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Duodenal-Mucosal Bacteria Associated with Celiac Disease in Children

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Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of cereal gluten proteins. This disorder is associated with imbalances in the gut microbiota composition that could be involved in the pathogenesis of CD. The aim of this study was to characterize the composition and diversity of the cultivable duodenal mucosa-associated bacteria of CD patients and control children. Duodenal biopsy specimens from patients with active disease on a gluten-containing diet ($n = 32$), patients with nonactive disease after adherence to a gluten-free diet ($n = 17$), and controls ($n = 8$) were homogenized and plated on plate count agar, Wilkins-Chalgren agar, brain heart agar, or yeast, Casitone, and fatty acid agar. The isolates were identified by partial 16S rRNA gene sequencing. Renyi diversity profiles showed the highest diversity values for active CD patients, followed by nonactive CD patients and control individuals. Members of the phylum *Proteobacteria* were more abundant in patients with active CD than in the other child groups, while those of the phylum *Firmicutes* were less abundant. Members of the families *Enterobacteriaceae* and *Staphylococcaceae*, particularly the species *Klebsiella oxytoca*, *Staphylococcus epidermidis*, and *Staphylococcus pasteuri*, were more abundant in patients with active disease than in controls. In contrast, members of the family *Streptococcaceae* were less abundant in patients with active CD than in controls. Furthermore, isolates of the *Streptococcus anginosus* and *Streptococcus mutans* groups were more abundant in controls than in both CD patient groups, regardless of inflammatory status. The findings indicated that the disease is associated with the overgrowth of possible pathobionts that exclude symbionts or commensals that are characteristic of the healthy small intestinal microbiota.

Celiac disease (CD) is a chronic intestinal disorder caused by a deregulated immune response to gluten proteins from wheat, rye, and barley and their cross-related varieties in genetically susceptible individuals. CD presents a set of diverse clinical features, which typically includes fatigue, weight loss, diarrhea, and anemia. Damage to the intestinal mucosa in patients with CD is characterized by intraepithelial lymphocytosis, crypt hyperplasia, and villous atrophy (1). In CD patients, the pathological response to gluten proteins involves both adaptive and innate immunity. It is known that gliadin-specific $CD4^+$ T cells develop an inflammatory reaction by production of Th1 cytokines (e.g., gamma interferon [IFN-γ]) at the mucosal level, which also induces $CD8^+$ cells to kill epithelial cells, contributing to tissue damage (2). In addition, a new subset of T cells, termed Th17 cells, was shown to contribute to CD pathogenesis by producing proinflammatory cytokines (such as interleukin-17 [IL-17], IFN-γ, and IL-21), although these cells can also produce mucosa-protective and regulatory factors (IL-22 and transforming growth factor β) (3, 4). Some gluten peptides that are not recognized by T cells can induce tissue damage by activating components of innate immunity; thus, peptide p31-43/49 activates the production of IL-15 and natural killer cell receptor-mediated cytotoxicity by intraepithelial lymphocytes, contributing to tissue injury (5–7). Improvement of the pathological lesions occurring in the intestinal mucosa of sensitive individuals is usually observed after gluten withdrawal from the diet; however, compliance with this dietary recommendation is complex, and other alternative strategies are being investigated (8).

HLA class II molecules DQ2 and DQ8 are the major risk factors predisposing individuals to CD and account for 35% of the genetic risk (9). Although the role of these molecules has been well established in the pathogenesis of CD, their frequency in the general population is approximately 30%, whereas only 1 to 3% of individuals actually develop the disease (10). These data would suggest that the presence of HLA molecules is a necessary factor but is not sufficient alone for disease development. Although gluten is the main environmental trigger of CD, its intake does not fully explain disease development, and thus, other environmental factors are thought to be involved. In recent years, early microbial infections (11, 12) and imbalances in the composition of the gastrointestinal microbiota (13–20) have also been associated with CD. Molecular techniques have shown that, compared to the fecal and duodenal microbiota of healthy individuals, the fecal and duodenal microbiota of CD patients is characterized by the presence of higher numbers of Gram-negative bacteria (bacteroides and enterobacteria) and lower numbers of Gram-positive bacteria, like bifidobacteria (19, 20). *In vitro* assays have shown that this altered microbiota and some enterobacteria isolated from CD patients could activate proinflammatory pathways, while some bifidobacteria could inhibit the inflammatory or toxic effects induced by the same isolated enterobacteria and gluten peptides (21–24). Alterations in the intestinal microbiota are also involved in the pathogenesis of chronic inflammatory bowel disease (IBD) (25, 26) and other immune-related disorders (27–29). For instance, IBD patients have altered duodenal bacterial populations in comparison to healthy controls (30–32). Nevertheless, neither the specific bacteria involved in pathologies affecting the small intestine nor their possible pathogenic modes of action are fully understood.

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TABLE 1 Clinical characteristics of study subjects[a]

Characteristic	Active CD ($n = 32$)	Nonactive CD ($n = 17$)	Control ($n = 8$)
Mean (SD) age (yr)	5.1 (3.2)	5.9 (1.2)	6.9 (4.2)
No. (%) of study subjects			
Sex (M/F)	14 (43.7)/18 (56.3)	8 (47.1)/9 (52.9)	4 (50)/4 (50)
Symptoms			
Abdominal pain	5 (15.6)	0 (0)	2 (25)
Diarrhea	3 (9.4)	0 (0)	5 (62.5)
Weight loss	5 (15.6)	3 (17.6)	1 (12.5)
Anemia	9 (28.1)	2 (11.8)	0 (0)
Iron deficiency	17 (53.1)	0 (0)	0 (0)
Presence of antigliadin antibodies (AGA^+)	32 (100)	0 (0)	0 (0)
Presence of antitransglutaminase antibodies (tTG^+)	32 (100)	0 (0)	0 (0)
Duodenal biopsy[b]			
M0-1	0 (0)	17 (100)	8 (100)
M3	32 (100)	0 (0)	0 (0)
HLA type DQ2 and DQ8	32 (100)	17 (100)	NA[c]

[a] Data are expressed as absolute numbers (percentages related to the total numbers) for all characteristics except age, which is expressed as the mean (standard deviation). M, male; F, female.

[b] Modified Marsh classification of CD (1): M0, normal mucosa; M0-1, infiltrative lesions, seen in patients on a gluten-free diet (suggesting that minimal amounts of gliadin are being ingested), patients with dermatitis herpetiformis, and family members of patients with CD; M2, hyperplastic type, occasionally seen in patients with dermatitis herpetiformis; M3, >40 intraepithelial lymphocytes per 100 enterocytes, crypts increased, and villi with atrophy (partial or complete villous atrophy), seen in cases of typical CD.

[c] NA, not applicable.

This study was designed to establish whether live culture-dependent bacteria associated with the duodenal mucosa of patients with active and nonactive CD and controls differ in composition and biodiversity, as reported in previous molecular studies, with a view to exploring their potentially pathogenic features in the future.

MATERIALS AND METHODS

Subjects. Biopsy samples from three groups of children were included in this study: 32 from patients with active CD (mean age, 5.1 years; range, 2 to 14 years) on a normal gluten-containing diet, 17 from patients with nonactive CD (mean age, 5.9 years; range, 3 to 8 years) after following a gluten-free diet for at least 2 years, and 8 from control children (mean age, 6.9 years; range, 3 to 13 years) with no known gluten intolerance. The control group consisted of children who were investigated for weight loss, growth retardation, or functional intestinal disorders of non-CD origin; and their non-CD status was confirmed by showing a normal villous structure by examination of the biopsy specimen. CD was diagnosed according to the criteria given by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (33). The children included in the study had not been treated with antibiotics for at least 1 month before sampling.

The study was conducted in accordance with the ethical rules of the Helsinki Declaration (Hong Kong revision, September 1989), according to EEC Good Clinical Practice guidelines (document 111/3976/88, July 1990), and under the guidelines of current Spanish law which regulates clinical research in humans (Royal Decree 561/1993). The study protocol was approved by the Committee on Ethical Practice from CSIC and the Hospital Universitario La Fe (Valencia, Spain). Written informed consent was obtained from the parents of the children included in the study. The clinical characteristics of the children are shown in Table 1.

Sample preparation and bacterial isolation. Duodenal biopsy specimens (approximately 10 mg) were obtained by capsule endoscopy, kept under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, United Kingdom), and analyzed in less than 2 h to avoid alterations in bacterial viability. Biopsy specimens were homogenized in 200 μl of a phosphate-buffered saline (PBS) solution (130 mM sodium chloride, 10 mM sodium phosphate, 0.05% cysteine, pH 7.2) by pipetting and thorough agitation in a vortex mixer (10 s). Each homogenized sample was randomly plated on two different culture media (100 μl).

The following media were used: plate count agar (PCA; Scharlau, Barcelona, Spain) (34), Wilkins-Chalgren agar (Scharlau, Barcelona, Spain) (35), brain heart agar (BH; Scharlau, Barcelona, Spain) (36), and yeast, Casitone, and fatty acid agar (YCFA) (37). PCA plates were incubated under aerobic conditions at 37°C for 48 h, whereas Wilkins-Chalgren, BH, and YFCA plates were incubated under anaerobic conditions at 37°C for 48 h using anaerobic jars and an AnaeroGen system (Oxoid, Hampshire, United Kingdom), which generates an atmosphere of <1% oxygen supplemented with carbon dioxide within 30 min, facilitating the culture of fastidious and obligate anaerobes. All the viable and cultivable bacteria recovered from duodenal biopsy samples (mucus and mucosa-associated bacteria) were isolated and restreaked onto the same agar media. For preliminary identification of the isolates, conventional microbiological methods were used, including analysis of colony and cellular morphology and Gram staining. All isolates were stored at −80°C in the presence of glycerol (20%, vol/vol) until use for further characterization.

DNA extraction. For DNA extraction, bacterial isolates were grown in the same isolation broth media and harvested at the late log growth phase. The bacterial suspensions were centrifuged for 5 min at 6,000 × *g*, the pellets were resuspended in 100 μl of suspension buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0) with lysozyme (50 mg/ml) (Sigma, St. Louis, MO), and the homogenates were incubated at 37°C for 1 h. The bacterial DNA extraction procedure was adapted from a standard cetyltrimethylammonium bromide (CTAB) DNA purification method (38). DNA samples were stored at −20°C until used as the templates for PCR.

Identification of bacterial isolates. The bacterial DNA of each isolate was partially amplified with 16S rRNA gene target primers 968f (5′-AACGCGAAGAACCTTA-3′) and 1401r (5′-CGGTGTGTACAAGACCC-3′) (39). When necessary, complete 16S rRNA amplification and sequencing were performed with the primers 27δ-f (5′-AGAGTTTGATCCTGGCTCAG-3′) (40) and 1401r. Amplification reactions were carried out in a 50-μl volume containing 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 1 μM each primer, 200 μM deoxynucleoside triphosphates, and 2.5 U of *Taq* polymerase (Ecotaq; Ecogen, Spain). The amplification program was 1 cycle at 94°C for 5 min; 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and finally, 1 cycle at 72°C for 7 min. The amplification products were subjected to gel electrophoresis in 1% agarose gels, purified using GFX PCR DNA and a Gel Band DNA purification kit (GE Healthcare, Buckinghamshire, United Kingdom), and sequenced in an ABI Prism-3130XL genetic analyzer (Applied Biosystems, CA). Search analy-

ses to determine the closest relatives of the partial 16S rRNA gene sequences retrieved were conducted in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm, and sequences with more than 97% similarity were considered to be of the same species.

Data and statistical analyses. The Renyi diversity index was used to explore differences in the mucosa-associated bacteria among active and nonactive CD patients and control children. This index provides three further diversity index values: species richness (S), the Shannon diversity index (H'), and the Simpson dominance index (1-D), which were determined using Paleontological Statistics (PAST) software (41).

Differences in the relative abundance of the duodenal mucosa-associated bacteria (estimated as isolates belonging to a specific taxon related to all isolates recovered from samples from each child group) were established by applying chi-square tests and, when appropriate, the two-tailed Fisher's exact test. Analyses were carried out with Statgraphics software (Manugistics, Rockville, MD), and statistical differences were established at a P value of less than 0.05.

RESULTS

Subjects. The clinical characteristics of the groups of children included in the study are shown in Table 1. No statistically significant differences in the gender ratio representation in the study were detected. Patients with active CD on a normal gluten-containing diet showed clinical symptoms of the disease, positive CD serology markers (antigliadin antibodies and antitransglutaminase antibodies), and signs of severe enteropathy by duodenal biopsy examination classified as type 3 according to the Marsh classification of CD (M3) (1). Patients with nonactive CD who had been on a gluten-free diet for at least 2 years showed negative CD serology markers and normal mucosa or infiltrative lesions classified as type 0-1 according to the Marsh classification of CD. The study included 32 biopsy specimens from children with active CD (mean age, 5.1 years), 17 biopsy specimens from children with nonactive CD (mean age, 5.9 years), and finally, 8 biopsy specimens from children without known gluten intolerance (mean age, 6.9 years) who were included in the control group for comparative purposes.

Influence of culture media on bacterial taxa recovered. Four different culture media, including PCA, Wilkins-Chalgren agar, BH, and YFCA, were used for isolating bacteria from biopsy specimens from the CD patients and controls. The same proportion of biopsy specimens (50%) from patients with active CD, patients with nonactive CD, and control children were cultured in each medium, and therefore, the suitability of each medium to recover duodenal bacteria could be analyzed independently of subject health status. A total of 29 CFU was recovered in PCA (1.0 ± 1.4 CFU/10 mg of biopsy specimen, on average), 52 CFU was recovered in Wilkins-Chalgren agar (1.9 ± 1.8 CFU/10 mg of biopsy specimen), 141 CFU was recovered in BH (4.4 ± 6.9 CFU/10 mg of biopsy specimen), and 81 CFU was recovered in YFCA (2.6 ± 3.8 CFU/10 mg of biopsy specimen).

The abundance of cultivable bacterial species associated with the mucosa of the subjects included in this study is shown in Table 2. Some differences in the bacterial phyla, genera, and species isolated from the different culture media were detected.

When the isolates were classified into different phyla, differences were found for *Proteobacteria*, whose members were more frequently recovered in PCA, followed by YFCA, Wilkins-Chalgren agar, and BH; significant differences were detected between PCA and BH ($P < 0.01$) and between YCFA and BH ($P = 0.02$). Differences among the culture media were not detected for isolates belonging to the phyla *Actinobacteria* and *Firmicutes*.

In relation to families and species, members of the family *Staphylococcaceae* were more frequently isolated in PCA and Wilkins-Chalgren agar than in BH ($P < 0.01$) and YCFA ($P = 0.01$ and $P = 0.03$, respectively). Of the staphylococcal species, *Staphylococcus epidermidis* was more frequently isolated in PCA and Wilkins-Chalgren agar than in YCFA ($P = 0.01$), and *Staphylococcus pasteuri* was isolated significantly more frequently in PCA than in BH ($P < 0.01$).

Members of the family *Streptococcaceae* were more frequently isolated in BH and YFCA than in PCA ($P < 01$ and $P = 0.02$, respectively) and in BH than in Wilkins-Chalgren agar ($P < 0.01$). Within this family, the *Streptococcus anginosus* group was significantly more abundant in biopsy samples cultured in BH than in those cultured in Wilkins-Chalgren agar ($P = 0.02$).

Finally, members of the *Clostridiaceae* family were more frequently isolated in Wilkins-Chalgren agar than in BH ($P = 0.02$), and those of the *Enterobacteriaceae* family were more frequently isolated in PCA and YFCA than in BH ($P = 0.03$ and $P = 0.02$, respectively).

The species richness (S), Shannon species diversity (H'), and Simpson species dominance (1-D) indexes were calculated for PCA ($S = 11$, $H' = 2.18$, and 1-D = 0.86), Wilkins-Chalgren agar ($S = 15$, $H' = 2.35$, and 1-D = 0.87), BH (S = 27, $H' = 2.69$, and 1-D = 0.91), and YFCA ($S = 22$, $H' = 2.71$, and 1-D = 0.91), in order to apply the Renyi index. Renyi diversity profiles showed that the use of PCA and Wilkins-Chalgren agar led to the recovery of bacteria with lower species diversity than the use of either BH or YFCA. Renyi diversity profiles also showed that the curves for PCA and Wilkins-Chalgren agar intersected each other, and the same was observed for the curves for BH and YFCA; therefore, the diversity of these pairs could not be compared (data not shown).

Duodenal mucosa-associated bacteria in CD patients and controls. The proportion of biopsy specimens inoculated in each culture medium was similar (~25%) for each group of individuals (patients with active CD, patients with nonactive CD, and controls), and therefore, the total number of bacteria recovered in the different media was considered to represent the differences among the study groups, regardless of the different culture media used. A total of 146 isolates were recovered from biopsy specimens from active CD patients (4.6 ± 4.8 CFU/10 mg of sample, on average), 84 were recovered from biopsy specimens from nonactive CD patients (5.1 ± 4.1 CFU/10 mg of sample), and 71 were recovered from biopsy specimens from the control group (8.9 ± 11.7 CFU/10 mg of sample).

The relative abundance of cultivable bacteria associated with the duodenal mucosa of the different child groups and the differences in abundance between groups are shown in Table 3. In relation to the phyla, members of the phylum *Proteobacteria* were more abundant in biopsy samples from patients with active CD than in those from controls ($P < 0.01$) and nonactive CD patients ($P < 0.01$), while the relative abundance of members of the *Firmicutes* in biopsy samples from patients with active CD was less than that in samples from controls ($P < 0.01$) and nonactive CD patients. Members of the phylum *Actinobacteria* were also more abundant in biopsy samples from patients with active CD than in samples from nonactive CD patients ($P = 0.02$).

In relation to families, members of the *Enterobacteriaceae* were more abundant in patients with active CD than in nonactive CD

TABLE 2 Cultivable bacterial taxa from active and nonactive CD patients and control subjects isolated in PCA, Wilkins-Chalgren, BH, and YCFA

Closest relative	No. (%) of clones[a]			
	PCA ($n = 29$)	Wilkins-Chalgren agar ($n = 50$)	BH ($n = 142$)	YCFA ($n = 81$)
Phylum *Actinobacteria*	2 (6.9)	1 (2.0)	12 (8.5)	6 (7.4)
Actinomycetaceae	0	0	7 (4.9)	4 (4.9)
Actinomyces odontolyticus	0	0	7 (4.9)	4 (4.9)
Corynebacteriaceae	0	0	1 (0.7)	0
Corynebacterium accolens	0	0	1 (0.7)	0
Micrococcaceae	2 (6.9)	1 (2.0)	3 (2.1)	1 (1.2)
Kocuria kristinae	2 (6.9)	1 (2.0)	1 (0.7)	1 (1.2)
Rothia mucilaginosa	0	0	2 (1.4)	0
Propionibacteriaceae	0	0	1 (0.7)	1 (1.2)
Propionibacterium acnes	0	0	1 (0.7)	1 (1.2)
Phylum *Firmicutes*	20 (69.0)	43 (86.0)	124 (87.3)	65 (79.0)
Carnobacteriaceae	0	1 (2.0)	5 (3.5)	1 (1.2)
Granulicatella adiacens	0	1 (2.0)	5 (3.5)	1 (1.2)
Clostridiaceae	0^{AB}	$5\ (10.2)^{A}$	$2\ (1.4)^{B}$	$2\ (2.5)^{AB}$
Clostridium bifermentans	0	3 (6.0)	1 (0.7)	0
Clostridium butyricum	0	2 (4.0)	0	0
Clostridium perfringens	0	0	1 (0.7)	2 (2.5)
Enterococcaceae	1 (3.5)	0	1 (0.7)	0
Enterococcus faecalis	1 (3.5)	0	1 (0.7)	0
Lactobacillaceae	0	0	0	2 (2.5)
Lactobacillus fermentum	0	0	0	2 (2.5)
Staphylococcaceae	$10\ (34.5)^{A}$	$14\ (28.0)^{A}$	$14\ (9.9)^{B}$	$9\ (11.1)^{B}$
Staphylococcus aureus	0	0	0	3 (3.7)
Staphylococcus epidermidis	$6\ (20.7)^{A}$	$9\ (18.0)^{A}$	$13\ (9.2)^{AB}$	$3\ (3.7)^{B}$
Staphylococcus hominis	0	2 (4.0)	0	1 (1.2)
Staphylococcus pasteuri	$4\ (13.8)^{A}$	$3\ (6.0)^{AB}$	$1\ (0.7)^{B}$	$2\ (2.5)^{AB}$
Streptococcaceae	$9\ (31.0)^{A}$	$22\ (44.0)^{A}$	$90\ (63.4)^{B}$	$40\ (49.4)^{AB}$
Streptococcus anginosus group	0^{AB}	0^{A}	$17\ (12.0)^{B}$	$8\ (9.9)^{AB}$
Streptococcus australis	0	0	4 (2.8)	0
Streptococcus bovis group	0	0	2 (1.4)	1 (1.2)
Streptococcus gallolyticus	0	0	1 (0.7)	0
Streptococcus mitis group	6 (20.7)	8 (16.0)	21 (14.8)	8 (9.9)
Streptococcus mutans group	0	0	1 (0.7)	4 (4.9)
Streptococcus pneumoniae	0	0	11 (7.8)	3 (3.7)
Streptococcus salivarius group	1 (3.5)	11 (22.0)	23 (16.2)	18 (22.22)
Streptococcus sanguinis group	2 (6.9)	3 (6.0)	14 (9.9)	6 (7.4)
Streptococcus suis	0	0	2 (1.4)	0
Veillonellaceae	0	0	3 (2.1)	2 (2.5)
Veillonella atypical	0	0	0	1 (1.2)
Veillonella dispar	0	0	1 (0.7)	0
Veillonella parvula	0	0	2 (1.4)	1 (1.2)
Unclassified *Bacillales*	0	1 (2.0)	3 (2.1)	0
Gemella haemolysans	0	1 (2.0)	2 (1.4)	0
Gemella sanguinis	0	0	1 (0.7)	0
Phylum *Proteobacteria*	$7\ (24.14)^{A}$	$6\ (12.0)^{AB}$	$5\ (3.5)^{B}$	$10\ (12.6)^{A}$
Burkholderiaceae	1 (3.5)	0	0	0
Burkholderia cepacia	1 (3.5)	0	0	0
Neisseriaceae	1 (3.5)	0	0	0
Neisseria flavescens	1 (3.5)	0	0	0
Enterobacteriaceae	$5\ (17.2)^{A}$	$5\ (10.0)^{AB}$	$5\ (3.5)^{B}$	$10\ (12.3)^{A}$
Enterobacter cloacae	2 (6.9)	2 (4.0)	0	2 (2.5)
Escherichia coli	0	0	3 (2.1)	3 (3.7)
Klebsiella oxytoca	3 (10.3)	3 (6.0)	3 (2.1)	6 (7.4)
Pseudomonadaceae	0	1 (2.0)	0	0
Pseudomonas stutzeri	0	1 (2.0)	0	0

[a] Data are expressed as absolute numbers of isolated clones belonging to one specific taxonomic group (phylum, family, or species) and, in parentheses, the percentage related to the total number of isolates from each culture medium (PCA, Wilkins-Chalgren agar, BH, and YCFA). Different letters within a row denote statistically significant differences at $P < 0.05$, estimated by using a two-by-two chi-square test and, when appropriate, Fisher's exact test.

TABLE 3 Relative abundance of cultivable bacterial taxa from biopsy specimens from patients with active and nonactive CD and control subjects isolated in PCA, Wilkins-Chalgren agar, BH, or YCFA

Closest relative	No. (%) of clones[a]		
	Active CD ($n = 146$)	Nonactive CD (n = 85)	Control ($n = 71$)
Phylum *Actinobacteria*	15 (10.6)^{A}	2 (2.4)^{B}	4 (5.6)^{AB}
Actinomycetaceae	9 (5.8)^{A}	0^{B}	2 (2.8)^{AB}
Actinomyces odontolyticus	9 (5.8)^{A}	0^{B}	2 (2.8)^{AB}
Corynebacteriaceae	1 (0.7)	0	0
Corynebacterium accolens	1 (0.7)	0	0
Micrococcaceae	5 (3.5)	0	2 (2.8)
Kocuria kristinae	3 (2.0)	0	2 (2.8)
Rothia mucilaginosa	2 (1.3)	0	0
Propionibacteriaceae	0	2 (2.4)	0
Propionibacterium acnes	0	2 (2.4)	0
Phylum *Firmicutes*	104 (73.2)^{A}	76 (91.6)^{B}	66 (93.0)^{B}
Carnobacteriaceae	2 (1.3)	4 (4.8)	1 (1.4)
Granulicatella adiacens	2 (1.3)	4 (4.8)	1 (1.4)
Clostridiaceae	4 (2.8)	2 (2.4)	3 (4.2)
Clostridium bifermentans	1 (0.7)	0	3 (4.2)
Clostridium butyricum	0	2 (2.4)	0
Clostridium perfringens	3 (2.0)	0	0
Enterococcaceae	2 (1.4)	0	0
Enterococcus faecalis	2 (1.4)	0	0
Lactobacillaceae	0	0	2 (2.8)
Lactobacillus fermentum	0	0	2 (2.8)
Staphylococcaceae	32 (22.5)^{A}	8 (9.6)^{B}	2 (2.8)^{B}
Staphylococcus aureus	3 (2.0)	0	0
Staphylococcus epidermidis	28 (18.2)^{A}	6 (7.1)^{B}	2 (2.8)^{B}
Staphylococcus hominis	1 (7.8)	2 (2.4)	0
Staphylococcus pasteuri	12 (6.9)^{A}	1 (1.2)^{B}	0^{B}
Streptococcaceae	59 (41.6)^{A}	58 (69.9)^{B}	58 (81.7)^{B}
Streptococcus anginosus group	0^{A}	0^{A}	25 (35.2)^{B}
Streptococcus australis	4 (2.6)	0	0
Streptococcus bovis group	0	3 (3.6)	0
Streptococcus gallolyticus	0	1 (1.2)	0
Streptococcus mitis group	14 (9.1)^{A}	21 (25.0)^{B}	8 (11.3)^{A}
Streptococcus mutans group	0^{A}	0^{A}	5 (7.0)^{B}
Streptococcus pneumoniae	7 (4.6)	6 (7.1)	1 (1.4)
Streptococcus salivarius group	25 (16.2)	19 (22.2)	9 (12.7)
Streptococcus sanguinis group	9 (5.8)	6 (7.1)	10 (14.1)
Streptococcus suis	0	2 (2.4)	0
Veillonellaceae	3 (2.1)	2 (2.4)	0
Veillonella atypica	1 (0.7)	0	0
Veillonella dispar	1 (0.7)	0	0
Veillonella parvula	1 (0.7)	2 (2.4)	0
Unclassified *Bacillales*	2 (1.4)	2 (2.4)	0
Gemella haemolysans	1 (0.7)	2 (2.4)	0
Gemella sanguinis	1 (0.7)	0	0
Phylum *Proteobacteria*	23 (16.2)^{A}	5 (6.0)^{B}	1 (1.4)^{B}
Burkholderiaceae	0	0	1 (1.4)
Burkholderia cepacia	0	0	1 (1.4)
Enterobacteriaceae	22 (15.5)^{A}	4 (4.8)^{B}	0^{B}
Enterobacter cloacae	6 (3.9)	0	0
Escherichia coli	5 (3.3)	0	0
Klebsiella oxytoca	11 (7.1)^{A}	4 (4.8)^{AB}	0^{B}
Neisseriaceae	0	1 (1.2)	0
Neisseria flavescens	0	1 (1.2)	0
Pseudomonadaceae	1 (0.7)	0	0
Pseudomonas stutzeri	1 (0.7)	0	0

[a] Data are expressed as the absolute numbers of isolated clones belonging to one specific taxonomic group (phylum, family, or species) and, in parentheses, the percentage related to the total number of isolates from each group of children (patients with active CD, patients with nonactive CD, and controls). Different letters within a row denote statistically significant differences at $P < 0.05$, estimated by using a two-by-two chi-square test and, when appropriate, Fisher's exact test.

patients and control children ($P = 0.03$ and $P < 0.01$, respectively). In particular, *Klebsiella oxytoca* isolates were more abundant in patients with active CD than in control children ($P = 0.02$). In addition, members of the family *Staphylococcaceae* were more abundant in patients with active CD than in patients with nonactive CD and control individuals ($P = 0.02$ and $P < 0.01$, respectively). In particular, *S. epidermidis* and *S. pasteuri* isolates were more abundant in patients with active CD than in patients with nonactive CD ($P = 0.03$ and $P = 0.04$, respectively) and in control children ($P < 0.01$ and $P = 0.01$, respectively). Furthermore, members of the family *Streptococcaceae* were less abundant in patients with active CD than in patients with nonactive CD and in control children ($P < 0.01$). Statistically significant differences in the abundance of some particular *Streptococcus* groups were also detected; thus, the *S. anginosus* and *Streptococcus mutans* groups were more abundant in control individuals than in patients with active CD ($P < 0.01$ and $P = 0.02$, respectively) and nonactive CD ($P < 0.01$ and $P = 0.02$, respectively), whereas members of the *Streptococcus mitis* group were more abundant in patients with nonactive CD patients than those with active CD ($P = 0.01$). In relation to the family *Actinomycetaceae*, the isolates of the only species of that family identified (*Actinomyces odontolyticus*) were more abundant in patients with active CD than in those with nonactive CD patients ($P = 0.04$).

The species richness (S), Shannon species diversity (H'), and Simpson species dominance (1-D) indexes were different between patients with active CD ($S = 27$, $H' = 2.73$, and 1-D = 0.91), patients with nonactive CD ($S = 17$, $H' = 2.35$, and 1-D = 0.82), and controls ($S = 13$, $H' = 2.06$, and 1-D = 0.82), indicating different species diversity between the child groups studied. Renyi diversity profiles showed that active CD patients had the highest biodiversity of duodenal cultivable bacteria, followed by nonactive CD patients and controls (data not shown).

DISCUSSION

The study reported herein demonstrates that the microbiota associated with the duodenal mucosa of CD patients has a characteristic deviation from the normal microbiota structure, which may characterize the disease. The alterations reported in the present study are partly consistent with those previously detected by molecular techniques using specific primers or probes (19, 20). Thus, our results support the hypothesis that normal components of the microbiota are excluded and replaced by others that could act as pathobionts in this specific disease environment. Although such associations do not demonstrate causality between the altered microbial groups and the disease, they provide a rationale for further studies on the possible pathogenic modes of action of such alterations and specific bacteria in CD.

To obtain bacterial isolates that are representative of those inhabiting the duodenal mucosa in both numbers and diversity, four different culture media previously described in the literature (34–37) were used. In general, the greatest species diversity and quantitative recovery of mucosa-associated bacteria were obtained using the BH and YFCA culture media. These differences could be linked to the high nutritional requirements of duodenal bacteria, which are better met by the compositions of these media; incubation conditions may also have been more appropriate, as they were more anaerobic than those used for PCA and Wilkins-Chalgren agar. The diverse morphology of the small intestine favors a precise spatial relationship for strains within particular intestinal nutritional and microaerobic environments (42), and therefore, it is rather complicated to completely reproduce the *in vivo* environmental conditions. Also, even though the duodenum environment is not strictly anaerobic, the possibility that some anaerobic bacteria were lost due to oxygen exposure during sample manipulation cannot be disregarded.

We also analyzed whether some of the media used proved better at isolation of specific bacteria. In this regard, PCA and Wilkins-Chalgren agar seemed to favor the growth and isolation of members of the family *Staphylococcaceae* but hindered the growth of members of the family *Streptococcaceae*. BH medium favored the growth of members of the family *Streptococcaceae* but hampered that of members of the family *Enterobacteriaceae*. Wilkins-Chalgren agar also favored the recovery of members of the family *Clostridiaceae* compared to the other media. We confirm that none of the media or incubation conditions tested were suitable for the recovery of all viable bacteria detected in the samples analyzed when used alone, and therefore, various media must be used to improve the recovery of bacteria that are representative of the live bacteria inhabiting the duodenum.

We observed an increased diversity of the cultivable mucosa-associated bacteria recovered from CD patients compared to the diversity of bacteria recovered from the controls, and these differences were restored after adherence to a gluten-free diet. In concordance with this finding, denaturing or temperature gradient gel electrophoresis (DGGE and TGGE, respectively) analysis of duodenal samples showed a higher bacterial diversity associated with the small intestinal microbiota of CD patients (13, 18). However, several recent molecular studies (43–46) have reported that reduced mucosal bacterial diversity is associated with inflammatory bowel disease (IBD), although the conditions and techniques used were not comparable to those used in the present study and the section of the intestinal tract studied was not the same.

Considering the isolates from all subject groups under study, our results show that the most abundant were those belonging to the phylum *Firmicutes*, followed by those of the phyla *Proteobacteria* and *Actinobacteria*. This is in concordance with the findings of a previous culture-independent study, where the same three phyla dominated the proximal small intestine of CD patients, followed by other phyla, such as *Bacteroidetes* or *Fusobacteria* (47). Although our previous culture-independent studies also detected increased numbers of duodenal and fecal *Bacteroides* spp. in CD patients compared with controls (19, 20, 48), this bacterial group was not isolated with the culture conditions applied, probably due to exposure to oxygen during the process of homogenization of biopsy specimens and the use of nonselective media for *Bacteroides*, which could have helped to limit the growth of less anaerobic and less nutritionally demanding bacteria. Culture-independent studies indicate that the members of the normal human gut microbiota mainly belong to two phyla, *Firmicutes* and *Bacteroidetes*, with a smaller number of bacteria belonging to the *Proteobacteria* and *Actinobacteria*, although these conclusions are mainly based on analyses of the fecal microbiota composition (45, 49). Previous data also suggest that only 12% of the total species richness was detected by applying both molecular and cultivation-based approaches (50). Remarkably, with both approaches, *Firmicutes* represented the most abundant group, *Proteobacteria* were relatively poorly detected by molecular approaches, and *Bacteroidetes* were less abundant when they were assessed with cultivation-based approaches than with molecular techniques (49–51).

In relation to CD, differences in phylum representation were identified, and in particular, isolates belonging to the *Proteobacteria* were more abundant in active CD patients than in nonactive CD patients and controls. In this context, other studies have also associated an increase in the *Proteobacteria* and, in particular, an increase in adherent-invasive *Escherichia coli*, *Campylobacter concisus*, and enterohepatic *Helicobacter* with IBD (52).

In addition, active and nonactive CD seemed to be associated with a decreased abundance of members of the family *Streptococcaceae*, specifically, the *S. anginosus* and *S. mutans* groups. The active phase of the disease was also associated with increased proportions of *Enterobacteriaceae* and *Staphylococcaceae* and, in particular, the species *Klebsiella oxytoca*, *S. epidermidis*, and *S. pasteuri*. In concordance with these observations, recent culture-independent studies indicate that the duodenal and fecal microbiotas of CD patients are characterized by higher numbers or proportions of *Escherichia coli* and *Staphylococcus* species (19, 20). Furthermore, previous studies using cultured-dependent techniques have shown increased levels of *S. epidermidis* (16) in feces from both active and nonactive CD patients in comparison with healthy controls and a lower prevalence of salivary *S. mutans* in association with CD (53). It seems that dominant genera in the normal microbiota of healthy individuals, which may act as symbionts, like *Streptococcus* spp., are replaced in the CD patient microbiota by potential pathobionts, like *Staphylococcus* spp. (*S. epidermidis*) and enterobacteria, which could contribute to breaking down the normal dynamics and balance of the ecosystem.

To our knowledge, this is the first time that cultivable mucosa-associated bacteria of patients with active and nonactive CD have been studied, because previous studies were focused on the characterization of CD microbiota using molecular tools, such as DGGE and TGGE (13, 15, 54), fluorescence *in situ* hybridization (FISH) (20), or real-time PCR (19). Culture-dependent studies are intrinsically biased by the culture media used, the impact of potential oxygen exposure, and the inability to detect viable but noncultivable bacteria present in biological samples; notwithstanding these limitations, the results obtained in the present study are coherent with those of previous studies based on molecular techniques, which overcome these limitations. Therefore, the use of culture-dependent techniques has allowed the characterization of the active fraction of the mucosal microbiota of CD patients and will facilitate future investigation into the possible pathogenic role that isolated bacteria play in the development of CD.

Conclusions. This study demonstrates that the duodenal-mucosal microbiota of CD patients presents alterations in the diversity and abundance of different cultivable bacterial taxa, which could be a consequence of the pathogenesis of CD, which involves massive destruction of the small bowel mucosa and the consequent release of intracellular contents and serum into the gut. In the active phase of the disease, the mucosa-associated microbiota was characterized by a higher abundance of members of the phylum *Proteobacteria* and the families *Enterobacteriaceae* and *Staphylococcaceae*, apparently excluding members of the phylum *Firmicutes* and the family *Streptococcaceae*, which are normal inhabitants of the healthy small intestine. These alterations are attenuated after long-term adherence to a gluten-free diet, but the microbiota is not completely restored; in particular, a reduced abundance of specific species of *Streptococcus* (*S. anginosus* and *S. mutans*) also characterizes the microbiota of CD patients with active and nonactive disease. These findings also suggest their potential use as hallmarks of CD, regardless of inflammatory status.

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