compared to that used by Schippa *et al.* (2013) for the V6–V8 regions, in order to improve the biodiversity indices. In our present study, further software analysis enabled identification of the discriminant bands of fingerprints in decreasing order as related to *Escherichia coli/Shigella*, *Blautia* spp., *Faecalibacterium prausnitzii*, *Collinsella aerofaciens*, *Dialister invisus*, *Eubacterium rectale* and *Bifidobacterium adolescentis* (Table 2).

The majority of discriminant bands belonged to the *Clostridium coccoides* group, which is consistent with prior reports (Duytschaever *et al.* 2013; Schippa *et al.* 2013). Thus, we analyzed this population in detail using PCR-DGGE. *Ruminococcus gnavus*, *Blautia luti*, *E. rectale* and *Bl. glucerasea* were more frequently identified in healthy controls than in patients with CF, while *Dorea longicatena* was indistinctly present in both groups (Fig. 2a). The fecal microbiota clusters did not correlate with the CLP level, FEV1 data, lung colonization or age. Healthy subjects and nine heterozygotes (delF508 and non-delF508) formed one group, while the remaining seven heterozygotes formed a separate cluster together with homozygotes (Fig. 2b).

We previously found that *Bacteroides uniformis* presence was remarkably discriminant for healthy profiles (Bruzzese *et al.* 2014); thus, we also evaluated the composition of the *Bacteroides-Prevotella* group using PCR-DGGE. A prior study reported that healthy subjects showed higher numbers of organisms from the *Bacteroides-Prevotella* group (obtained by count plates) compared to their siblings with CF (Duytschaever *et al.* 2011). However, the PCR-DGGE profiles did not reveal particular differences in terms of the number of bands, and the intensity of *Ba. uniformis*-related amplicons did not discriminate healthy subjects from patients with CF.

Depletion of butyrate-producing bacteria such as *F. prausnitzii*, *E. rectale* and *Roseburia* spp. may have negative effects on the supply of butyrate to gut epithelial cells and also on the luminal pH owing to their ability to utilize lactate. Most butyrate producers within the gut use the butyryl-CoA:acetate CoA-transferase route which depends on acetate (Barcenilla *et al.* 2000). Acetate is in turn produced by acetogens such as some species belonging to the genera *Ruminococcus* and *Blautia* (Bernalier *et al.* 1996). Other important acetate producers are members of *Bifidobacterium* and *Bacteroides*

Table 2. Sequence analysis of the bands indicated in Fig 1a (numerical ID) and 2a (capital letters ID).

Band ID	% Blast similarity	Nearest species	Accession number	Presence in profiles of healthy controls (%)	Presence in profiles of CF patients (%)
1	100	*Bifidobacterium adolescentis*	KP256215.1	62.5	23.3
2	100	*Faecalibacterium prausnitzii*	AJ270469	100	60
3	99	*Collinsella aerofaciens*	NR113316.1	100	30
4	100	*Bifidobacterium catenulatum*	KP256217.1	50	26.7
	100	*Bifidobacterium pseudocatenulatum*	AP012330.1		
5	100	*Bifidobacterium adolescentis*	KP256215.1	87.5	30
6	100	*Collinsella aerofaciens*	NR113316.1	75	33.3
7	99	*Eubacterium rectale*	AY804151	87.5	53.3
8	100	*Collinsella aerofaciens*	NR113316.1	50	33.3
9	100	*Bifidobacterium coccoides*	NR104700.1	100	63.3
	100	*Blautia luti*	NR041960.1		
10	99	*Escherichia coli*	LN867523.1	50	90
	9	*Shigella flexneri*	NR026331.1		
	99	*Escherichia fergusonii*	NE074902.1		
11	99	*Dorea invisus*	NR113355.1	75	50
A	100	*Eubacterium rectale*	NR 074634.1	100	66.7
B	95	Uncultured *Roseburia*	JX 230356.1	87.5	16.7
C	99	*Blautia glucerasea*	NR 113231.1	100	73.3
D	97	*Ruminococcus gnavus*	AB 910745.1	100	62.5
E	98	*Blautia luti*	NR 041960.1	88	33.0
F	99	*Dorea longicatena*	NR 028883.1	100	44.3

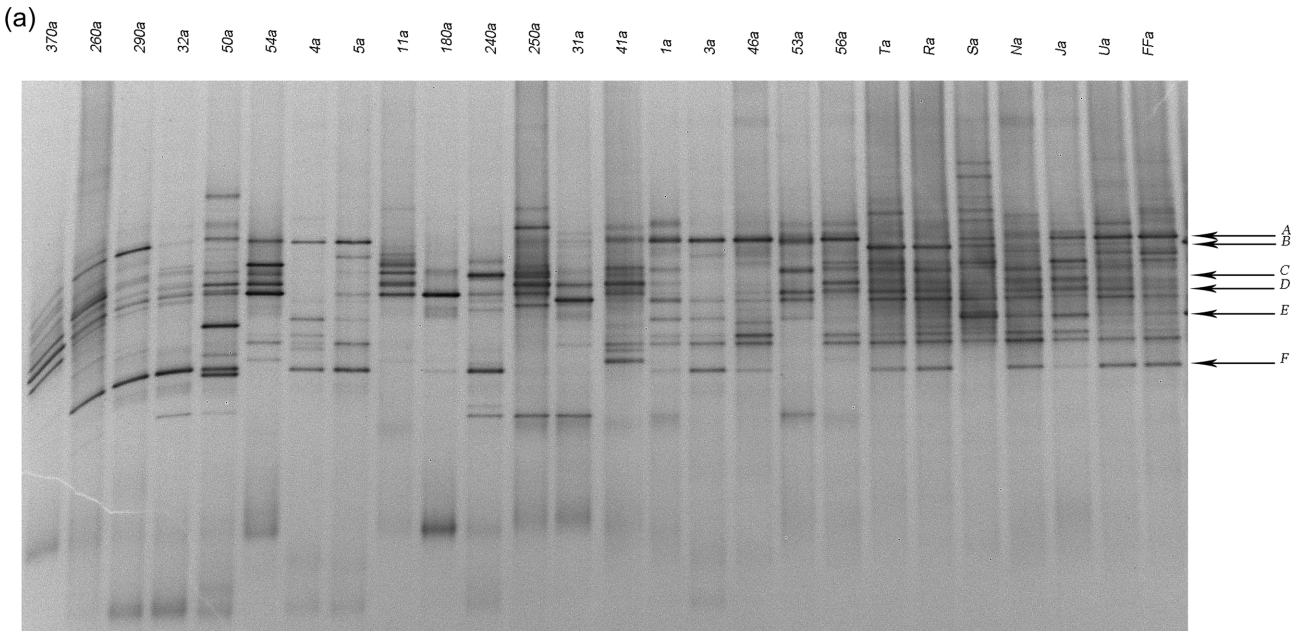

Figure 2. (a) DGGE profiles obtained using *Cl. coccoides* group-specific primers. CF samples are indicated by a number, while healthy subjects are indicated by capital letters. **(b)** Dendrogram constructed from analysis of DGGE gels using Pearson's correlation coefficient and the unweighted-pair group method. Red squares indicate homozygotes, green squares heterozygotes and light blue squares healthy subjects. Next to each patient's identification code, their age and CLP value are reported.

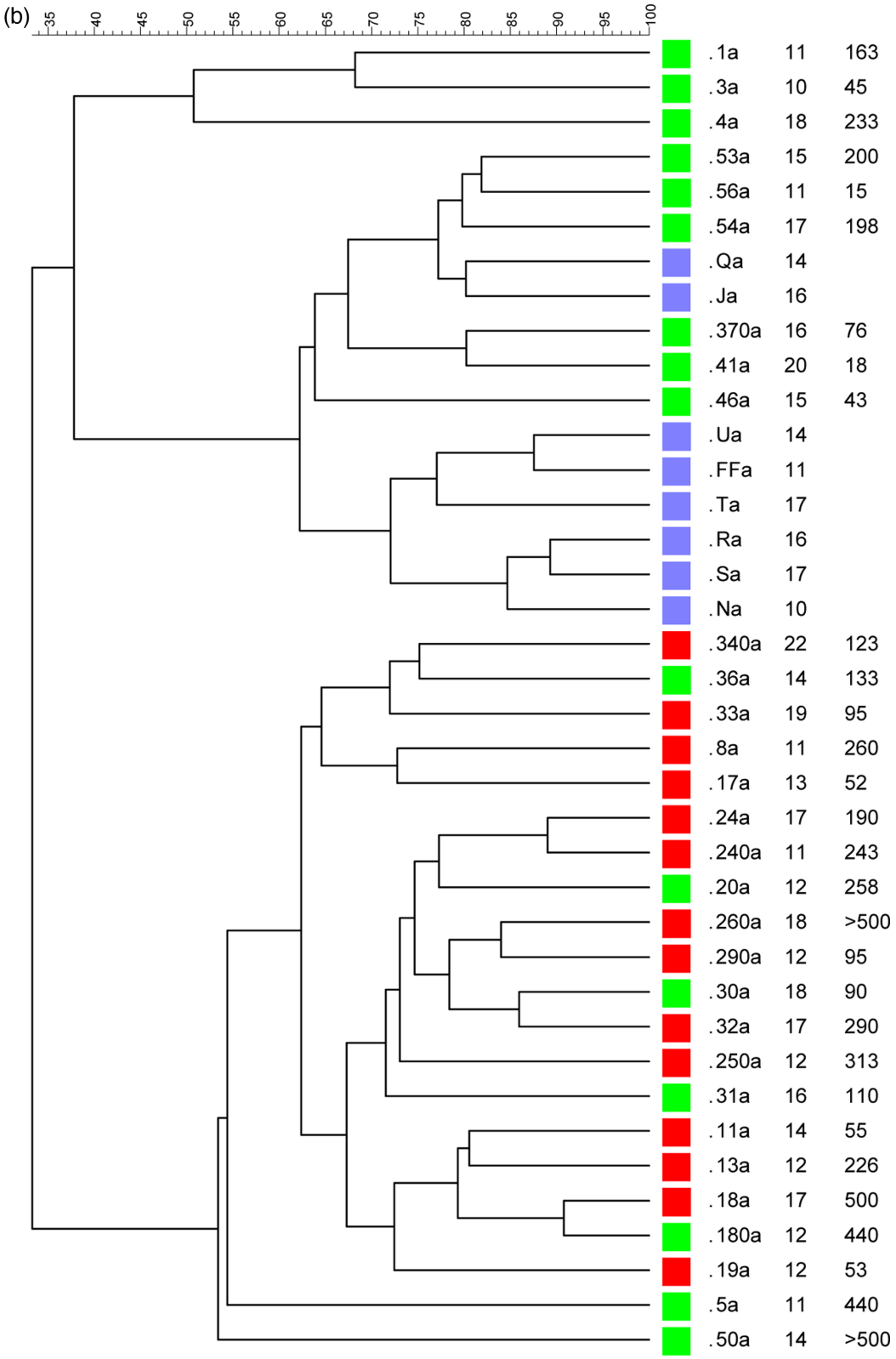

Figure 2. (*Continued*).

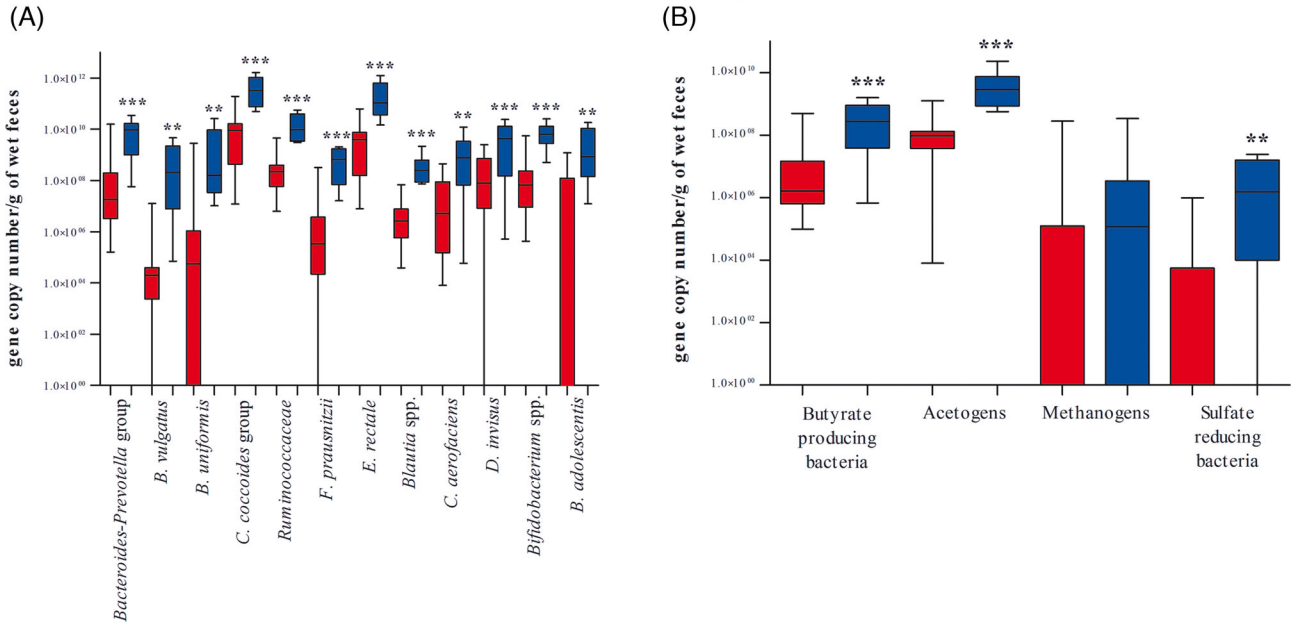

Figure 3. (A) Box plot of qPCR data (expressed as gene copy number per gram of wet feces) from patients with CF (red) and healthy controls (blue). The boxes show the first and third quartiles, with a horizontal line inside to represent the median. The whiskers represent the maximum and minimum data values, without outliers. $^{**}P < 0.050$ and $^{***}P < 0.001$. **(A)** qPCR data of species/groups of bacteria significantly different between CF and control individuals. **(B)** qPCR results for *BcoAT*, *acs*, *mcr* and *dsr* functional genes.

genera (Rajilić-Stojanović and de Vos 2014). Since the concentration of SCFAs in the intestinal lumen depends on the balance of production, microbial utilization and mucosal uptake, reduction of key bacteria groups has important effects on gut ecology and host mucosa.

qPCR

To confirm the results of qualitative analysis, we performed qPCR targeting the different bacterial genera or species that emerged as discriminant in PCR-DGGE analysis (Fig. 3A). Compared with healthy controls, patients with CF showed significantly reduced contents of *D. invisus* ($P = 0.0001$), *B. adolescentis* ($P = 0.0011$), *Bifidobacterium* spp. ($P < 0.0001$) and *C. aerofaciens* ($P = 0.0025$). Our results concerning *Bifidobacterium* spp. are consistent with previous findings (Scanlan *et al.* 2012; Duytschaever *et al.* 2013; Schippa *et al.* 2013). A search of the literature revealed no information concerning *B. adolescentis* in patients with CF, but *B. adolescentis*, *C. aerofaciens* and *D. invisus* are constituents of the human gut microbiota (Lyra *et al.* 2009; Qin *et al.* 2010; Agans *et al.* 2011; Hakansson and Molin 2011) and their abundance is reportedly reduced in the microbiota of patients with Crohn's disease (Suchodolski *et al.* 2012).

Enterobacteriaceae (Debyser *et al.* 2016), particularly *Es. coli* (Hoffman *et al.* 2014), were more abundant in CF fecal samples, although according to our data this difference did not reach statistical significance ($P = 0.6278$). We performed further quantitative analyses targeting bacteria belonging to the *Cl. coccoides* group, specifically *F. prausnitzii*, *E. rectale*, *Blautia* spp. and the *Ruminococcaceae* family. All of these groups were significantly reduced among adolescents with CF ($P \leq 0.0002$). Persistent use of broad-spectrum antibiotics, frequent in patients with CF, can reduce bacterial diversity promoting or inhibiting specific indigenous taxa (Modi, Collins and Relman 2014). For example, a marked decrease of butyrate-producing bacteria, especially *F. prausnitzii*, and of *Bifidobacterium* spp. was reported after 4 days of amoxicillin-clavulanic acid administration (Young and Schmidt 2004). Reduced abundance of *Cl. coccoides* group in CF gut microbiota could be due to an altered intestinal environment since these populations are extremely sensitive to pH variations (Flint *et al.* 2007). Moreover, butyrate-producing bacteria, and in particular *Roseburia intestinalis* and *E. rectale*, were able to colonize mucins in an *in vitro* study (Van den Abbeele *et al.* 2013). So it is possible that the very viscous mucus present in the gastrointestinal tract of patients with CF could entrap these strong colonizers removing them from the intestinal lumen. On the other hand, *R. gnavus* abundance did not statistically differ ($P = 0.4225$) between patients with CF and the control group. These results differ from the findings of a recent study that applied a metaproteomic approach to analyze CF fecal samples, and reported reduced proteins taxonomically attributed to butyrate-producing bacteria and increased proteins associated with *R. gnavus* and *Clostridia* species (Debyser *et al.* 2016). Further investigations are required to explain these discrepancies.

Eubacterium rectale and *F. prausnitzii* are butyrate-producing bacteria that are considered important markers of inflammatory status (Sokol *et al.* 2008; Gupta *et al.* 2014). Reductions of this bacterial group could substantially impact enterocyte nutrition and the maintenance of intestinal health (Flint *et al.* 2007). With regard to the *Bacteroides-Prevotella* group, *Ba. vulgatus* and *Ba. uniformis* were found to be significantly reduced in patients with CF compared to healthy subjects ($P = 0.0002$, $P = 0.0015$ and $P = 0.0025$, respectively). These modifications of the *Bacteroides-Prevotella* group contents were unrelated to age, since we previously found the same results among children with CF (Bruzzese *et al.* 2014). A previous study used culture-based counts to compare the gut microbiota of children with CF to that of their healthy siblings, and found a borderline significant difference in *Bacteroides-Prevotella* presence ($P = 0.07$) (Duytschaever *et al.* 2011). This discrepancy could be due to the difference in methodological approaches, and further investigation into this subject is needed.

We additionally quantified butyrate-producing bacteria, acetogens, methanogenic archaea and SRB by qPCR using primers targeting the functional genes *BcoAT*, *acs*, *mcr* and *dsr* (Fig. 3B). The *BcoAT* gene has been demonstrated to be prevalent in the human gut (Louis *et al.* 2004). The *acs* gene encodes for an enzyme exclusive of the acetyl-CoA pathway (Ragsdale 1991), *mcr* is highly conserved among methanogens (Luton *et al.* 2002) and *dsr* is common to all SRB (Christophersen, Morrison and Conlon 2011). Thus, amplification of these genes represents a suitable approach for specific detection of these metabolic groups.

Compared to healthy subjects, patients with CF exhibited significantly decreased butyrate-producing bacteria and acetogens ($P = 0.0002$ and $P = 0.0003$, respectively). The observed reduction of butyrate-producing bacteria is consistent with previous findings (Duytschaever *et al.* 2013; Schippa *et al.* 2013), while no prior study has investigated acetogens presence in CF disease. SRB were detected in eight CF subjects (26.7%) with equal distribution among homozygotes and heterozygotes, compared to in 87.5% of healthy subjects. In subjects with detectable SRB, the amounts ranged from 10^4–10^7 gene copy number per gram of wet feces in healthy subjects and 10^4–10^5 gene copy number per gram of wet feces in patients with CF. Overall, SRB prevalence and abundance were significantly reduced in patients with CF ($P = 0.0011$), which is in contrast to other bowel diseases (Gibson, Cummings and Macfarlane 1991; Loubinoux *et al.* 2002). Prior studies have described H_2S involvement in retaining both proinflammatory and anti-inflammatory properties depending on the specific work (Singh and Lin 2015), and thus its precise role in intestinal inflammation remains unclear.

Archaea were detected in 62.5% of healthy subjects, compared to in 33.3% of patients with CF, all of which were heterozygotes. Within the subgroup of heterozygotes with CF, the prevalence of archaea was 62.5%, the same proportion as in healthy individuals. Archaea were present in both delF508 and non-delF508 heterozygote patients with CF. Methanogens were less abundant in CF fecal samples than in healthy controls, although this difference did not reach statistical significance ($P = 0.2572$). In contrast, a statistically significant difference was found between homozygotes and healthy subjects ($P < 0.05$), and between homozygotes and heterozygotes ($P < 0.01$). *Methanobrevibacter* spp. were the predominant genera in both healthy subjects and patients with CF, with amounts comparable with the total archaea content. As expected, *Methanobrevibacter smithii* was the most abundant methanogen, with quantification data comparable with the amounts of *Methanobrevibacter* spp., ranging from 10^4 to 10^9 gene copy number per gram of wet feces in both healthy and CF subjects. *Methanosphaera stadtmanae* was detected in 40% of healthy subjects and 20% of patients with CF, and the abundance of this species ranged from 10^4 to 10^5 gene copy number per gram of wet feces. Our results regarding *M. stadtmanae* prevalence and abundance for both groups are in accordance with those previously described in healthy subjects (Mihajlovski *et al.* 2010; Dridi *et al.* 2012).

It can be speculated that the reduced colonization of SRB and archaea in CF subjects (mainly in homozygotes) may be due to disease-related changes in the intestinal environment that interfere with these microorganisms' ability to thrive. The reduction of SRB in CF subjects could be due to an altered lumen environment in which the availability of SCFAs and proteins is somewhat reduced. This hypothesis is based on the ability of SRB to use not only molecular hydrogen but also a wide range of substrates such as SCFAs or proteins as electron donors. In the human colon, the predominant SRB are those able to oxidize organic acids, mainly lactate and proteins (Newton *et al.* 1998) whereas the H_2/CO_2 utilizers are less represented (Gibson, Macfarlane and Cummings 1988). In addition, one possible explanation for the reductions of both methanogens and SRB could be an alteration of the pH gradient from the right to left colon that enables distribution of these bacterial groups in accordance with their physiological requirements in healthy subjects (Nakamura *et al.* 2010). Unfortunately, no detailed data are available regarding colon pH in patients with CF to support our hypothesis. Further investigations are required to elucidate the causes and effects of the unbalanced hydrogenotrophic populations in CF microbiota.

Figure 4a shows the results of PCA of qPCR data, and Fig. 4b shows the eigenvalues between all considered variables. The first three PC explained 66% of the total variation: 42% for PC1, 13% for PC2 and 11% for PC3. PC1 could efficiently separate the samples according to CF disease (Fig. 4a). Ordination analysis showed no discrimination between heterozygotes and homozygotes, and indicated higher interindividual biodiversity among healthy subjects compared with the microbiota of patients with CF. The abundance fluctuations of *Ba. uniformis*, *Ruminococcaceae*, *F. prausnitzii*, *Blautia* spp., acetogens and SRB were the predominant variables producing a positive impact on PC1 (Fig. 4b). The CF disease seemed to be the most important factor affecting this grouping since ANOVA revealed no correlation between bacterial population levels and lung colonization, FEV1 data, CLP concentration or genetic background ($P = 0.7$). Furthermore, stepwise regression analysis did not identify any group/bacterial species as being associated with CLP concentration, showing no link between intestinal inflammation and key bacterial groups in patients with CF.

Spearman's correlation analysis identified fewer correlations in healthy subjects (see Fig. S1A, Supporting Information) compared to among CF samples (see Fig. S1B, Supporting Information), possibly due to there being fewer subjects in the control group. In patients with CF, *Ruminococcaceae* and *Blautia* spp. were positively correlated with *E. rectale* and *F. prausnitzii*. Increased numbers of these butyrate-producing bacteria were correlated to higher abundance of *Ba. uniformis* and/or *Ba. vulgatus*. One possible metabolic interaction could be acetate production by members of the *Bacteroides-Prevotella* group and *Blautia* genera, which is then metabolized by butyrate-producing bacteria (Chassard and Bernalier-Donadille 2006). *Bifidobacterium adolescentis* was positively correlated with *F. prausnitzii*, confirming previous reports that this species was correlated to butyrate-producing bacteria (Belenguer *et al.* 2006). This analysis also revealed a positive correlation of *D. invisus* with members of the *Cl. coccoides* group, *Ba. vulgatus*, *Ba. uniformis* and *Ba. catenulatum*. Archaea were negatively correlated with the *Bacteroides-Prevotella* group and positively correlated with *Blautia* spp. Previous findings have shown a negative correlation between *Bacteroides* and *Methanobrevibacter* (Hoffman *et al.* 2014), as well as positive correlations between *Methanobacteriales* and *Bifidobacterium* spp., members of *Lachnospiraceae*, *Dialister* spp. and *Blautia* spp. (Vanderhaeghen, Lacroix and Schwab 2015). The presently observed low prevalence of SRB in CF patient samples prevent detailed analysis of their possible correlations with other groups of bacteria.

It is well recognized that a balance between different functional groups is crucial for maintaining efficient degradation of organic matter in the gastrointestinal tract. Disruption of this balance can affect bacterial community stability, leading to impaired host health. In our present study, patients with CF exhibited reduced abundance of several dominant bacterial groups, including *C. coccoides*, *Bacteroides-Prevotella* group and the

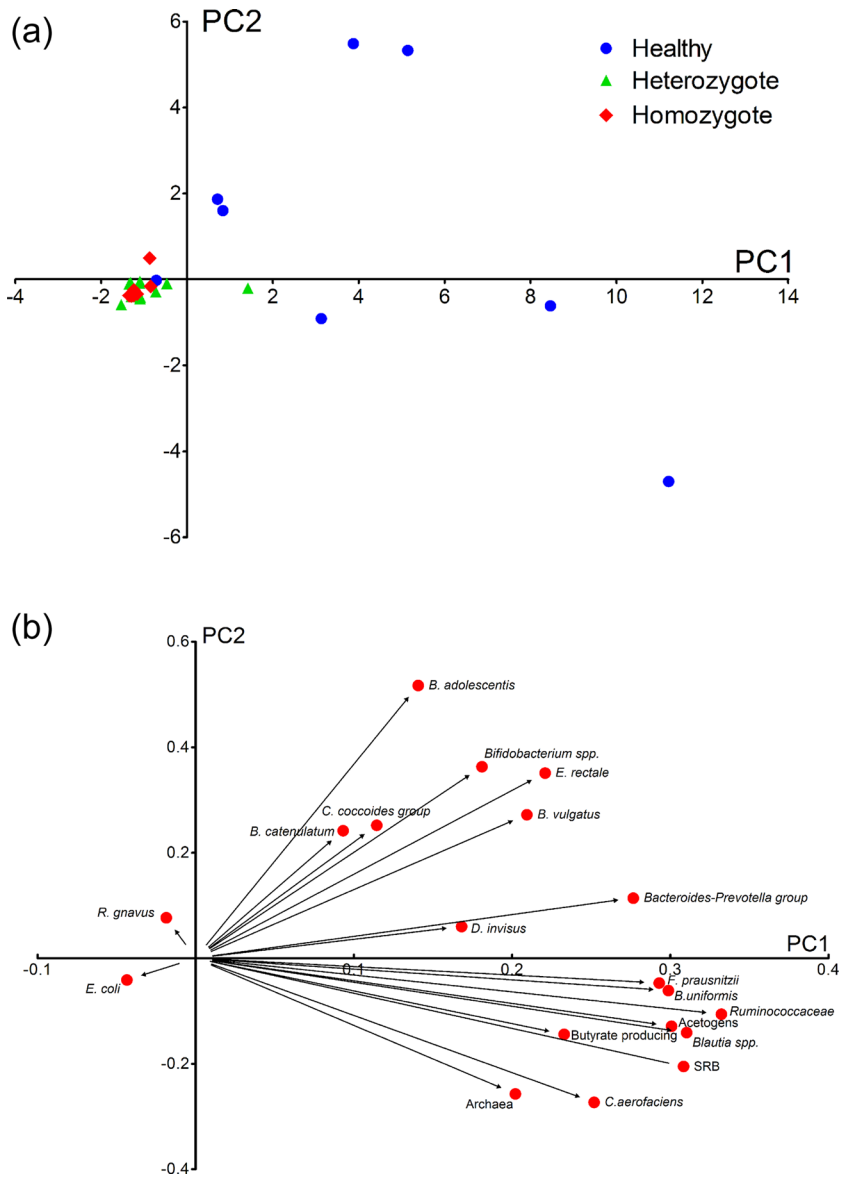

Figure 4. (a) Principal component analysis (PCA) plots of the qPCR dataset, indicating healthy subjects (blue dots), heterozygote patients with CF (green triangles) and homozygote patients with CF (red rhombus). **(b)** Graphical representation of the eigenvectors between qPCR data and PC1 and PC2, explaining 55% of the total variation (42% and 13% for PC1 and PC2, respectively).

Bifidobacterium genera. Patients with CF also showed strong modifications in the abundance of several functional groups, particularly acetogens, methanogens and SRB. We did not identify associations among bacterial groups, CLP values, lung colonization and genetic backgrounds. It is possible that the intestinal environment in CF disease leads to very strong selection of the more resilient enteric bacteria, as well as archaea and SRB, which showed low prevalence in homozygote patients with CF.

Considering the many CF-associated factors that could influence the gut microbiota composition and function, a major limitation of this study is represented by the limited number of recruited patients. A further limitation consists in the use of fecal samples in place of mucosal specimens. The gut bacteria community composition changes along the gastrointestinal tract and microbial communities living in the gut lumen actually differs from those adhering to the intestinal mucosa. Since mucosa-associated bacteria are in close contact with the epithelial tissues they could interact with the host more strongly than luminal microorganisms. Moreover, mucosal samples as those obtained by biopsies could be more representative of the inflammatory processes eventually occurring in the gut. To address some of these limitations, a larger number of patients with CF should be enrolled to increase the power of future studies. Clinical and nutritional parameters evaluation, together with the use of metagenomic approaches, could improve our understanding of the ecological relationships between gut microorganisms and their impact on CF disease.

CONCLUSION

The present work even if with some limitations represents the pioneer study investigating the balance of hydrogenotrophic bacteria in CF fecal microbiota. Our results confirm that the gut microbiota composition of patients with CF is significantly

altered compared with that of healthy controls, although no correlation was found with either gut inflammation or host genotype. The reduction of species belonging to *Clostridium coccoides* group, *Bacteroides-Prevotella* group and *Bifidobacterium* genera could influence the metabolic potential of the microbiota in CF. Decrease of key butyrate-producers such as *Faecalibacterium prausnitzii* and *Eubacteriumrectale* or important functional bacteria groups as *Blautia* spp., *E. rectale Ruminococcaceae* and *Bacteroides* can have important effects on gut ecology and host cell metabolism. Hydrogenotrophic microorganisms, which resulted to be significantly less abundant in CF compared with controls, can have important ecological effects on the gut microbiota influencing the hydrogen economy of the colon. Our results warrant further investigation to highlight the link between these microbiota differences and disease states.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

REFERENCES

Agans R, Rigsbee L, Kenche H *et al*. Distal gut microbiota of adolescent children is different from that of adults. *FEMS Microbiol Ecol* 2011;**77**:404–12.

Barcenilla A, Pryde SE, Martin JC *et al*. Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microb* 2000;**66**:1654–61.

Belenguer A, Duncan SH, Calder AG *et al*. Two routes of metabolic cross-feeding between. *Society* 2006;**72**:3593–9.

Bernalier A, Rochet V, Leclerc M *et al*. Diversity of H2/CO2-utilizing acetogenic bacteria from feces of non-methane-producing humans. *Curr Microbiol* 1996;**33**:94–9.

Bruzzese E, Callegari ML, Raia V *et al*. Disrupted intestinal microbiota and intestinal inflammation in children with cystic fibrosis and its restoration with *Lactobacillus* GG: A randomised clinical trial. *PLoS One* 2014;**9**:e87796.

Carbonero F, Benefiel AC, Gaskins HR. Contributions of the microbial hydrogen economy to colonic homeostasis. *Nat Rev Gastroentero* 2012;**9**:504–18.

Castellani C, Cuppens H, Macek M *et al*. Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. *J Cyst Fibros* 2008;**7**:179–96.

Chassard C, Bernalier-Donadille A. H2 and acetate transfers during xylan fermentation between a butyrate-producing xylanolytic species and hydrogenotrophic microorganisms from the human gut. *FEMS Microbiol Lett* 2006;**254**:116–22.

Christophersen CT, Morrison M, Conlon MA. Overestimation of the abundance of sulfate-reducing bacteria in human feces by quantitative PCR targeting the Desulfovibrio 16S rRNA gene. *Appl Environ Microb* 2011;**77**:3544–6.

Corey M, Edwards L, Levison H *et al*. Longitudinal analysis of pulmonary function decline in patients with cystic fibrosis. *J Pediatr* 1997;**131**:809–14.

De Lisle RC, Borowitz D. The cystic fibrosis intestine. *Cold Spring Harb Perspect Med* 2013;**3**:a009753.

Debyser G, Mesuere B, Clement L *et al*. Faecal proteomics: a tool to investigate dysbiosis and inflammation in patients with cystic fibrosis. *J Cyst Fibros* 2016;**15**:242–50.

Deng B, Shen CH, Shan XH *et al*. PCR-DGGE analysis on microbial communities in pit mud of cellars used for different periods of time. *J Inst Brew* 2012;**118**:120–6.

Denman SE, Tomkins NW, McSweeney CS. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol* 2007;**62**:313–22.

Dhaliwal J, Leach S, Katz T *et al*. Intestinal inflammation and impact on growth in children with cystic fibrosis. *J Pediatr Gastr Nutr* 2015;**60**:521–6.

Dridi B, Henry M, El Khéchine A *et al*. High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *PLoS One* 2009;**4**:e7063.

Dridi B, Henry M, Richet H *et al*. Age-related prevalence of *Methanomassiliicoccus luminyensis* in the human gut microbiome. *APMIS* 2012;**120**:773–7.

Duytschaever G, Huys G, Bekaert M *et al*. Cross-sectional and longitudinal comparisons of the predominant fecal microbiota compositions of a group of pediatric patients with cystic fibrosis and their healthy siblings. *Appl Environ Microb* 2011;**77**:8015–24.

Duytschaever G, Huys G, Bekaert M *et al*. Dysbiosis of bifidobacteria and Clostridium cluster XIVa in the cystic fibrosis fecal microbiota. *J Cyst Fibros* 2013;**12**:206–15.

Fagerberg UL, Lööf L, Merzoug RD *et al*. Fecal calprotectin levels in healthy children studied with an improved assay. *J Pediatr Gastr Nutr* 2003;**37**:468–72.

Federici S, Miragoli F, Pisacane V *et al*. Archaeal microbiota population in piglet feces shifts in response to weaning: *Methanobrevibacter smithii* is replaced with *Methanobrevibacter boviskoreani*. *FEMS Microbiol Lett* 2015;**362**:1–7.

Flint HJ, Duncan SH, Scott KP *et al*. Interactions and competition within the microbial community of the human colon: links between diet and health: minireview. *Environ Microbiol* 2007;**9**:1101–11.

Freudenberg F, Broderick AL, Yu BB *et al*. Pathophysiological basis of liver disease in cystic fibrosis employing a DeltaF508 mouse model. *Am J Physiol Gastr L* 2008;**294**:G1411–20.

Gagen EJ, Denman SE, Padmanabha J *et al*. Functional gene analysis suggests different acetogen populations in the bovine rumen and tammar wallaby forestomach. *Appl Environ Microbiol* 2010;**76**:7785–95.

Garcia MAS, Yang N, Quinton PM. Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion. *J Clin Invest* 2009;**119**:2613–22.

Garcia-Mazcorro JF, Suchodolski JS, Jones KR *et al*. Effect of the proton pump inhibitor omeprazole on the gastrointestinal bacterial microbiota of healthy dogs. *FEMS Microbiol Ecol* 2012;**80**:624–36.

Geets J, Borremans B, Diels L *et al*. DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. *J Microbiol Methods* 2006;**66**:194–205.

Gibson GR, Cummings JH, Macfarlane GT. Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis. *FEMS Microbiol Lett* 1991;**86**:103–11.

Gibson GR, Macfarlane GT, Cummings JH. Occurrence of sulphate-reducing bacteria in human faeces and the

relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. *J Appl Bacteriol* 1988;**65**:103–11.

Gupta A, Kang S, Wagner J *et al.* Analysis of mucosal microbiota in inflammatory bowel disease using a custom phylogenetic microarray. *Austin J Gastroenterol* 2014;**1**:1020.

Hakansson A, Molin G. Gut microbiota and inflammation. *Nutrients* 2011;**3**:637–87.

Hoffman LR, Pope CE, Hayden HS *et al.* *Escherichia coli* dysbiosis correlates with gastrointestinal dysfunction in children with cystic fibrosis. *Clin Infect Dis* 2014;**58**:396–9.

Houwen RH, van der Doef HP, Sermet I *et al.* Defining DIOS and constipation in cystic fibrosis with a multicentre study on the incidence, characteristics, and treatment of DIOS. *J Pediatr Gastr Nutr* 2010;**50**:38–42.

Hradsky O, Ohem J, Mitrova K *et al.* Fecal calprotectin levels in children is more tightly associated with histological than with macroscopic endoscopy findings. *Clin Lab* 2014;**60**:1993–2000.

Huys G, Vanhoutte T, Vandamme P. Application of sequence-dependent electrophoresis fingerprinting in exploring biodiversity and population dynamics of human intestinal microbiota: what can be revealed? *Interdiscip Perspect Infect Dis* 2008;**2008**:597603.

Jakobsson HE, Jernberg C, Andersson AF *et al.* Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* 2010;**5**:e9836.

Jernberg C, Lofmark S, Edlund C *et al.* Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J* 2007;**1**:56–66.

Jernberg C, Å Sullivan, Edlund C *et al.* Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. *Appl Environ Microb* 2005;**71**:501–6.

Joossens M, Huys G, Cnockaert M *et al.* Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* 2011;**60**:631–7.

Kassinen A, Krogius-Kurikka L, Mäkivuokko H *et al.* The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007;**133**:24–33.

Kim SC, Tonkonogy SL, Albright CA *et al.* Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology* 2005;**128**:891–906.

Kondo R, Nedwell DB, Purdy KJ *et al.* Detection and enumeration of sulphate-reducing bacteria in estuarine sediments by competitive PCR. *Geomicrobiol J* 2004;**21**:145–57.

Kumar PS, Griffen AL, Barton JA *et al.* New bacterial species associated with chronic periodontitis. *J Dent Res* 2003;**82**:338–44.

Li L, Somerset S. The clinical significance of the gut microbiota in cystic fibrosis and the potential for dietary therapies. *Clin Nutr* 2014;**33**:571–80.

Loubinoux J, Bronowicki JP, Pereira IAC *et al.* Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases. *FEMS Microbiol Ecol* 2002;**40**:107–12.

Louis P, Duncan SH, McCrae SI *et al.* Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. *J Bacteriol* 2004;**186**:2099–106.

Louis P, Flint HJ. Development of a semiquantitative degenerate real-time PCR-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. *Appl Environ Microb* 2007;**73**:2009–12.

Luton PE, Wayne JM, Sharp RJ *et al.* The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* 2002;**148**:3521–30.

Lyra A, Rinttilä T, Nikkilä J *et al.* Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. *World J Gastroenterol* 2009;**15**:5936–45.

Maidak BL, Larsen N, McCaughey MJ *et al.* The ribosomal database project. *Nucleic Acids Res* 1994;**22**:3485–7.

Maukonen J, Satokari R, Mättö J *et al.* Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria. *J Med Microbiol* 2006;**55**:625–33.

Mihajlovski A, Doré J, Levenez F *et al.* Molecular evaluation of the human gut methanogenic archaeal microbiota reveals an age-associated increase of the diversity. *Environ Microbiol Rep* 2010;**2**:272–80.

Modi SR, Collins JJ, Relman DA. Antibiotics and the gut microbiota. *J Clin Invest* 2014;**124**:4212–8.

Morgan XC, Tickle TL, Sokol H *et al.* Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* 2012;**13**:R79.

Mosca A, Leclerc M, Hugot JP. Gut microbiota diversity and human diseases: should we reintroduce key predators in our ecosystem? *Front Microbiol* 2016;**7**:455.

Nakamura N, Lin HC, McSweeney CS *et al.* Mechanisms of microbial hydrogen disposal in the human colon and implications for health and disease. *Annu Rev Food Sci Technol* 2010;**1**:363–95.

Newton DF, Cummings JH, Macfarlane S *et al.* Growth of a human intestinal *Desulfovibrio desulfuricans* in continuous cultures containing defined populations of saccharolytic and amino acid fermenting bacteria. *J Appl Microbiol* 1998;**85**:372–80.

Pitcher MCL. The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. *Gut* 2000;**46**:64–72.

Qin J, Li R, Raes J *et al.* A human gut microbial gene catalog established by metagenomic sequencing. *Nature* 2010;**464**:59–65.

Ragsdale SW. Enzymology of the acetyl-CoA pathway of CO2 fixation. *Crit Rev Biochem Mol* 1991;**26**:261–300.

Rajilić-Stojanović M, de Vos WM. The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiol Rev* 2014;**38**:996–1047.

Rinttilä T, Kassinen A, Malinen E *et al.* Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004;**97**:1166–77.

Scanlan PD, Buckling A, Kong W *et al.* Gut dysbiosis in cystic fibrosis. *J Cyst Fibros* 2012;**11**:454–5.

Schippa S, Iebba V, Santangelo F *et al.* Cystic fibrosis transmembrane conductance regulator (CFTR) allelic variants relate to shifts in faecal microbiota of cystic fibrosis patients. *PLoS One* 2013;**8**:e61176.

Singh S, Lin H. Hydrogen sulfide in physiology and diseases of the digestive tract. *Microorganisms* 2015;**3**:866–89.

Skillman LC, Evans PN, Naylor GE *et al.* 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. *Anaerobe* 2004;**10**:277–85.

Sokol H, Pigneur B, Watterlot L *et al.* *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by

gut microbiota analysis of Crohn disease patients. *P Natl Acad Sci USA* 2008;**105**:16731–6.

Stephenson AL, Mannik LA, Walsh S *et al.* Longitudinal trends in nutritional status and the relation between lung function and BMl in cystic fibrosis: a population-based cohort. *Am J Clin Nutr* 2013;**97**:872–7.

Suchodolski JS, Markel ME, Garcia-Mazcorro JF *et al.* The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One* 2012;**7**:e51907.

Takaishi H, Matsuki T, Nakazawa A *et al.* Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. *Int J Med Microbiol* 2008;**298**:463–72.

Van den Abbeele P, Belzer C, Goossens M *et al.* Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an in vitro gut model. *ISME J* 2013;**7**: 949–61.

Vanderhaeghen S, Lacroix C, Schwab C. Methanogen communities in stools of humans of different age and health status and co-occurrence with bacteria. *FEMS Microbiol Lett* 2015;**362**, DOI: 10.1093/femsec/fnv092.

Young VB, Schmidt TM. Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the fecal microbiota. *J Clin Microbiol* 2004;**42**:1203–6.

Yu Y, Lee C, Kim J *et al.* Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng* 2005;**89**:670–9.

Yu Z, Morrison M. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl Environ Microb* 2004;**70**:4800–6.

Zielenski J. Genotype and phenotype in cystic fibrosis. *Respiration* 2000;**67**:117–33.