TITLE: DYSBIOSIS OF BIFIDOBACTERIA AND *CLOSTRIDIUM* CLUSTER XIVA IN THE CYSTIC FIBROSIS FAECAL MICROBIOTA

RUNNING TITLE: Intestinal dysbiosis in cystic fibrosis

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ABSTRACT

BACKGROUND: Recurrent antimicrobial interventions and disease-related intestinal dysfunction are suspected to contribute to the dysbiosis of the gastrointestinal microbial ecosystem in patients with cystic fibrosis (CF). The present study set out to detect and identify microbial discriminants in the gut microbiota composition that are associated with CF-related intestinal dysbiosis.

METHODS: An in-depth description of CF-associated gut dysbiosis was obtained by screening denaturing gradient gel electrophoresis (DGGE) fingerprints for potentially discriminating bacterial species, and quantification by means of real-time PCR analyses using group-specific primers.

RESULTS: A total of 8 DGGE band-classes assigned to the genus *Bifidobacterium* (n=3), and members of *Clostridium* clusters XIVa (n=3) and IV (n=2), were significantly ($p<0.05$) underrepresented in samples of patients with CF. Real-time PCR analyses confirmed a significantly lower abundance and temporal stability of bifidobacteria and *Clostridium* cluster XIVa in the faecal microbiota of patients with CF.

CONCLUSION: This study is the first to report specific microbial determinants of dysbiosis in patients with CF.

1. INTRODUCTION

Chronic pulmonary infections in patients with cystic fibrosis (CF) are controlled with frequent antimicrobial administration. Although essential to restrain lung function decline, antimicrobial therapies disturb the balance of the gastrointestinal (GI) microbial ecosystem, also known as GI dysbiosis.

In populations without underlying chronic disease the selective pressure of prolonged antibiotic treatment can trigger a substantial reduction of metabolically important bacterial groups [1, 2], favour selection of antimicrobial resistant strains [3] and decrease colonization resistance, eliciting overgrowth by potentially pathogenic microorganisms [4]. Moreover, several studies have reported a long-term post-treatment perturbation [5-8]. In young children, antibiotic-associated GI dysbiosis might have severe consequences for their quality of life by disturbing the maturation of the mucosa-associated lymphoid system [9-12] and inadequate immune development [13, 14], thus increasing the risk for acquiring infections.

In addition to antimicrobial interventions, dysbiosis in patients with CF could also result from the specific dietary regimen. Several studies have indicated that a positive correlation exists between alimentary habits early in life and improvement of CF lung function through stimulation of the mucosal immune defense maturation [15-17]. Furthermore, it has been shown that GI dysbiosis can be induced by a high-fat, calorie-rich diet [18].

Using a culture dependent approach combined with molecular fingerprinting we previously found evidence for a compositional perturbation and substantially decreased temporal stability of the faecal microbiota in a group of patients with CF [19]. We now present the results of a follow-up study aiming at identifying specific

bacterial groups that can be considered determinants for intestinal dysbiosis in patients with CF.

2. MATERIALS AND METHODS

2.1 Ethics statement. All eligible candidates for this survey and their parents received written information detailing the intent of the study. As the study only included a non-invasive sampling procedure for which the participants' willingness to provide the samples was mandatory, the Ethics Committee of the University of Leuven, Belgium, approved verbal informed consent obtained from both parents/guidance and participating children (no. ML4698).

2.2 Participants. Faecal samples of 21 family units were analysed in this study. A family unit was defined as one patient with CF and one to two healthy siblings. All patients had a history of antibiotic treatment prior to and/or during the sampling period [19]. For 9 of these family units, 4 to 8 faecal samples collected between 2007 and 2009 were analysed in a longitudinal study (table 1); in addition, one sample of each of the study participants was analysed in a cross-sectional study.

2.3 Sample preparation. Stool samples were thawed at room temperature, and 1 g (wet weight) was homogenized in 9 ml Peptone Buffered Saline (PBS) (0.1 % w/v bacteriological peptone [catalog no. L37; Oxoid, Basingstoke, UK], 0.85 % w/v NaCl). 1 ml of the resulting faecal solution was used for DNA extraction as previously described [20, 21].

2.4 Denaturing Gradient Gel Electrophoresis (DGGE). Cross-sectional study DGGE fingerprints of 21 patients with CF and 24 healthy siblings, and longitudinal study DGGE fingerprints of 2 family units were available from a previous study [19]. Novel DGGE-PCR experiments were performed as previously described [19], on longitudinal samples from 7 additional family units using universal bacterial primers targeting the hyper-variable V3 region of the 16S rRNA gene. The resulting 16S

rRNA amplicons were separated by DGGE fingerprinting using the D-code System (Bio-Rad, Nazareth, Belgium) with a 35-70% linear gradient (100% denaturing polyacrylamide solution containing 7M urea (EC-605, National Diagnostics) and 40% formamide (F-9037, Sigma, St. Louis, USA)).

2.5 DGGE gel band matching, extraction and sequencing. Upon normalization, all fingerprint profiles were subjected to band-class analysis as described by Joossens and coworkers [22]. Non-parametric Mann-Whitney-U tests were performed to screen for potentially discriminating band-classes in fingerprint profiles of patients with CF and healthy siblings. P-values of less than 0.05 were considered significant and multiple-testing errors were corrected by using the adapted Benjamini-Hochberg method [23]. Bands representing potentially discriminating band-classes were excised from the DGGE gel and sequenced as previously described [24]. The web-based EzTaxon Server v2.1, a manually annotated and curated database of 16S ribosomal RNA gene sequences for bacterial type strains with validly described species names, was used to allocate sequenced bands to species based on 16S rRNA gene sequence similarities [25]. The V_3-16S rRNA gene sequences determined in this study have been deposited in the EMBL database under accession numbers HE617671 to HE617678.

2.6 Real-time PCR. Based on the results of DGGE band discriminant analysis, concentrations of faecal bifidobacteria and members of *Clostridium* cluster XIVa were determined with real-time PCR using the LightCycler system I (Roche, Mannheim, Germany) and the SensiMix^{TM} Capillary Kit (QT405-05, Bioline, London, UK). Bifidobacteria were quantified using *Bifidobacterium*-specific primers g-Bifid-F (5' CTCCTGGAAACGGGTGG 3') and g-Bifid-R (5' GGTGTTCTTCCCGATATCTACA 3')

[26], and a calibration curve was constructed based on a serial dilution of DNA from *Bifidobacterium breve* LMG 13208^T from which anaerobic plate counts were obtained on Modified Columbia agar [27]. For quantification of *Clostridium* cluster XIVa members, previously described group-specific primers g-Ccoc-F (5' AAA TGA CGG TAC CTG ACT AA 3') and g-Ccoc-R (5' CTT TGA GTT TCA TTC TTG CGA A 3') were used [28] and anaerobic enumeration of *Ruminococcus torques* strain L2-14 onto medium YCFAGSC [29] was performed for construction of the calibration curve. The 20 µl reaction mixture contained 4 µl SensiMix^{TM} Lite (Bioline Ltd., London, UK), 0.4 µl 50*SYBR® Green I solution (Bioline Ltd.), 1.5 µl enzyme mix (Bioline Ltd.), 8.1 µl MQ water, 5 µM of each primer (Sigma Aldrich, Bornem, Belgium) and 2 µl bacterial DNA. The real-time PCR program consisted of initial denaturation at 94℃ for 5 min, followed by 40 cycles of denaturation for 20 s at 94℃, annealing at 65℃ (genus *Bifidobacterium*) or 55℃ (*Clostridium* cluster XIVa) for 20 s and elongation at 72℃ for 50 s. SYBR green fluorescence was detected at the end of each amplification step. Melting curve analyses were performed by slowly increasing the temperature from 60℃ to 95℃ (genus *Bifidobacterium*) or 75℃ to 95℃ (*Clostridium* cluster XIVa). Measurements were performed in triplicate, and were repeated when variation between measurements exceeded 0.5 C_t.

2.8 Statistical analyses of real-time PCR data. The longitudinal datasets were analysed with a linear mixed model that accounts for the temporal variation between consecutive samples of each subject nested within a family unit. Samples of the cross-sectional study were compared with parametric one-tailed paired *t*-tests. In addition, the effect of several clinical parameters including forced expiratory volume in one second (FEV_1), forced vital capacity (FCV), age, weight for height and gender was assessed. Statistical analyses were performed using SAS version 9.4 with p-

values of less than 0.05 considered significant. Faecal concentrations were expressed as $\log_{10}$ colony forming units (CFU) per gram wet weight stool.

3. RESULTS

3.1 DGGE fingerprint analysis of longitudinal samples. The DGGE study of nine family units, in which per subject 4 to 8 samples obtained over a two year period were included, yielded a total of 103 DGGE profiles which were investigated for the presence of potentially discriminating band-classes. The total number of band-classes present in more than half of the CF samples or sibling samples, ranged from 6 to 24 per family unit. Statistical analysis of these band-classes revealed significant differences between samples of patients with CF and healthy siblings (Mann-Whitney-U test, $p<0.05$) in six family units, with each unit displaying 1 to 7 potentially discriminating bands. Benjamini-Hochberg correction for multiple testing reduced these results to five family units with the number of significant band-classes ranging from 1 to 6 per family unit (table 2). For each of these discriminating band-classes, bands were significantly more represented in sibling samples compared to samples of the corresponding patient with CF (figure 1). In total 8 different band-classes displayed a significant difference between samples of patients with CF and healthy siblings: band-class 8.90 was significantly underrepresented in faecal samples of patients in five out of the six family units, band-class 16.27 was discriminative in three family units, band-classes 10.22 and 14.02 were underrepresented in patient samples of 2 family units, and band-classes 3.79, 9.46, 11.46 and 16.05 were underrepresented in one family unit only.

Sequence analysis of DNA amplicons corresponding to band-classes 14.02, 16.05 and 16.27 revealed 100% 16S rRNA gene sequence identity with *Bifidobacterium longum, Bifidobacterium catenulatum/ Bifidobacterium pseudocatenulatum/ Bifidobacterium kashiwanohense* (the 140-144 bp V_3-16S rRNA

fragment does not allow differentiation of closely related bacterial species) and *Bifidobacterium adolescentis/ Bifidobacterium ruminantium/ Bifidobacterium stercoris,* respectively (table 3). Subsequent DGGE band position analysis [30] with type and reference strains of these *Bifidobacterium* species, indicated that only *B. adolescentis* co-migrated with band-class 16.27, and that only *B. catenulatum* and *B. pseudocatenulatum* co-migrated with band-class 16.05 (figure S1). We therefore tentatively identified the bacteria represented by band-classes 14.02, 16.05 and 16.27 as *B. longum, B. catenulatum/B. pseudocatenulatum* and *B. adolescentis,* respectively.

In addition, DNA amplicons corresponding to band-classes 3.79 and 11.46 showed the highest sequence similarity with *Ruminococcus bromii* (97.8%) and *Faecalibacterium prausnitzii* (98.5%), respectively (table 3). Both species are members of *Clostridium* cluster IV [31]. DNA amplicons corresponding to band-class 8.90 showed 100% sequence similarity with *Eubacterium contortum, Clostridium oroticum* and *Eubacterium fissicatena;* DNA amplicons of band-class 9.46 showed 100% sequence similarity to five species of the genus *Blautia* (table 3), and DNA amplicons corresponding to band-class 10.22 showed 99.3% sequence similarity to five different species, namely *Ruminococcus lactaris, Ruminococcus gauvreauii, Eubacterium xylanophilum, Clostridium herbivorans* and *Acetovibrio ethanogignens,* of which the latter three have not been isolated from human stool before. Band-classes 8.90, 9.46 and 10.22 each represent members of *Clostridium* cluster XIVa [31].

3.2 DGGE fingerprint analysis of cross-sectional samples. In parallel with the longitudinal study, a cross-sectional dataset consisting of 42 samples from 20 patients with CF and 22 siblings was screened for potentially discriminating band-classes. However, none of the band-classes was found to represent a significant difference between both groups.

3.3 Real-time PCR analyses. The 8 discriminative DGGE band-classes identified in this study represent three major components of the human GI microbiota, namely the genus *Bifidobacterium* and *Clostridium* clusters IV and XIVa [31]. Subsequent quantification experiments did not include *Clostridium* cluster IV as it was represented by two band-classes, each detected in only one family unit., whereas *Bifidobacterium* band-classes (i.e. 14.00, 16.05 and 16.27) were significantly underrepresented in faecal samples of patients with CF in 5 out of the 6 family units and *Clostridium* cluster XIVa band-classes (i.e. 8.90, 9.46 and 10.22) were significantly underrepresented in samples of patients with CF in all but one family unit.

Figure 2 shows the temporal variation of bifidobacterial abundance in the five family units where bifidobacteria were significantly underrepresented as determined by band-class analysis. CF samples consistently displayed lower bifidobacterial concentrations (figure 2). In addition, although not statistically significant (linear mixed model, p=0.16) visual inspection of these data revealed a stronger temporal variation in bifidobacterial abundance in samples of patients with CF compared to healthy siblings throughout (figure 2).

Furthermore, a cross-sectional analysis was conducted investigating the first sample of 21 family units. Although DGGE band-class analysis did not reveal any discriminatory potential of bifidobacteria, real-time PCR analysis revealed a

significantly higher *Bifidobacterium* abundance in samples from healthy siblings (8.71±0.13 mean $\log_{10}$ CFU/g stool) compared to patient samples (7.97±0.26 mean $\log_{10}$ CFU/g stool) (one-tailed paired *t*-test, p=0.003) (figure 3).

Figure 4 shows the temporal variation of the *Clostridium* cluster XIVa abundance in the five family units where these bacteria were consistently underrepresented in samples of patients with CF. In addition, analysis of the temporal variation of *Clostridium* cluster XIVa abundance per family unit again revealed a higher temporal variation between the different sampling points of the patients with CF compared to their healthy siblings (figure 4), which however, was not significant between both groups (linear mixed model, p=0.39).

Although members of *Clostridium* cluster XIVa were not discriminatory in the cross-sectional dataset using DGGE band class analysis, real-time PCR analysis revealed a significantly lower abundance of these bacteria in samples of patients with CF (6.51±0.17 mean $\log_{10}$ CFU/g stool) compared to samples of healthy siblings (7.14±0.09 mean $\log_{10}$ CFU/g stool) (one-tailed paired *t*-test, p=0.006) (figure 3). Moreover, for both groups *Clostridium* cluster XIVa abundance and the weight for height parameter were positively correlated (linear mixed model, p=0.03) (figure 5). This correlation, however, diminished with age (linear mixed model, p=0.041). In addition, patients with a low weight for height parameter revealed much lower abundance of *Clostridium* cluster XIVa compared to healthy siblings with a low weight for height parameter (figure 5).

DISCUSSION

We previously reported that the predominant faecal microbiota of children with CF is characterized by a compositional disturbance of the total faecal microbiota and decreased temporal stability [19]. The present study expanded the 2-year longitudinal study from two to nine familial units in order to obtain more statistically sound results, identified the microbial components of the dysbiosis using band-class analysis of the community fingerprints, and applied real-time PCR to confirm and quantify the most important observations. We showed that members of the genus *Bifidobacterium* were temporary unstable and significantly underrepresented in the faecal microbiota of patients with CF in comparison to their healthy siblings. Several studies, including one of CF patients [32], reported a substantial decline in the number of GI bifidobacteria during and following antimicrobial supplementation [33-37]. The observed reduction of bifidobacteria could be the result of their high antimicrobial susceptibility and reduced adhesion capacity to inflamed mucosa [38, 39]. The results of our DGGE fingerprint analyses revealed a significant reduction of bands assigned to *B. longum, B. catenulatum/ B. pseudocatenulatum* and *B. adolescentis*. These species are considered among the most important members of the bifidobacterial community in the GI tract [40]. High bifidobacterial species richness rather than high abundance is positively correlated with the maturation of the mucosal immune system [11] but an overall reduction of the bifidobacteria in children with CF could influence extra-intestinal disorders such as respiratory inflammation and even infection. Given the health-promoting features attributed to bifidobacteria [38, 39], their intestinal abundance is also often used as a biomarker for a well-balanced gut microbiota. Therefore, the significant decline observed in the present study could have severe repercussions for the host's health.

We also report for the first time that bacteria of *Clostridium* cluster XIVa [31] are significantly underrepresented in the faecal microbiota of CF patients. Furthermore, in siblings as well as patients with CF *Clostridium* cluster XIVa abundance and weight for height were positively correlated. However, patients with poorer nutritional status revealed a much lower abundance of *Clostridium* cluster XIVa compared to healthy siblings with poorer nutritional status (figure 5). Finally, band-classes 3.79 and 11.46 were underrepresented in samples of patients with CF. These band-classes were tentatively assigned to *Ruminococcus bromii* and *Faecalibacterium prausnitzii*, respectively, representing *Clostridium* cluster IV bacteria [31]. Many *Clostridium* clusters IV and XIVa bacteria produce butyrate as a result of carbohydrate fermentation. This metabolite has various important biological functions such as providing energy for colonocytes, eliciting an anti-inflammatory response, establishment and maintenance of the GI barrier, and reduction of intestinal permeability, and is presumed to be involved in the prevention of colorectal cancer [41-43]. Yet, the GI microbial ecosystem has a high level of functional redundancy and a broad phylogenetic spectrum of bacteria is capable of synthesizing butyrate. It is therefore unclear to which extent a significant reduction of these bacterial groups may have clinical consequences.

Frequent use of high doses of antibiotics such as amoxicillin-clavulanate likely contributed to the reported dysbiosis. In a study by Young and Schmidt [44] a considerable decrease in members of the *Clostridium* cluster XIVa and of bifidobacteria was noted in the faecal microbiota of a male patient after treatment with amoxicillin-clavulanate. Strikingly, whereas the levels of the former were restored after cessation of antibiotic therapy, *Bifidobacterium* concentrations were not. Furthermore, decreased levels of members of *Clostridium* clusters IV and XIVa have

also been reported in patients with inflammatory bowel disease [24, 45, 46], which could suggest that also CF-associated GI inflammation [47-49] may be involved in the observed dysbiosis.

Although intensive antimicrobial treatment courses are likely to contribute to the dysbiosis in patients with CF, highly personalized antibiotic therapies did not allow correlating the use of specific antimicrobial compounds with a significant reduction of the bacterial groups. Furthermore, no significant correlation was found between faecal bacteria and FEV_1, FCV or gender. However, it should be noted that the cohorts in the present study were not age-matched, gender-matched, thus limiting our evaluations.

To our knowledge, this is the first study that identifies specific bacterial groups as main determinants for a dysbiosis in patients with CF. The observed underrepresentation and temporal instability of members of the genus *Bifidobacterium* and *Clostridium* clusters IV and XIVa in patients with CF could be the result from the disease-related impairment of essential gastrointestinal functions as well as the detrimental effects of intensive antimicrobial treatment courses. Further exploration of this dysbiosis at the functional level is needed to help establishing the role of these bacterial groups and may lead the way to alternative nutritional interventions with functional foods.

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Figure 1. DGGE fingerprint profiles of the longitudinal study. DGGE fingerprint profiles showing the predominant faecal microbiota of a) family unit 3; b) family unit 6; c) family unit 11; d) family unit 14; e) family unit 16; f) family unit 18, and discriminating band-classes.

Bc: Band-class, S1 to 8: Sample 1 to 8, CF: Patient with CF; SIBLING: Healthy sibling.

Figure 2. Temporal variation of bifidobacterial abundance. Temporal variation of the genus *Bifidobacterium* in the five family units displaying one or more potentially discriminating bands assigned to this group. The error bars represent standard errors. The asterisks indicate significant differences between patients with CF and healthy siblings (linear mixed model, $p<0.05$). The intervals between the consecutive sampling points are ±3 months.

Figure 3. Bifidobacterial and *Clostridium* cluster XIVa abundance in cross-sectional study. Graphical representation of bacterial population differences as boxplots. Left: Boxplot displaying *Bifidobacterium* abundance in faecal samples of patients with CF (n=21) and healthy siblings (n=22) from the cross-sectional dataset. The open circle indicates an outlier value. Right: Bloxplot displaying *Clostridium* cluster XIVa abundance in faecal samples of patients with CF (n=21) and healthy siblings (n=22) from the cross-sectional dataset. The p-values are based on the one-tailed paired *t*-test.

Figure 4. Temporal variation of *Clostridium* cluster XIVa abundance. Temporal variation of members of *Clostridium* cluster XIVa in the five family units displaying one or

more potentially discriminating bands assigned to this group. The error bars represent standard errors. The asterisks indicate significant differences between patients with CF and healthy siblings (linear mixed model, $p<0.05$). The intervals between the consecutive sampling points are ±3 months.

Figure 5. *Clostridium* cluster XIVa abundance and weight for height. Scatterplot displaying the correlation between *Clostridium* cluster XIVa abundance and weight for height (%) (linear mixed model, $p=0.03$).