The Duodenal Microbiota Composition of Adult Celiac Disease Patients Is Associated with the Clinical Manifestation of the Disease

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Background: Celiac disease is classically manifested in the gastrointestinal (GI) tract but extraintestinal symptoms, such as dermatitis herpetiformis (DH), are also common. Besides several well-known shared genetic risk factors and an environmental trigger, gliadin, factors determining the clinical outcome of the disease are not known. In this study, the role of duodenal microbiota in the celiac disease outcome was studied by analyzing mucosa-associated microbiota in celiac disease patients with a variety of intestinal and extraintestinal symptoms.

Methods: Microbiota in duodenal biopsy samples obtained from 33 patients with celiac disease with GI, DH, anemia, or mixed symptoms, as well as screen-detected asymptomatic celiac disease and 18 control subjects were analyzed using PCR denaturing gradient gel electrophoresis and a subset of samples additionally by the 16S ribosomal RNA gene sequencing.

Results: The composition and diversity of mucosal microbiota was associated with the manifestation of celiac disease when analyzed using PCR denaturing gradient gel electrophoresis and the 16S ribosomal RNA gene sequencing. The patients with celiac disease with GI symptoms or anemia had lower microbial diversity than those with DH. Moreover, the patients with GI symptoms had different intestinal microbiota composition and structure, dominated by Proteobacteria, in comparison to those with DH or control subjects (patients with dyspepsia). The relatively similar intestinal microbiota composition in the control subjects and those with DH was characterized by the high abundance of Firmicutes.

Conclusions: The two common outcomes of celiac disease, classical GI and extraintestinal manifestations, had marked differences on the diversity and composition of intestinal microbiota. This association suggested that intestinal microbiota may have a role in the manifestation of the disease.

Celiac disease is a chronic inflammatory enteropathy occurring in genetically predisposed individuals after dietary gluten consumption, affecting 1% to 2% of Caucasian individuals.[1,2] Currently, celiac disease with a classical gastrointestinal (GI) manifestation is diagnosed by detecting mucosal villous atrophy with crypt hyperplasia and increased inflammation in an intestinal biopsy. The serological test, especially IgA-class endomysial and transglutaminase 2 antibodies, can support the diagnosis. Celiac disease cannot be cured, but the symptoms can disappear, and small bowel mucosal damage, inflammation, and epithelial integrity are improved by commitment to a lifelong gluten-free diet.

Nowadays, the clinical picture of celiac disease is highly variable. The most typical symptoms are classical GI complaints, such as diarrhea and abdominal pain, as well as malabsorption with weight loss or anemia. However, although the disease primarily affects the GI tract, a considerable number of patients diagnosed with celiac disease present only with extraintestinal symptoms including a bullous rash (dermatitis herpetiformis, DH), infertility, as well as neurologic and psychiatric problems.[3] Patients with celiac disease may also be asymptomatic. Asymptomatic celiac disease is typically diagnosed by screening at-risk individuals such as first-degree relatives of those affected with celiac disease.

Celiac disease is strongly associated with histocompatibility complex II class HLA-DQ02 and HLA-DQ08,[4] which are present in more than 95% to 99% of patients with celiac disease[5] and with several other genetic polymorphisms.[6–8] Although they are relevant as risk factors, their presence is not sufficient for the development of the disease.[9,10] Analysis of genetic polymorphisms have shown that even though genetic loci specific to the two major outcomes of celiac disease, classical GI symptoms and DH, have been identified,[11] they do not explain the discordance of celiac disease phenotypes detected in twin pairs[12] or siblings.[13]

Thus, other factors such as environmental factors or intestinal microbiota may play a role in the diversification of the manifestation. Several risk factors, such as the introduction of gluten to the diet at an early age,[15] certain infections,[16] and formula feeding,[14] which may affect the intestinal microbiota composition, have been reported. Indeed, evidence for the dysbiosis of intestinal commensal microbiota in celiac disease has been presented in studies of pediatric patients with celiac disease[17–22] and in a single study of adult patients with celiac disease.[23]

The role of intestinal microbiota on different clinical manifestations of celiac disease has not been studied yet. To address this question, we analyzed the duodenal microbiota composition of adult patients with celiac disease in relation to a range of intestinal and extraintestinal symptoms of the disease. Microbiota composition in duodenal biopsy samples of 33 untreated patients with celiac disease and 18 control subjects were analyzed by the denaturing gradient gel electrophoresis (DGGE) and a subset of samples also by the 16S ribosomal RNA (rRNA) gene sequencing. This study showed that the composition and diversity of duodenal microbiota differed between the patients with the GI and those with extra-intestinal outcomes of celiac disease, indicating that microbiota dysbiosis may have a role in the manifestation of the disease.

MATERIALS AND METHODS

Study Subjects and Sampling

The study group comprised 33 adults with untreated celiac disease at the time of diagnosis (24 females and 9 males; mean age, 39 years; range, 18–67 years). Eight of these patients had GI symptoms (ie, diarrhea, abdominal pain), six had DH, and seven had anemia. Eight patients with celiac disease diagnosed when screening celiac disease family members were asymptomatic. Furthermore, an additional 4 patients with celiac disease had combinations of the above-mentioned symptoms or other complaints (Fig. 1). The small bowel biopsy and serum samples of the 33 patients were taken at the time of the diagnosis of celiac disease, and thus, all patients were consuming normal Western gluten-containing diet on sample collection. The biopsy and serum samples of 18 subjects without celiac disease with a similar age and sex distribution experiencing dyspepsia served as control subjects (Fig. 1).

All the patients and control subjects had undergone an upper GI endoscopy at the Department of Gastroenterology and Alimentary Tract Surgery. On the endoscopy, seven forceps biopsy specimens were taken from the distal part of the duodenum. Two to three small bowel biopsy specimens were freshly embedded in an optimal cutting temperature compound (Tissue-Tek, Miles; Elkhart, IN), snap frozen in liquid nitrogen, and stored at −70°C until used. The rest of the biopsies were used for diagnostic purposes. The diagnosis of celiac disease was based on the presence of small bowel mucosal, severe, partial, or subtotal villous atrophy with crypt hyperplasia.[24] To diagnose DH, a skin biopsy was taken from the uninvolved perilesional skin, and the diagnosis was based on the demonstration of pathognomonic granular IgA deposits in the
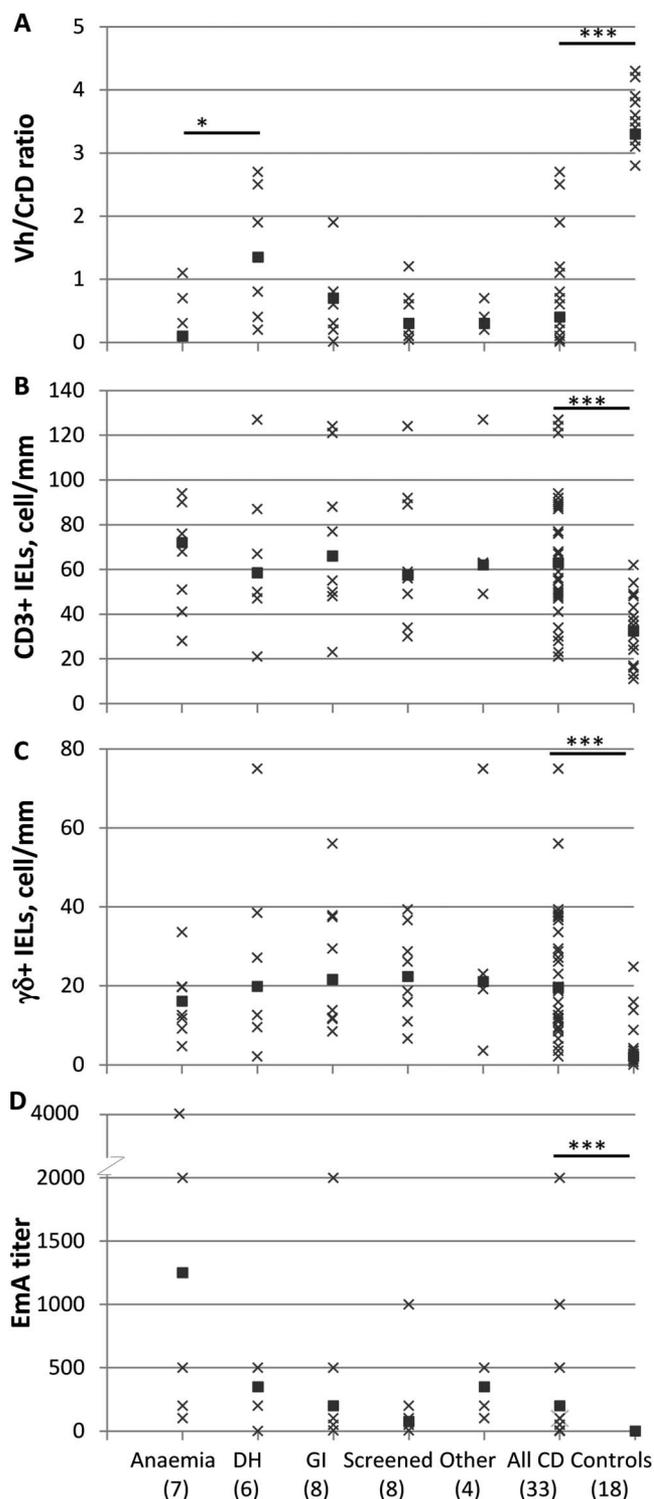
FIGURE 1. A–D, Distribution and median (indicated by square) of clinical parameters. The group "other" contained patients with a combination of DH and GI symptoms (2), weight loss (1), and a combination of mild GI symptoms and dementia (1). CD, celiac disease. The *P* values between different CD symptom groups and between all CD patients and controls in Mann–Whitney *U* test are indicated in the graph: *$P < 0.05$, ***$P < 0.001$.

dermal papillae revealed by a direct immunofluorescence examination.[25] Even if the majority of patients with DH evince small bowel mucosal villous atrophy, a proportion of the patients only present with mild enteropathy. The small bowel mucosal villous height to crypt depth ratio (Vh/CrD), and densities of CD3+ and γδ+ intra-epithelial lymphocytes (IEL) were analyzed from biopsy samples and endomysial antibody (EmA) titers were measured from the serum samples, as described earlier.[26] Mann–Whitney *U* test in StatsDirect version 2.5.6 (StatsDirect Ltd, Cheshire, United Kingdom) was applied to calculate statistically significant differences between the clinical parameters.

DNA Extraction

The total DNA was extracted from the biopsy samples using the QIAamp Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with minor modifications. Briefly, the biopsy samples were lysed by incubating the sample in ATL lysis buffer with proteinase K overnight at 56°C, purified with spin columns, and then eluted with 400 μL of buffer AE. The DNA concentrations were determined with NanoDrop 1000 (Thermo Scientific, Wilmington, DE). The extracted DNAs were stored at −20°C.

PCR-DGGE

The similarity and diversity of microbiota in the biopsy samples of the study subjects was analyzed by the nested PCR-DGGE. The partial 16S rRNA gene was first amplified by PCR with universal bacterial primers 7F (5'-AGAGTTTGA-TYMTGGCTCAG-3') and U1401R (5'-CGGTGTGTACAA-GACCC-3').[27] In second PCR, the PCR product of the first PCR reaction was amplified with primers, U968F +GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGG-GGGAACGCGAAGAACCTTA-3') and U1401R, as described in the study by Mättö et al.[28] A 1 μL of template DNA was used for both PCRs. A volume of 20 μL of the PCR product was separated in 8% polyacrylamide gel with a denaturing gradient of urea and formamide ranging from 38% to 60%.[29] The DGGE gels were run at 70 V for 960 minutes using the DCode universal mutation detection system (Bio-Rad, Hercules, CA). The gels were stained and documented, as described in Wacklin et al.[29] Despite several attempts, the amplification was not successful for the four samples belonging to the GI (1), anemia (2) symptom groups, and a control subject (1) probably because of the low amount of bacterial DNA in comparison to human DNA or PCR inhibitors in the sample. These samples were excluded from the DGGE analysis.

The digitalized DGGE gel images were imported to the Bionumerics program version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium) for normalization and band detection, as described in Wacklin et al.[29] Matrices based on band intensities were exported from Bionumerics and used for the calculation of Shannon diversity indexes. Shannon diversity index, H', was calculated using the equation $H' = -(\Sigma p_i \ln(p_i))$, where p_i was the proportion of each species (ie, DGGE band intensity) in the sample. The richness was calculated as a number of detected bands in the DGGE profile of the sample. Principal component analysis (PCA) based on the band intensities of all the patients with celiac disease and control subjects was calculated as implemented in Bionumerics, version 5.0. Statistical significance in the diversity between symptom groups was tested with *t* test.

Cloning of the 16S rRNA Gene

Cloning was performed for a subset of 20 samples including randomly selected samples of the control subjects (n = 5), and the patients with celiac disease with GI symptoms (n = 4), DH (n = 6), anemia (n = 4), and other symptoms (n = 1). The samples were cloned using the pGEM-T Vector system II (Promega, Madison, WI) kit according to the manufacturer's instructions. The 16S rRNA gene fragment was amplified similarly to the second PCR reaction in DGGE analysis, except primers without GC-rich clamps were used. Positive clones containing the insert were selected using Luria agar with isopropyl-2-D-galactopyranoside/X-Gal/ampicillin. The clones with the correct insert size were sequenced in Eurofins MWG Operon (Ebersberg, Germany).

The sequences shorter than 200 base pair or a nonbacterial origin according to Blast[30] were removed from further analysis. Of all the anemia samples, only 43 sequences were obtained, and they were therefore excluded from the further data-analysis. The rest of the sequences were assigned to bacterial taxa using the Classifier tool in Ribosomal database Project[31] and aligned by Mothur.[32] Rarefaction curves and diversity indexes were calculated in mothur using 0.03 dissimilarity threshold. Principal coordinate analysis and Unifrac analysis were performed by Fast Unifrac software.[33] A maximum likelihood tree for the Unifrac analysis was inferred by RAxML using the gamma model of rate heterogeneity (http://phylobench.vital-it.ch/raxml-bb/).

RESULTS

Clinical Data

All the patients with celiac disease, except three patients with DH, had both villous atrophy and an increased titer of serum EmA (Fig. 1). Two patients with DH showed mild enteropathy and had increased lymphocyte and EmA levels, whereas one patient with DH had villous atrophy, but normal levels of lymphocytes. The small bowel mucosal structure was normal, and serum EmA was negative for all the control subjects (Fig. 1). The medians of all measured clinical parameters differed between control subjects and patients with celiac disease (Mann–Whitney *U* test, *P* >0.0001), as expected (Fig. 1). The patients with DH had a higher median of Vh/CrD than those with celiac disease with anemia (Mann–Whitney *U* test, *P* = 0.03) (Fig. 1). Other clinical parameters did not differ between the celiac disease symptom groups. Thus, the clinical parameters indicated the presence of intestinal inflammation and mucosal damage, albeit mild, in two patients with DH in the celiac patient group.
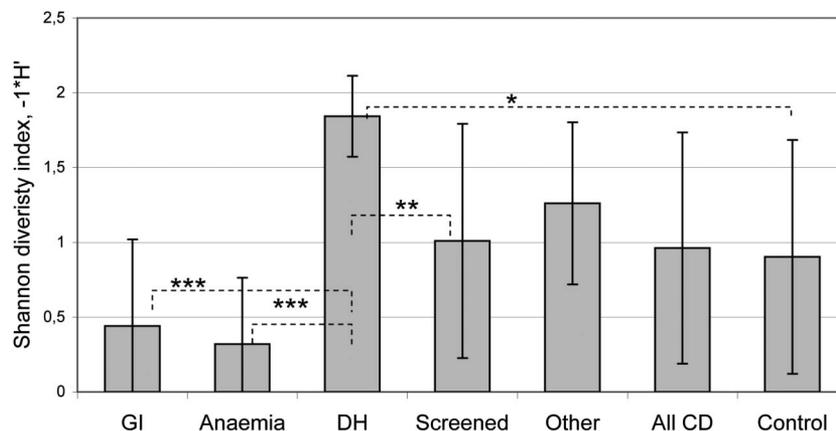
FIGURE 2. The microbial diversity and richness based on an intensity matrix of DGGE profiles in the control subjects and the patients with celiac disease (CD) with different clinical manifestations. The *P* values in ANOVA are indicated in the graph. *$P < 0.05$, **$P < 0.02$, ***$P < 0.001$.

Microbiota Diversity and Composition by DGGE

The PCR-DGGE analysis of dominant mucosa-associated microbiota showed that the patients with celiac disease presenting DH had a higher microbial diversity and richness than the control subjects (analysis of variance [ANOVA], $P < 0.05$) and screened asymptotic patients (ANOVA, $P < 0.02$), especially in comparison with the microbiota of the patients with celiac disease with anemia (ANOVA, $P < 0.0002$) or GI symptoms (ANOVA, $P < 0.0006$) (Fig. 2).

The patients with celiac disease with different symptoms (GI, anemia, DH) were clustered separately in PCA of the DGGE profiles (Fig. 3). The patients with DH (including the two patients with mild enteropathy) clearly shared different microbiota as compared with more closely clustered patients with GI symptoms and anemia

(Fig 3), both of which indicate abnormal functionality of the GI track. This indicated that the composition of mucosa-associated microbiota in the duodenum of the patients differed depending on the manifestation of celiac disease. Interestingly, the samples of patients reporting both GI and DH symptoms (n = 2) were clustered with the samples of patients with GI symptoms (data not shown), and the sample of the patient with intestinal malabsorption and weight loss was clustered with the samples of the anemia symptom group. The celiac patient group as a whole or the asymptomatic celiac patient group, which was diagnosed in the screening of risk individuals, was not separable from the control subjects in the PCA of PCR-DGGE profiles (Fig. 3).

Microbiota Diversity and Composition by the 16S rRNA Sequence Analysis

To extend the microbiota analysis to phylum and genus levels, the microbial composition in the control subjects and those patients with DH, GI, and anemia symptoms was studied using
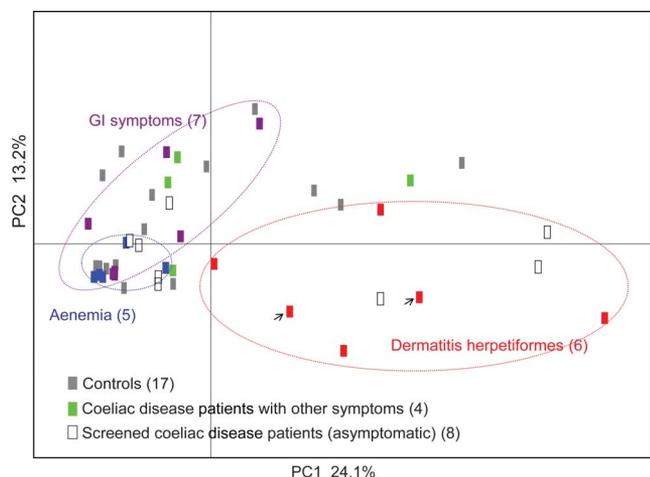

FIGURE 3. PCA based on the DGGE profiles of the samples of patients with celiac disease and control subjects. The samples from the patients with celiac disease manifesting different symptoms and control subjects are indicated by different colors, and the number of samples in each symptom group is in parentheses. The two samples of the patients with DH presenting mild enteropathy are indicated by arrows.
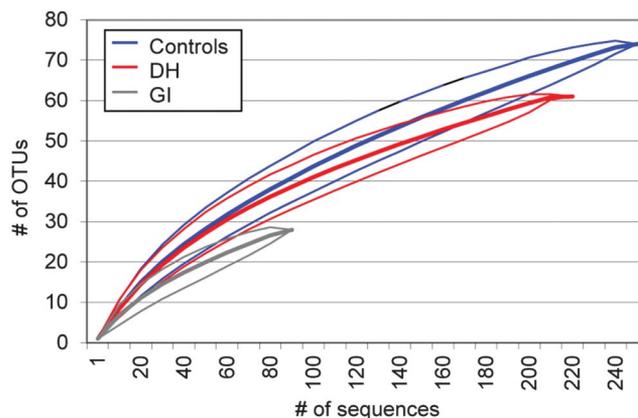

FIGURE 4. Rarefaction curves of the 16S rRNA gene sequences for the control subjects, for those patients with celiac disease presenting GI symptoms, and for those patients with celiac disease presenting DH. The thin lines indicate 95% confidence intervals. Operational taxonomic units were defined at a 97% similarity level.

TABLE 1. Diversity and Richness Estimators of the 16S rRNA Gene Clone Libraries of the Control Subjects, Patients With Celiac Disease, and Two Disease Subgroups (Patients With GI Symptoms and Patients With DH Symptoms)

Study Group	No. of Samples	No. of Clones	No. of OTUs (Mean)	Shannon Diversity Index (Mean)
Controls	5	245	23	2.71[a,b]
Patients with celiac disease	10	298	13	2.08[a]
GI	4	87	10	1.71[b]
DH	6	211	16	2.33

Operational taxonomic units (OTUs) at 97% similarity.
[a]Statistical significance at $P = 0.08$ using *t* test, control subjects versus patients with celiac disease.
[b]Statistical significance at $P = 0.04$ using *t* test, control subjects versus patients with celiac disease with GI symptoms.

the 16S rRNA gene sequencing. Altogether, the 16S rRNA gene clone libraries were performed for 20 biopsy samples. We obtained 725 clone sequences in total. The four samples from the patients with anemia, for which only eight to 13 clones were obtained per sample, as well as 75 short sequences were excluded from the analysis. Rarefaction curves showed a decreasing rate of operational taxonomic units (defined by 0.03 dissimilarity threshold) at the end of most curves (Fig. 4), demonstrating that a large part of diversity was achieved.

Based on the sequence analysis, the microbial diversity and richness of samples differed depending on the symptoms of the patient with celiac disease. Microbial richness based on the rarefaction curve analysis and diversity was slightly lower in the GI symptom group than in the DH symptom group (Shannon diversity, ANOVA, $P < 0.04$) (Fig. 4; Table 1). The low diversity and richness in the patients with GI symptoms was also detected in DGGE, thus confirming the results. In contrast to the DGGE results, the patients with DH and control subjects shared a rather similar diversity according to the sequence analysis. The Shannon diversity index showed a trend toward higher diversity in the control subjects in comparison with the patients with celiac disease (ANOVA, $P < 0.08$) (Table 1). The rarefaction curves for the control subjects and the all patients with celiac disease did not differ (Fig. 4).

Taxonomic assignments of the sequences showed that the mucosa-associated microbiota in the duodenum consisted of Firmicutes, Bacteroides, and Proteobacteria and Actinobacteria phyla. Firmicutes-related and Bacteroides-related sequences were abundant in the control subjects and patients with DH, whereas Proteobacteria-related sequences dominated (70%) in the patients with GI symptoms (Fig. 5). The altered duodenal microbiota composition of the patients with GI symptoms was also evident at the genus level (Fig. 5). Several proteobacterial genera were abundant in the samples of the GI symptom group, *Acinetobacter* (25%) and *Neisseria* (12%) being the most abundant. The sequences related to *Streptococcus* and *Prevotella* were the most abundant in the duodenum of the patients with DH (29% and 18%) and control subjects (14% and 18%). In total, we detected 45 bacterial genera in the data set, 32 genera being detected in the control samples, and 31 genera in the celiac disease patient samples.

The samples of the patients with GI symptoms were clustered separately from the samples of the control subjects in the PCA of the 16S rRNA gene clone sequences (Fig. 6). The samples of patients with DH (including the two samples with mild enteropathy) were located between the control subjects and the patients with GI symptoms (Fig. 6). Accordingly, the weighted and unweighted Unifrac analysis indicated that the microbial composition ($P < 0.002$) and structure ($P < 0.002$) of the GI symptom group were significantly different from the control group. In addition, the microbial structure differed between the DH and the GI symptom groups ($P < 0.002$) in the weighted Unifrac analysis. The

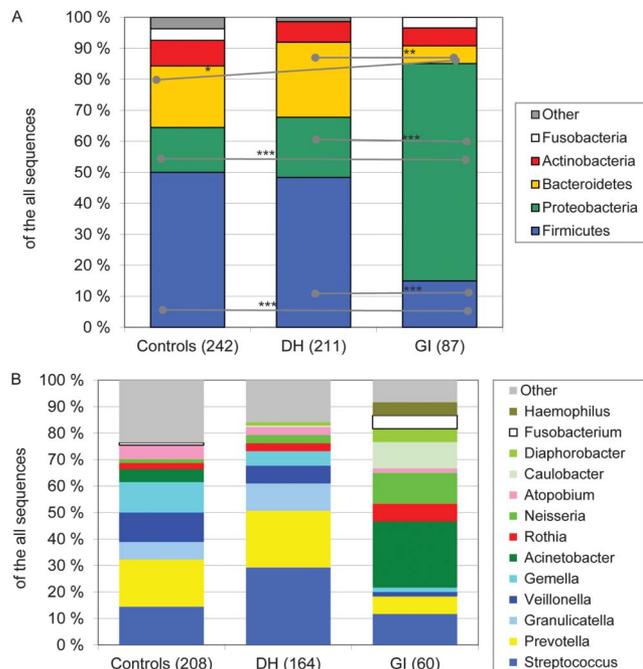FIGURE 5. Relative abundances of the sequences from the control subjects, the patients with celiac disease presenting GI symptoms, and those with DH at the phyla level (A) and genera level (B). The numbers in parentheses show the total number of sequences identified in each study group at phyla and genera levels. The significant phyla-level differences between the study groups: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.
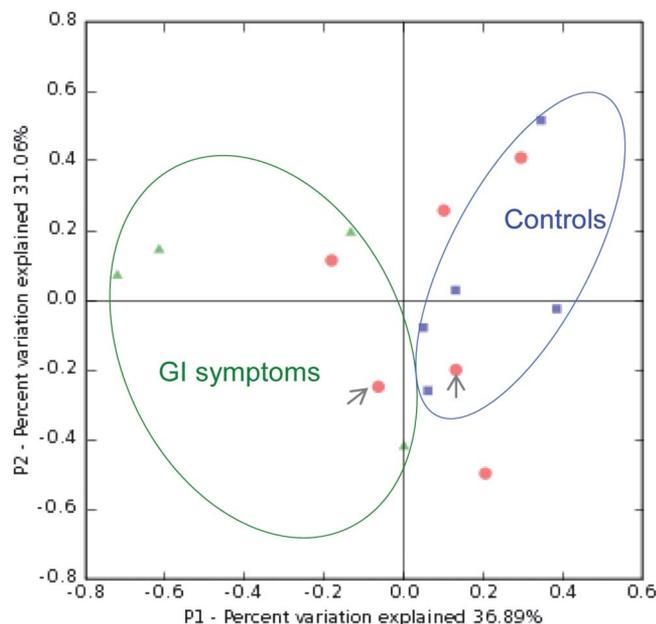
FIGURE 6. Principal coordinate analysis of the samples was associated with the symptoms of celiac disease. The samples of the patients with celiac disease with GI symptoms are indicated with green triangles, patients with DH with red circles, and controls with blue squares. The two samples of the patients with DH with mild enteropathy are indicated by arrows.

microbial composition or structure between the control subjects and patients with DH did not differ. The results indicate that the composition of the mucosa-associated microbiota in the duodenum of the patients with celiac disease presenting GI symptoms was altered, as compared with the control subjects and patients with DH.

DISCUSSION

Commensal microbiota interacts with the host immune system, and the disturbance in the interaction caused by internal or environmental factors may lead to dysbiosis and inflammation. This hypothesis has been suggested in inflammatory bowel diseases (IBD),[34] but it may also be extended to intestinal inflammation in general. Microbiota may have a role in the development or manifestation of celiac disease, as shown by the present study. Our results demonstrate that the composition, structure, and diversity of microbiota differed depending on the manifestation of celiac disease, especially between classical intestinal symptoms (ie, GI and anemia) and extraintestinal symptoms (ie, DH). The dysbiosis of microbiota indicated by the altered microbiota composition is a common phenomenon in several intestinal inflammation disorders. For example, microbial composition in Crohn's disease[35,36] and irritable bowel syndrome[28,37] have been shown to differ depending on the disease phenotypes. Recently, altered intestinal microbiota populations and/or diversities have also been reported in patients with type 2 diabetes[38] or obese subjects,[39] both characterized by a low grade of intestinal inflammation.

Our study indicated that the patients with celiac disease presenting DH symptoms shared more similar microbial composition with the control subjects than those with other clinical manifestations of celiac disease. The patients with DH had biopsy-proven mucosal damage/mild enteropathy and increased lymphocyte counts and EmA titer. As compared with the other celiac disease symptom groups, the Vh/CrD ratio in the patients with DH was closer to the ratio detected in the control subjects. However, it did not differ statistically from the other celiac disease symptom groups, except for the anemia symptom group. The two patients with DH showed mild enteropathy. The microbiota profiles of these patients clustered tightly with those from the other patients with DH. Thus, the Vh/CrD ratio cannot explain the higher similarity of the microbial composition of the control subjects and patients with DH. The finding of unexpectedly low richness and distinct clustering of duodenal microbiota profiles in the patients with anemia symptoms also suggests a possible role of microbiota in the outcome of celiac disease and warrants further studies with a larger patient cohort. Interestingly, the samples of the patients showing both DH and GI symptoms were clustered with the samples of the GI symptom group, demonstrating the strong effect of the GI symptoms on the intestinal microbiota. These findings indicate that a more detailed patient segmentation based on the symptoms is reasonable in further studies of microbiota in patients with celiac disease.

In contrast to the colon, the microbiota composition of the small intestine has been infrequently studied. The few studies performed to profile microbiota in the small intestine have revealed that Firmicutes and Bacteroides are dominant phyla also in the small intestine,[35,40,41] as also supported by the present study. The patients with a classical celiac disease manifestation, GI symptoms, had a higher amount of Proteobacteria than the patients with another manifestation of the disease or the control subjects. Similar to our results of patients with celiac disease, the small intestines of patients with IBD were characterized by the parallel increase of cell wall–associated Proteobacteria and the decrease of Firmicutes in comparison to the patients without IBD.[35] The genera (mostly *Streptococcus* and *Prevotella)* observed in the present study were largely matching to the genera detected by Nistal et al,[23] another study on the microbiota composition of adult patients with celiac disease. In fact, the microbiota composition of the duodenum resembles more the microbiota in the esophagus[42] or the oral cavity[43] than the distal parts of the intestine. In future studies, it may be informative to detect the oral disease status and oral microbiota composition and study whether it is reflected on the duodenal microbiota composition.

Although the dysbiosis of microbiota observed in patients with celiac disease may be a consequence of the disease, it is possible that the spectrum of a patient's intestinal microbes has a role in the actual clinical symptoms of celiac disease caused by gliadin. The comparison of active and untreated pediatric patients with celiac disease has shown that certain bacterial groups (ie, a decreased number of Bifidobacteria, Bacteroides, and virulent *Escherichia coli*) are associated with both active and treated celiac

disease, suggesting that microbial composition changes are not just a consequence of the disease.[44] Nevertheless, no specific bacterial agent or pathogen has been linked to the development or manifestation of celiac disease, the situation being similar in IBD.[45] Intestinal and extraintestinal manifestations of celiac disease can occur discordantly among family members[13] and even among identical twins.[11] Although few host genetic loci have been associated with the manifestation of celiac disease,[11] it has been suggested that yet unknown susceptibility genes or environmental factors or their combination play a role in the determination of the outcome of celiac disease.[13] Based on our results, it is tempting to speculate that microbiota composition could be one such factor. Recently, Rausch et al[46] and we[29] have shown that host gene polymorphisms in *FUT2* (coding for the histoblood group secretor status) have an effect on intestinal microbiota composition. Interestingly, *FUT2* association with celiac disease was detected by Dickey et al[47] but not by Heneghan et al.[48] Thus, it is possible that host genes could indirectly, by means of microbiota composition, be involved in the manifestation of the disease. Alternatively, bacteria, for example, those belonging to Proteobacteria, may contribute to the development of the disease. In this study, an increased abundance of proteobacteria was associated with the commonly occurring GI manifestation of the disease but not with DH. The combined effect of gliadin and *Shigella*, a member of proteobacteria, has been shown to enhance the gut epithelial barrier disruption in a rat model,[49] and Sanz et al[44] suggested that intestinal bacteria could be factors enhancing immunologic responses to gliadin.[35] Moreover, proteobacteria with adherent, invasive properties have been suggested to promote inflammation leading to IBD in genetically or immunologically predisposed subjects.[50]

The different clinical manifestations of celiac disease showed characteristic compositions of the intestinal microbiota. Hence, it is possible that the spectrum of a patient's intestinal microbes has a role in the actual clinical symptoms of celiac disease caused by gliadin. The differences in microbiota depending on the manifestation of celiac disease also demonstrate the importance of patient stratification in the microbiome studies of celiac disease.

ACKNOWLEDGMENTS

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