Differences of Small Intestinal Bacteria Populations in Adults and Children with/without Celiac Disease: Effect of Age, Gluten Diet, and Disease

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Background: Scientific evidence has revealed microecological changes in the intestinal tract of celiac infants. The objective of this work is the study of bacterial differences in the upper small intestine in both adults (healthy, untreated celiac disease [CD], and CD treated with a gluten-free diet) and children (healthy and untreated CD).

Methods: Intestinal bacterial communities were identified by 16S rRNA gene sequencing of DNA extracted from duodenal biopsies.

Results: Analysis of the sequences from adults and children showed that this niche was colonized by bacteria affiliated mainly with three phyla: Firmicutes, Proteobacteria, and Bacteroidetes. In total, 89 different genera were identified in adults and 46 in children. Bacterial richness was significantly lower in the children than in the adults. A global principal component analysis of the bacterial communities of both healthy and untreated CD patient groups (including both children and adults) revealed a strong effect of age in principal component 1—clustering all adults and children separately—and a possible effect of the disease in adults with untreated patients clustering separately.

Conclusions: There are bacterial differences in the upper small intestine between untreated children CD patients and untreated CD adults due to age. There are bacterial differences in the upper small bacteria microbiota between treated and untreated CD adults due to treatment with a gluten-free diet.

Key Words: celiac disease, duodenal microbiota, molecular ecology, clone library

Celiac disease (CD) is a gluten-sensitive enteropathy that develops in genetically susceptible individuals following exposure to dietary wheat gluten and similar proteins in barley and rye.1 Susceptibility to celiac disease is strongly associated with the MHC class II molecules HLA-DQ2 and HLA-DQ8.2 Although high-risk HLA alleles and exposure to gluten are frequent, most people with genetic susceptibility and gluten intake in their diets do not develop CD.3 Thus, other environmental factors might be involved in the development or the pathogenesis of this disease.

A role for the dimorphic fungus Candida albicans in CD based on sequence similarities between a hyphal wall protein and several T-cell epitopes from α-gliadins and γ-gliadins was proposed.4 Stene et al3 found a relationship between the high frequency of rotavirus infections and an increase in the risk of developing CD in genetically predisposed children. However, the finding of a rod-shaped bacterium attached to the small intestinal epithelium of some untreated CD patients but not to the epithelium of healthy controls suggested that bacteria are involved in the pathogenesis of CD.5,6

Tjellstrom et al,7 who studied the microbial metabolic activity of intestinal microbiota in children, found a different pattern of short chain fatty acids in patients with CD both at presentation and after adhering to a gluten-free diet...
ous studies have reported that the duodenal and fecal intestine microbiota of two groups of children (healthy and CD, and treated CD) and compare it with the upper small intestine in three groups of adults (healthy, untreated CD, and adults. Two mucosal samples were obtained and were stored in saline solution at 

Recent studies have described the microbiota of the small intestine in children with CD, but no differences were found compared with healthy children.10 However, previous studies have reported that the duodenal and fecal microbiota are unbalanced in children with CD and are only partially restored after long-term treatment with a GFD.11,12 Most of the studies dealing with the role of bacteria in CD have been performed in children. Although CD has been traditionally considered a disease that develops during childhood, several screening studies have found similar prevalence rates in adults.13 In this study our aim was to characterize the bacterial differences in the upper small intestine in three groups of adults (healthy, untreated CD, and treated CD) and compare it with the upper small intestine microbiota of two groups of children (healthy and untreated CD).

PATIENTS AND METHODS

Patients and Biopsies

Duodenal biopsies were collected from children and adults at the Department of Gastroenterology at the Hospital de Leon, Spain. Thirteen children were included in this study: eight untreated CD children (UCDC) on a normal gluten-containing diet (mean age 3.75 years, range 1–10 years; three boys and five girls) and five healthy children (HC) without any known food intolerances (mean age 7.2 years, range 3–12 years; two girls and three boys).

Fifteen adults were included in this study: five untreated CD adults (UCDA) (mean age 31.4 years, range 26–38 years; three men and two women); five treated CD adults on a gluten-free diet (TCDA) (mean age 18.8 years, range 16–21 years; two men and three women); and five healthy adults (HA) (mean age 29.2 years, range 15–40 years; three women and two men). None of the patients included in the study had been treated with antibiotics for at least 1 month prior to the sampling time. Duodenal biopsy specimens were collected by upper endoscopy in both children and adults. Two mucosal samples were obtained and were stored in saline solution at −80°C.

Informed consent was obtained from the adult patients and from the parents of the children. The study was approved by the local Research Ethics Committee of the Hospital de Leon.

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification of 16S rRNA Genes

DNA was extracted from duodenal biopsies using the NucleoSpin Tissue XS kit (Macherey-Nagel). The DNA concentration was determined in a NanoDrop ND-1000 spectrophotometer (Saveen & Werner, Limhamn, Sweden). Amplification of 16S rRNA genes was performed using the universal primers F968 (5’-GAACGCGAAGACCTTAAC-3’) and R1401 (5’-GGTGTGATACAGCCGC-3’). The PCR conditions used were 4 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 47°C, and 1 minute at 72°C; followed by 7 minutes at 72°C. Each reaction mixture (25 μL) contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 μM of each primer, 50 ng of DNA, and 1 U of Taq DNA polymerase (GE Healthcare, Milwaukee, WI). We performed four individual PCR reactions for each sample after all the PCR reactions were mixed. The mixed PCR products were cloned with the Stratagene PCR Cloning Kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. From each sample, over 50 transformants were randomly selected for 16S rRNA gene analysis.

DNA Sequencing and Phylogenetic Analysis

Transformants were checked for the presence of an insert of the expected size (≈450 bp) by agarose gel electrophoresis after restriction cleavage of the plasmid. Plasmid DNA from each transformant was purified with the Illustra plasmidPrep Mini Spin Kit (GE Healthcare). Sequences were obtained from the sequencing service at the University of Leon. All sequences were edited, transferred to a suitable format, and examined using the program BioEdit (Biological sequence alignment editor for winXP, Tom Hall, Ibis Biosciences, Carlsbad, CA). Each sequence obtained was used to perform sequence alignment searches using the BLASTN method14 for all nonredundant nucleotide databases. Sequences were also classified with RDP (Ribosomal Database Project release 10).15 Similar results were obtained with both of them. Sequences with ≥98% identity to sequences of known bacteria genera after the phylogenetic analysis were classified as belonging to that genus. Sequences that did not match any known genus in the database were considered unknown bacteria and were denoted Operational Taxonomic Units (OTUs) with different numbers. The identified sequences were aligned with the CLUSTAL W computer program14 according to the standard parameters of the program’s instructions. Phylogenetic and molecular evolutionary analyses were conducted using MEGA v. 4.0.16 Distance matrices were calculated using the Tamura-Nei model (because of the different GC content in the sequences) and phylogenetic trees were constructed under the minimum evolution criterion.

Sequences have been deposited in the GenBank database under the accession numbers JF332168-JF333585.

Data Analysis

UniFrac17 was used to study the differences among communities. The UniFrac online server (http://bmf2.colorado.edu/unifrac/index.psp) estimates the distance between communities as the fraction of the branch length of the phylogenetic tree that leads to descendants from either one sample or another, but not both. The analysis was weighted using the
P-value generated from the UniFrac significance test, which can determine whether microbial communities are significantly different. The values reported were Bonferroni-corrected for the number of sequences used in the comparison. The UniFrac Web application also permits many samples to be compared simultaneously using principal component analysis (PCA).

Collection and rarefaction curves and richness observed were determined by the MOTHUR package\(^1\) using the distance matrices as input. MOTHUR allows community sequence data to be analyzed and evaluates whether the sampling effort was sufficient or incomplete. All the statistical analyses were performed using SPSS 17.0 software (Chicago, IL). Significant differences between the bacterial communities were evaluated by means of an analysis of variance (ANOVA) with a post-hoc test (Scheffé test).

RESULTS

Bacteria of the Upper Small Intestine in Children and Adults Belong Mainly to the Phyla Firmicutes, Proteobacteria, and Bacteroidetes

An average of 50 sequences per subject were obtained (range 47–54 sequences) in this study. Ninety-eight percent of the 753 bacterial sequences identified in the proximal small intestine of adults (including healthy adults, untreated CD patients, and treated CD patients) were from known and unknown bacteria classified within five phyla: Firmicutes (38%), Proteobacteria (29%), Bacteroidetes (17%), Actinobacteria (10%), and Fusobacteria (4%). After sequencing and analysis of the 667 sequences from the group of children (including healthy children and untreated CD patients), the majority (＞99%) of the sequences were from a limited number of phyla: Proteobacteria (38%), Firmicutes (34%), Bacteroidetes (13%), Actinobacteria (4%), Deinococcus-Thermus (2.7%), Fusobacteria (2.9%), and unknown phylum sequences (5%).

Major Changes in Known Duodenal Bacteria Between Healthy Adults (HA), Untreated CD Adults (UCDA), and Treated CD Adults (TCD\(A\)) Are Found in Clones of Streptococcus spp. and Prevotella spp.

Sequence analysis identified the presence of 61 different genera of known bacteria in the upper small intestine of adults. Fifteen genera were present in more than 60% of the population; these genera are shown in Figure 1. Most of the sequences of known bacteria were classified within two genera: Streptococcus and Prevotella. The most important differences observed between the HA, UCDA, and TCD\(A\) groups were the number of sequences in the Streptococcus and Prevotella spp. The Mann–Whitney U-test showed that differences between healthy and celiac patients in the number of Streptococcus spp. sequences were significant (\(P < 0.05\)) according to the diagnosis. There were also significant changes in the number of Mycobacterium and Methylobacterium spp. sequences according to diet (\(P = 0.03, P = 0.01\), respectively).

FIGURE 1. Number of sequences of known bacterial genera found in the upper intestinal biopsies of healthy adults (H\(A\)), untreated CD adults (UCD\(A\)), and treated CD adults (TCD\(A\)). The 15 genera represented in the graph appeared in more than 60% of the subjects studied.
Prevotella spp. and Streptococcus spp. Show the Same Pattern in Children and Adults: Higher Detection in Healthy Children and Lower Detection in Celiac Children

We identified 36 different genera of known bacteria in children, who exhibited less bacteria richness than did the adults. Most of the sequences (Fig. 2) of known bacteria belonged to the genera Streptococcus, Prevotella, Neisseria, Haemophilus, Granulicatella, and Acinetobacter. All of these genera were present in at least 60% of the children in the analyzed groups. As shown in Figure 2, sequences of the genus Streptococcus and Prevotella showed higher detection in healthy children (HC) than in CD children (UCDc). Between HC and untreated CD children (UCDc), there were also changes in the number of sequences in the genera Neisseria and Haemophilus. These differences were not significant.

HAs and UCDAs Share an Unknown Duodenal Bacterial Microbiota that Is Not Detected in TCDAs

Analysis of the 753 sequences from the adult population showed the presence of 28 different sequences classified as unknown bacteria that have never been cultured (Table 1). These sequences were denominated by OTUs and could not be assigned to any known genus. Fourteen different OTUs (Fig. 3A) were found in healthy adults, 16 in UCDAs, and six in TCDAs. HAs and UCDAs share seven OTUs represented by more than 70 sequences (Fig. 3B), indicating a consistent population of unknown bacteria between HAs and UCDAs. By contrast, HAs and TCDAs shared only one OTU, which was present in only two sequences. Unshared OTUs were found when comparing UCDAs with TCDAs. Moreover, no common OTUs were found between the three adult populations (HAs, UCDAs, and TCDAs). These results suggest that treatment of CD patients drives an important change in the unknown bacterial population.

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<th>OTU</th>
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<td>OTU24a</td>
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<td>Nitrospira</td>
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<td>OTU1,a OTU2,a OTU4,a OTU5,a OTU7,a OTU8,a OTU9,a OTU13,a OTU15,a OTU16,a OTU17,a OTU22,a OTU28,a OTU41,b OTU46b</td>
<td>Proteobacteria</td>
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<td>OTU6,a,b OTU10,a OTU21,a OTU45b</td>
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*OTUs related to adults.

*OTUs related to children.

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FIGURE 2. Number of sequences of known bacterial genera found in the upper intestinal biopsies of healthy children (HC) and untreated CD children (UCDc). The 12 genera represented in the graph appeared in more than 60% of the subjects studied.

TABLE 1. Bacterial Phyla of the Different OTUs that Could Not Be Assigned to Any Known Genus in Adults and Children

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*OTUs related to children.
Healthy Children Have an Unknown Bacterium (OTU45) that Is Not Detected in the Upper Small Intestinal Mucosa of Celiac Children

Analysis of the 667 sequences from the group of children showed the presence of 10 different sequences classified as unknown genera that were also designated OTUs (OTUs 3, 6, 18, and 19 were shared with unknown bacteria of some adults; OTUs 41, 42, 43, 44, 45, and 46 were found only in children; Table 1). Healthy children showed the presence of four different unknown bacteria; most of these OTUs were detected only at rates lower than one sequence/HC. However, the noncultured bacterium OTU45 was present in 60% of the healthy children at an average of six sequences per child (Fig. 4); this was the most abundant OTU detected in children. In the celiac children we detected eight different OTUs, but most of them were present in only one sequence and in only one child. OTU45 was detected only in one sequence of one child with CD.

Bacterial Richness in the Upper Small Intestinal Mucosa Is Higher in Adults Than in Children

Based on rarefaction curve analysis (Supplementary Figure) the phylogenetic distances greater than or equal to 0.1 was considered adequate to evaluate the community’s richness. Hence, a phylogenetic distance of 0.1 was selected for the following richness analysis.

We assessed bacterial richness in three groups of adults: healthy adults, untreated celiac patients, and treated celiac patients. Bacterial richness analysis showed (Fig. 5) the presence of an average of 21 different clusters (similar at the number of genera taxa) per subject in the upper small intestine of HAs, 24 in UCDAs, and reduced richness in TCDAs (17 different clusters).

Bacterial richness was also assessed in upper small intestine biopsies in the two groups of children: healthy children and untreated celiac patients. The results showed (Fig. 5) that bacterial richness was lower than in the adults, with an average of 13 different clusters of bacteria per individual in HC and 16 in UCD.

The statistical analysis showed that there were significant differences in the bacterial richness between the groups (Fig. 5). ANOVA ($P \leq 0.05$) revealed that the differences in richness between the bacterial communities can be explained by two factors: subject age and maintenance of a gluten-free diet. Thus, there were significant richness differences between the adult groups and the juvenile groups (HAs vs. HC; UCDAs vs. UCD; HAs vs. UCDs; HAs vs. UCDAs vs. HC), highlighting age as the main factor. There were also significant differences between UCDAs and TCDAs (reflecting an effect of either inflammation in the gut or the gluten content of the diet).
Significant Differences Were Detected Between the Bacterial Communities of the Three Groups of Adults

To investigate whether there were differences in global bacterial composition among the three groups of adults we used the UniFrac significance test. All comparisons between HAs, UCDAs, and TCDAs revealed statistically significant differences ($P < 0.03$ after Bonferroni correction).

Comparison of the bacterial communities in 15 adults using UniFrac PCA showed a separation of the bacterial communities according to principal component 1 (PC1) (Fig. 6). The bacterial communities of UCDAs clustered separately from the clustered bacterial communities of TCDAs. The bacterial communities of HAs were dispersed along the PC1 component. The PC1 score plot (23.71%) revealed a clear separation of bacterial communities according to the presence of treatment in celiac patients.

Bacterial Community Composition of Adults Differs Significantly from the Bacterial Community Composition of Children

We finally studied whether there were significant differences between the bacterial communities in the juvenile groups. The UniFrac significance test showed that the global bacterial communities in UCDcs and Hcs were significantly different ($P \leq 0.01$ after Bonferroni correction).

We also compared the bacterial communities present in children and adults. The UniFrac significance showed that the global bacterial communities of UCDAs, HAs, UCDcs, and Hcs were significantly different ($P \leq 0.01$).

A comparison between 10 adults (H$_A$ and UCD$_A$) and 13 children (H$_C$ and UCD$_C$) using UniFrac PCA (Fig. 7) showed an age-specific pattern according to the PC1 component. This component accounted for 17.92% of the total variance. The PCA of microbial community shows distinct clusters for adults and children. However, the PC2 component suggests a cluster of the bacterial communities of UCD$_A$s within the adult group (Fig. 7).

**DISCUSSION**

Traditionally, the upper small intestine was considered to be an inhospitable habitat for microorganisms because of its low pH, its rapid peristalsis, the flushing action of chyme, and the presence of antimicrobial compounds such as bile and proteolytic enzymes. By culture-based methods it was shown that the upper small intestinal microbiota of adults is dominated by oropharyngeal microorganisms; *Streptococcus* and *Haemophilus* were found to be present only in healthy individuals and not in others with various gastrointestinal pathologies. However, most of the bacteria are not easily cultured and, therefore, to obtain an accurate picture of the intestinal microbiota molecular methods are required.

In this study we used culture-independent techniques to study the bacterial microbiota of the upper small intestine in healthy children, healthy adults, and CD patients. This report is the first (to our knowledge) of a difference between the microbiota of juvenile and adult CD patients. Using molecular techniques based on 16S rRNA gene libraries we identified 89 different genera in the upper...
small intestinal mucosa of adults, whereas children exhibited less richness, with 46 different genera. Interestingly, bacterial richness was much lower in treated adults (17 different genera) than in either untreated adults (24 genera) or healthy controls (21 genera). This richness is higher than that shown in previous studies based on culture methods.\textsuperscript{19,20} In our study of adults we found that two genera predominated the sequences: \textit{Streptococcus} and \textit{Prevotella}; these have also been frequently detected by culture-based methods.\textsuperscript{20} In addition, we found genera (such as \textit{Veillonella}, \textit{Neisseria}, \textit{Haemophilus}, \textit{Methylobacterium}, and \textit{Mycobacterium}) that seem to be part of the adult small intestinal mucosa microbiota, as well as a number of other genera detected less frequently. We also found that the microbiota of the upper small intestine includes several unknown bacteria. We detected seven unknown bacteria that are frequently detected in the small intestinal mucosa of H\textsubscript{A} and UCD\textsubscript{A}. These unknown bacteria have not been previously described as part of the microbiota of the upper small intestine of adults. These OTU populations change due to a GFD because most of them are not detected in biopsies from TCD\textsubscript{AS}. The GFD in treated CD patients has a strong effect on the small intestine microbiota as indicated by the following: 1) significant changes in richness between UCD\textsubscript{AS} and TCD\textsubscript{AS}; 2) the bacterial communities of UCD\textsubscript{AS} clustered separately from those of TCD\textsubscript{AS} by the UniFrac PCA analysis; and 3) the differences in unknown bacteria in TCD\textsubscript{AS}. There have also been reports of differences in the fecal microbiota of healthy adults adhering to a GFD.\textsuperscript{22} At least part of these changes could be a direct consequence of the GFD because diet is an obvious potential source of niche-determining factors in the gut.\textsuperscript{23} The fact that H\textsubscript{AS} and UCD\textsubscript{AS} share an OTU bacterial population that is not detected in TCD\textsubscript{AS} strongly suggests a gluten influence in the diet. However, another part of the changes in the bacterial community could be secondary to changes in the microenvironment such as restoration of the lost homeostasis or (more likely) gut healing. Perhaps healing affects the composition of mucus and thereby changes which bacteria are attached to it.\textsuperscript{24} If it is the case that bacterial communities are responding to gut healing or restoration of homeostasis, one might expect to see more similarity in microbial communities among H\textsubscript{A} and TCD\textsubscript{A} (compared to H\textsubscript{A} and UCD\textsubscript{A}). In the known bacterial population is observed a reduced detection of \textit{Prevotella} in UCD\textsubscript{A}, while TCD\textsubscript{A} had similar higher rates of \textit{Prevotella} detection as H\textsubscript{A}. As well, \textit{Streptococcus} showed a similar pattern, reduced \textit{Streptococcus} detection in UCD\textsubscript{A} and higher detection in H\textsubscript{A} and TCD\textsubscript{A}. Interestingly, \textit{Prevotella} and \textit{Streptococcus} show the same pattern in juvenile subjects: higher detection in Hc and lower detection in UCDc, suggesting that these bacterial populations are modified due to changes in the upper small intestine ecosystem. Other taxa may also display this pattern but the sequencing effort is not enough to really evaluate these patterns.

Does CD itself provoke a change in the upper small intestine microbiota? We expected to find a change in the microbiota because active CD patients have a small intestinal environment different from that of healthy individuals: cryptic hyperplasia and inflammation should lead to a change in the microbial community.\textsuperscript{25–27} In adults, we found significant differences in the number of \textit{Streptococcus} spp. sequences due to diagnosis between healthy and CD patients. According to the UniFrac significance test, the global bacterial communities in adults are significantly different. UniFrac PCA analysis shows a cluster of bacterial communities related to UCD\textsubscript{A} that appears separate from the dispersed bacterial communities of healthy adults with PC2. These controls are really non-CD patients who had some intestinal symptoms; perhaps this is the reason for the scattering of their bacterial communities in the PCA analysis.

Some reports have searched for changes in the specific bacterial groups in the small intestinal mucosa of children with CD.\textsuperscript{11,12} In our study we found that children had significantly lower bacterial richness than adults and that their bacterial communities clustered separately from the bacterial communities in adults. In fact, the adult-type and infant-type microbiomes have distinct gene complements to optimize nutrient acquisition with respect to different diets.\textsuperscript{28} According to our results the microbiota of children with active disease was richer than that of healthy children, although these differences were not significant. In addition, Schippa et al\textsuperscript{29} used temporal temperature gradient gel electrophoresis (TTGE) to assess the dominant duodenal microbiota in children with CD; they found greater duodenal microbiota richness in CD patients. They proposed that this increase could have a harmful impact on duodenal homeostasis. In children, the microbiota sequences detected were dominated by the genera \textit{Streptococcus}, \textit{Prevotella}, and \textit{Neisseria} and, to a lesser extent, \textit{Veillonella}, \textit{Granulicatella}, \textit{Acinetobacter}, and \textit{Haemophilus}. There are some differences from the upper small intestine microbiota of adults (like \textit{Acinetobacter}), but in general the dominant genera are very similar. The previously reported microbiota in the proximal small intestine of Swedish infants\textsuperscript{10} is also quite similar to the genera identified in our work with Spanish children. We found a lower diversity of unknown bacteria in Spanish children than in adults; one unknown bacterium (OTU45) occurs frequently in healthy children but it is not detected in CD children. This unknown microorganism could act as a probiotic or a health-promoting microorganism.

In conclusion, we detected differences in the bacterial communities of children and adults due to age, including in celiac patients (i.e., between UCD\textsubscript{CS} and UCD\textsubscript{AS}). There were very important differences in the communities of
treated and untreated CD patients as well differences in the bacterial communities according to the diagnosis in adults are suggested. The microbial communities of treated patients share some patterns with the known microbial communities of healthy adults, although the patterns in the unknown bacteria communities are dissimilar; something is still missing from the restoration of the “normal bacteria microbiota.” More experiments are required to assess the importance of some of these bacteria for CD patients, such as the unknown bacterium not detected in TCDAS, and the role of the OTU45 in healthy children.

ACKNOWLEDGMENT

The authors thank David Bernardo for his role in the revision of the article.

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