RESEARCH ARTICLE

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

Francesco Miragoli¹,†, Sara Federici¹,‡, Susanna Ferrari¹, Andrea Minuti², Annalisa Rebecchi¹, Eugenia Bruzzese³, Vittoria Buccigrossi³, Alfredo Guarino³ and Maria Luisa Callegari¹,*

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

*Corresponding author: Istituto di Microbiologia, Università 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 H₂-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...
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 H₂S, 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 Huot 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 CO₂ and H₂. Since high hydrogen levels can inhibit normal fermentative reactions (Carbonero, Benešel and Gaskins 2012), mechanisms involved in H₂ disposal are required for proper function of the intestinal ecosystem. A low H₂ partial pressure is maintained in the colon by both H₂ 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 cocoides group        | 16S rRNA             | Rinttilä et al. (2004)           |
| Ruminococcus gnavus               | 16S rRNA             | Joossens et al. (2011)           |
| Methanobrevibacter smithii        | 16S rRNA             | Dridi et al. (2009)              |
| Methanospirillum 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)sulphite 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 cocoides 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, B. 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)sulphite 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 patient (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, FEV1 data 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 clustering 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.](image-url)
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 cocoides 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 gnaeus, 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 *B. 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       | 99                 | *Shigella flexneri*              | NR026331.1       |                                             |                                        |
| 99      | 99                 | *Escherichia fergusonii*         | NEO074902.1      |                                             |                                        |
| 11      | 99                 | *Dorea invisus*                  | NR113355.1       | 75                                          | 50                                     |
| A       | 100                | *Eubacterium rectale*            | NR074634.1       | 100                                         | 66.7                                   |
| B       | 95                 | *Uncultured Roseburia*           | JX20356.1        | 87.5                                        | 16.7                                   |
| C       | 99                 | *Blautia glucerasea*             | NR113231.1       | 100                                         | 73.3                                   |
| D       | 97                 | *Ruminococcus gnavus*            | AB310745.1       | 100                                         | 62.5                                   |
| E       | 98                 | *Blautia luti*                   | NR041960.1       | 88                                          | 33.0                                   |
| F       | 99                 | *Dorea longicatena*              | NR028883.1       | 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)
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. inuis (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. inuis 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 qualitative 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 < 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 Abbeeke 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 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 (Bruzese 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 \(\text{Box}A\), \(\text{acs}\), \(\text{mcr}\) and \(\text{dsr}\) (Fig. 3B).

The \(\text{BoxAT}\) gene has been demonstrated to be prevalent in the human gut (Louis et al. 2004). The \(\text{acs}\) gene encodes for an enzyme exclusive of the acetyl-CoA pathway (Ragsdale 1991), \(\text{mcr}\) is highly conserved among methanogens (Luton et al. 2002) and \(\text{dsr}\) 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 \(10^{11}\) to \(10^{7}\) gene copy number per gram of wet feces in healthy subjects and \(10^{4}\) to \(10^{5}\) 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 \(\text{H}_{2}\)-\(\text{S}\) 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 heteroygotes. 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 \(10^{5}\) 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 \(\text{H}_{2}/\text{CO}_{2}\) 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 microflora.

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 \(\text{Ba. uniformis}\), \(\text{Ruminococcaceae}\), \(\text{F. prausnitzii}\), \(\text{Blautia spp.}\), 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, \(\text{Ruminococcaceae}\) and \(\text{Blautia spp.}\) were positively correlated with \(E.\) rectale and \(F.\) prausnitzii. Increased numbers of these butyrate-producing bacteria were correlated to higher abundance of \(\text{Ba. uniformis}\) and/or \(\text{Ba. vulgatus}\). One possible metabolic interaction could be acetate production by members of the \(\text{Bacteroides-Prevotella}\) group and \(\text{Blautia}\) genera, which is then metabolized by butyrate-producing bacteria (Chassard and Bernalier-Donadille 2006). \(\text{Bifidobacterium ado-

lescentis}\) 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 \(D.\) inuis with members of the \(\text{Cl. coccoides}\) group, \(\text{Ba. vulgatus}\), \(\text{Ba. uniformis}\) and \(\text{Ba. catenula-

tum}\). Archaea were negatively correlated with the \(\text{Bacteroides-Prevotella}\) group and positively correlated with \(\text{Blautia}\) spp. Previous findings have shown a negative correlation between \(\text{Bacteroides}\) and \(\text{Methanobrevibacter}\) (Hoffman et al. 2014), as well as positive correlations between \(\text{Methanobacteriales}\) and \(\text{Bifidobacteri-

um}\) spp., members of \(\text{Lachnospiraceae}\), \(\text{Dialister}\) spp. and \(\text{Blautia}\) spp. (Vanderhaegen, 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 \(\text{C. coccoides}\), \(\text{Bacteroides-Prevotella}\) group and the
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 cocoides 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 Eubacterium rectale or important functional bacterial 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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