Fecal Microbiota Composition Differs Between Children With β-Cell Autoimmunity and Those Without

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The role of the intestinal microbiota as a regulator of autoimmune diabetes in animal models is well-established, but data on human type 1 diabetes are tentative and based on studies including only a few study subjects. To exclude secondary effects of diabetes and HLA risk genotype on gut microbiota, we compared the intestinal microbiota composition in children with at least two diabetes-associated autoantibodies (n = 18) with autoantibody-negative children matched for age, sex, early feeding history, and HLA risk genotype using pyrosequencing. Principal component analysis indicated that a low abundance of lactate-producing and butyrate-producing species was associated with β-cell autoimmunity. In addition, a dearth of the two most dominant *Bifidobacterium* species, *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum*, and an increased abundance of the *Bacteroides* genus were observed in the children with β-cell autoimmunity. We did not find increased fecal calprotectin or IgA as marker of inflammation in children with β-cell autoimmunity. Functional studies related to the observed alterations in the gut microbiome are warranted because the low abundance of bifidobacteria and butyrate-producing species could adversely affect the intestinal epithelial barrier function and inflammation, whereas the apparent importance of the *Bacteroides* genus in development of type 1 diabetes is insufficiently understood. ***Diabetes*** **62:1238–1244, 2013**

Type 1 diabetes (T1D) is caused by the destruction of the pancreatic β-cells in genetically susceptible individuals. The disease is considered to be immune mediated, and the appearance of circulating autoantibodies against β-cells is seen years before the diagnosis along with a significant reduction in β-cell mass (1,2). Environmental factors associated with the activation of the gut immune system, such as early exposure to dietary antigens (cow's milk and gluten), have been associated with the induction of this process (3–5). The role of the gut immune system in the pathogenesis of T1D has been supported by studies showing an immunological link between the pancreas and the gastrointestinal tract. It has been demonstrated that oral antigens are capable of activating antigen-specific T cells in pancreatic lymph nodes (6) and that the interaction between endothelium and T cells is controlled by shared homing receptors in inflamed islets and in the gut (7). The development of autoimmune diabetes in animal models is regulated by factors affecting the function of the gut immune system, such as dietary factors and microbial stimuli, which further affect the intestinal mucosal barrier and immune responsiveness (8). The effects of intestinal microbes may not be restricted to barrier mechanisms, but gut microbiota seems to play a key role in the regulation of the T-cell populations in the gut, including regulatory T cells, T helper 1, and T helper 17 cells (9).

Several animal studies indicate that alterations in the intestinal microbiota are associated with the development of autoimmune diabetes. Nonobese diabetic mice lacking MyD88, an essential signal transducer in Toll-like receptor signaling, did not have development of diabetes (10), which emphasizes the role of intestinal microbiota as a regulator of autoimmune diabetes. There are differences in the gut microbiota between bio-breeding (BB) diabetes-prone (DP), and diabetes-resistant rats before the diagnosis of diabetes. Antibiotics also can prevent autoimmune diabetes in BB-DP rats (11). Furthermore, it has been reported that stool from BB diabetes-resistant rats contained more probiotic-like bacteria, whereas *Bacteroides*, *Eubacterium*, and *Ruminococcus* were more prevalent in BB-DP rats (12). *Lactobacillus johnsonii* prevented diabetes when administered to BB-DP rats (13). There are only a few studies of the intestinal microbiota in relation to T1D in humans, but the results of a follow-up study including four children with development of T1D suggested that the *Bacteroidetes*-to-*Firmicutes* ratio increased over time in those children with eventual progression to clinical T1D, whereas it decreased in children who remained nondiabetic (14).

The aim of this study was to compare the composition of the gut microbiota between children with β-cell autoimmunity and autoantibody-negative children matched for age, sex, HLA risk genotype, and early feeding history using pyrosequencing as the method of choice.

RESEARCH DESIGN AND METHODS

The current study included 18 children with HLA-conferred susceptibility to T1D who had development of signs of progressive β-cell autoimmunity, i.e., tested positive for at least two diabetes-associated autoantibodies (cases). Eighteen control children were matched for age, sex, and HLA-DQB1 genotype, as well as for the time of exposure to and the type of infant formula. The characteristics of the children recruited to the gut microbiota study are shown

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in Table 1 and are shown in detail in Supplementary Table 1. The study subjects were recruited from the study population of two intervention trials performed in Finland: the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) pilot (n = 20) or the Finnish Dietary Intervention Trial for Prevention of Type 1 Diabetes (FINDIA) pilot study (n = 16) (3,15). In the TRIGR pilot study, autoantibody positivity was monitored until the age of 10 years and in the ongoing FINDIA pilot study and follow-up time for autoantibodies varied from 3 to 6 years. Eighteen of 26 children who had development of at least two autoantibodies in these intervention studies but who had not yet had progression to overt T1D participated in the current study. The fecal samples from the study subjects recruited from the FINDIA and TRIGR studies were similarly collected between March 2009 and February 2010. Fecal samples from children were collected using stool collection vials and immediately stored in home freezers (−20°C). Families delivered the frozen sample to the study center, and the sample was stored at −80°C until processing. The fecal samples were collected at a point in time when the study subjects did not have gastroenteritis and had not received any antibiotic treatment during the past 3 months. Four children had developed T1D after the fecal samples were collected. The control children remained negative for T1D and for all four autoantibodies analyzed. The study was approved by the ethics committees of the participating hospitals and the families gave their written informed consent.

In the TRIGR pilot study, infants with a first-degree relative affected by T1D were randomized to receive either a regular cow's milk formula (Enfamil; Mead Johnson, Evansville, IN) or an extensively hydrolyzed casein-based test formula (Nutramigen; Mead Johnson) until the age of 6–8 months. In the FINDIA study, infants were randomized to receive a standard cow's milk–based formula (Tutteli; Valio, Helsinki, Finland), a whey-based hydrolyzed formula (Peptidi-Tutteli), or a whey-based FINDIA formula from which bovine insulin was removed. In both studies exclusive breastfeeding was encouraged.

Autoantibody assays. Insulin autoantibodies, autoantibodies against the 65-kDa isoform of glutamic acid decarboxylase, and autoantibodies against the protein tyrosinase phosphatase–related IA-2 molecule (IA-2A) were measured by specific radiobinding assays, and islet cell antibodies were measured by a standard immunofluorescence assay as described previously (16). Six out of 18 cases tested positive for four autoantibodies, seven were positive for three autoantibodies, and five were positive for two autoantibodies (Table 1).

TABLE 1
Characteristics of the study subjects

Characteristics (n)	Case children (n = 18)	Control children (n = 18)
Female/male	7/11	7/11
T1D in first-degree relative	10	10
Age (years)		
TRIGR pilot study	13.3 (11.7−14.2)	12.8 (11.9−13.6)
FINDIA pilot study	5.1 (4.9−6.0)	5.0 (3.9–7.0)
HLA-DQB1 genotype		
*02/03:02	7	7
*03:02/x	8	8
*02(DQA1*05)/y	2	2
*02(DQA1*02:01)	1	1
Study formula		
CM	10	10
HC	4	4
HW	3	3
FINDIA	1	1
Duration of exclusive BF (mo)	2.9 (0−5.5)	4.0 (0.1−6.0)
Total duration of BF (mo)	8.1 (1.6–16.5)	10.5 (5.1–19.3), P = 0.03
Cesarean delivery	4	3

Cases are children positive for at least two diabetes-associated autoantibodies and control subjects are negative for β-cell autoantibodies. The study subjects were participants in the TRIGR and FINDIA pilot studies. Data are number or medians (with range). x ≠ *03:01 or *06:02, y ≠ *03:01, *06:02, or *06:03. BF, breastfeeding; CM, cow's milk formula; FINDIA, insulin-free whey-based formula; HC, hydrolyzed casein-based formula; HW, hydrolyzed whey-based formula.

HLA genotyping. HLA genotyping was performed according to the screening protocol in the TRIGR and FINDIA studies. The initial HLA-DQB1 typing for risk-associated (DQB1*02, DQB1*03:02) and protective (DQB1*03:01, DQB1*06:02, and DQB1*06:03) alleles was complemented with DQA1 typing for DQA1*02:01 and DQA1*05 alleles in those with DQB1*02 without protective alleles or the major risk allele DQB1*03:02. This two-step screening technique is based on the hybridization of PCR products with lanthanide-labeled probes detected by time-resolved fluorometry as described previously (17,18).

DNA extraction. Total DNA was extracted from 0.25 g fecal sample using the repeated bead beating method described in detail by Yu et al. (19), with a number of modifications. In brief, four 3-mm glass beads were added during the homogenization step, whereas 0.5-mm glass beads were not used at all. Bead beating was performed using a Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) at 5.5 ms^{-1} in three rounds of 1 min each with 30-s pauses at room temperature in between. The incubation temperature after the bead beating was increased from 70°C to 95°C. Importantly, protein precipitation with 260 μL ammonium acetate was performed twice. Elution of DNA from the purification columns was performed twice. Columns from the QiaAmp Stool kit were replaced by those from the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Pyrosequencing. From each sample, the 16S rRNA genes were amplified using a primer set corresponding to primers 27F-degS (20) and 534-R (21). These PCR primers target the V1, V2, and V3 hypervariable regions of the 16 S rRNA; 27-degS was chosen in particular because it appears to provide a more complete assessment of actinobacterial abundance (20). Pyrosequencing was performed using a Roche FLX Genome Sequencer at DNAvision (Liège, Belgium) using their standard protocol (22).

Sequencing quality control. Pyrosequencing produced a total of 461,874 reads of 16S rDNA. Sequences were assigned to samples according to sample-specific barcodes. Using the Galaxy Tools web site (23), SFF files from the 454 Genome Sequencer FLX were converted into FASTA files and FASTA quality files. FASTA formatted files contained an average (± SD) of 12,830 ± 4,888 reads per sample. The RDP pyrosequencing pipeline (24) (RDP 10 database, update 17) was subsequently used to check the FASTA sequence files for the same criteria as described by De Filippo et al. (22) and to check that the average experimental quality score was at least 20. After this quality check, the FASTA files contained an average (± SD) of 8,024 ± 3,136 high-quality reads.

Classification. Taxonomy (phylum, family, and genus level) was assigned using RDP classifier 2.01 (25). Richness and diversity analyses were performed as described by De Filippo et al. (22). Identification to the species level was performed using ARB software (26). For this, SSU reference database (SSURef_106_ SILVA_19_03_11) was downloaded from the SILVA web site (27). From this database, only sequences of cultured and identified isolates were used. From these sequences, a "PT-server" database was built, which was subsequently used to find the closest match for each of our high-quality sequences imported from the FASTA files. For this, the "search next relatives of listed species in PT-server" function was applied with the following settings: oligo length, 12; mismatches, 0; match score, relative; and minimum score, 10. The average (± SD) match score was 75.2 ± 18.5. Sequences that were identified as being from different strains but belonging to the same species were grouped together. Species that represented >0.005% of all sequences were taken along for statistical analysis, together representing 99.2 ± 0.35% of all high-quality FASTA reads per sample.

Assay of IgA and calprotectin in stool samples. A beaker in the bottom cap of the extraction device was filled with thawed and homogenized stool sample, avoiding seeds and grains (~100 mg). The extraction tube was filled with 4.9 mL extraction buffer and vortexed for 30 s. Mixing was continued in a shaker at 1,000 rpm for 3 min. The particles were allowed to settle before 10-min centrifugation at 10,000g at room temperature. Supernatant was collected and stored at −20°C.

IgA values were measured with the modified ELISA method described by Lehtonen et al. (28). Calprotectin levels in stool samples were determined using Calprolab calprotectin ELISA tests (Lot CALP-Pilot3; Calpro AS, Lysaker, Norway); 20 μL extract was mixed with 980 μL of sample dilution buffer.

Statistical analysis. Principal component analysis (PCA) was performed to find clusters of similar groups of samples or species. PCA is an ordination method based on multivariate statistical analysis that maps the samples in different dimensions. All tests were performed with PASW Statistics 18 (SPSS, Chicago, IL). Initial analysis of the samples showed that bacterial populations were most often not normally distributed. Mann-Whitney U and Spearman ρ and χ^2 tests were used. All tests were two-tailed. $P < 0.05$ was considered to indicate statistical significance.

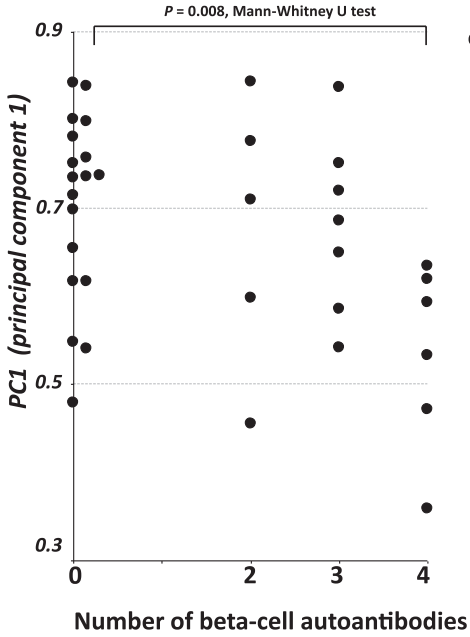
RESULTS

Comparison of sequence diversity at phylum, family, and genus levels. The analysis of the high-quality reads of all samples at the phylum level showed that *Firmicutes* (58.1%), *Actinobacteria* (36.2%), and *Bacteroidetes* (3.4%) were the most dominant phyla. On the family level, the *Bifidobacteriaceae* (32.8%; *Actinobacteria*), the *Lachnospiraceae* (18.4%; *Firmicutes*), and *Ruminococcaceae* (17.1%; *Firmicutes*) were the most common. On the genus level, the *Bifidobacterium genus* was the most frequent (34.2%). The most interesting finding was that the *Bacteroidetes* phylum, the *Bacteroidaceae* family (2.5%), and the *Bacteroides* genus (3.1%) were more common in autoantibody-positive children than in autoantibody-negative peers (4.6 vs. 2.2%, 3.5 vs. 1.5%, and 4.3 vs. 2.0%, respectively; P = 0.035, 0.022, and 0.031, respectively; Mann-Whitney U test).

Species level PCA. PCA analysis of the species level revealed various correlations with β-cell autoimmunity. The first principal component (PC1) (46.0%) correlated positively with a number of important short-chain fatty acid–producing species ($P \leq 0.01$ for all species; Spearman ρ test), such as *Bifidobacterium adolescentis* (11%), *Faecalibacterium prausnitzii* (5.6%), *Clostridium clostridioforme* (2.3%), and *Roseburia faecis* (0.94%), as shown in Fig. 1. This PC1 was inversely related to the number of diabetes-associated autoantibodies in children (Fig. 1; P = 0.018; Spearman ρ test). The children with four autoantibodies had a significantly lower PC1 score, i.e., they had lower numbers of short-chain fatty acid producers than the control children (P = 0.008; Mann-Whitney U test). PC1 score was especially lower in IA-2A–positive children than in children negative for IA-2A (P = 0.004; Mann-Whitney U test).

A remarkable chevron-like distribution of dots in the second PC (PC2) and third PC (PC3) provided another important observation illustrated in Fig. 2. PC2 showed an inverse correlation with the abundance of *B. adolescentis*, but a positive correlation with the abundance of *Bifidobacterium pseudocatenulatum* ($P = 1 \times 10^{-9}$ and $P = 1 \times 10^{-6}$; Spearman ρ test). *B. adolescentis* and *B. pseudocatenulatum* represented the two most commonly identified species (11.0 and 9.1%, respectively). The PC2 score differed significantly between the children from the TRIGR study representing older children and the FINDIA children who were younger (P = 0.001; Mann Whitney U test). *B. adolescentis* was the most common species (15.8%) among the children in the TRIGR pilot study, and their samples were clustered in the left leg of the chevron-like distribution, whereas *B. pseudocatenulatum* was most frequent (15.8%) in the children from the FINDIA study, and their samples were clustered in the right leg. PC3 was inversely related to the abundance of both *B. adolescentis* and *B. pseudocatenulatum* (P = 0.023 and P = 0.002; Spearman ρ test), and it is characterized by the sum of *B. adolescentis* and *B. pseudocatenulatum* ($P = 1 \times 10^{-18}$; Spearman ρ test). Various bacteria were inversely associated with the combined count of these two bifidobacteria, especially the members of the *Clostridium* cluster XI ($P = 6 \times 10^{-4}$).The apex, encompassed by a circle in Fig. 2, can be described as comprising those samples in which the combined abundance of *B. adolescentis* and *B. pseudocatenulatum* is <12%. The children with β-cell autoimmunity were over-represented in the apex when compared with control children (10/18 vs. 4/18; P = 0.040; χ^2 test).

Species-level analysis. The association of single bacterial species with autoantibody positivity is shown in Table 2. *Roseburia faecis* (0.94%) was more abundant in autoantibody-negative than autoantibody-positive children (P = 0.009; Mann-Whitney U test), whereas *Clostridium perfringens* (0.03%) were more abundant in children with β-cell autoimmunity than in those without (P = 0.18; Mann-Whitney



Correlation of bacterial groups with principal component 1 (Spearman's rho test)

Variable	%	P
**Faecalibacterium prausnitzii*	5.6	5×10^{-4} (+)
**Roseburia faecis*	0.94	0.001 (+)
**Gemmiger formicilis*	0.19	9×10^{-5} (+)
**Eubacterium desmolans*	0.14	0.002 (+)
†Bifidobacterium adolescentis	11	3×10^{-4} (+)
†Clostridium clostridioforme	2.3	0.006 (+)
†Clostridium clariflavum	0.44	0.010 (+)
†Clostridium bolteae	0.38	0.001 (+)
‡Ruminococcus albus	0.52	0.011 (+)
‡Ruminococcus callidus	0.25	0.008 (+)

(+) or (-) indicates a positive or negative correlation

* Producers of butyrate

† Producers of acetate and lactate

‡ Degraders of complex polysaccharides

FIG. 1. The association of PC1 with autoantibody positivity was demonstrated as an inverse correlation between PC1 and number of β-cell autoantibodies in the study cohort (P = 0.018; Spearman ρ test) and as a difference in PC1 score between the children positive for four autoantibodies and control children negative for autoantibodies (P = 0.008; Mann-Whitney test). Control children are indicated by being positive for no autoantibodies and cases are positive for two, three, or four autoantibodies (x-axis). Correlations between PC1 and bacterial groups are shown in right panel.

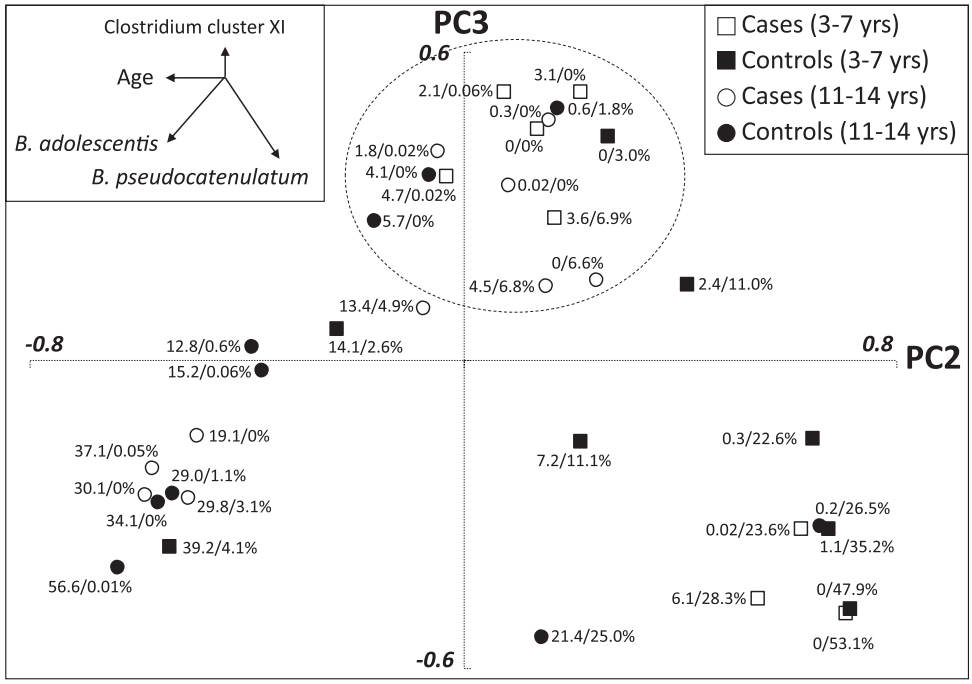


FIG. 2. The distribution of children positive for β-cell autoantibodies (case subjects with open symbols) and autoantibody-negative children (control subjects with filled symbols) according to PC2 (*x*-axis) and PC3 (*y*-axis). PC2 shows an inverse correlation with the abundance of *B. adolescentis* and a positive correlation with *B. pseudocatenulatum*, whereas PC3 shows an inverse correlation with both *B. adolescentis* and *B. pseudocatenulatum*. Indicated percentages indicate the prevalence of *B. adolescentis* and *B. pseudocatenulatum*, respectively. Age of the children is associated with PC2, whereas autoantibody positivity is associated with PC3. The children 3 to 7 years of age (circle) from the FINDIA pilot have higher numbers of *B. pseudocatenulatum* and lower numbers of *B. adolescentis* than the children from the TRIGR pilot 11 to 14 years of age (square). The main correlations with PC2 and PC3 are depicted with vectors in the top left. Children with a dearth of both *B. adolescentis* and *B. pseudocatenulatum* (sum <12%) are encompassed by the dashed circle near the top and the number of autoantibody-positive children is higher than the number of control subjects at the apex (10/18 vs. 4/18; $P = 0.040$; χ^2 test).

U test). The *Bacteroides* genus was associated with autoantibody positivity, but only a few of its many members were observed to be related on the species level. Interestingly, the correlation of the *Bacteroides* genus with autoantibody positivity was significant only in males, not in females ($P = 0.005$ vs. 0.655, respectively).

TABLE 2
Association between single bacterial species and signs of β-cell autoimmunity

Variable	%	Mann-Whitney *U* test
**Roseburia faecis* (−)	0.94	0.009
**Clostridium xylanovorans* (−)	0.03	0.031
**Peptoniphilus gorbachii* (−)	0.006	0.008
**Eubacterium hallii* (−)	6.0	0.088
**Eubacterium desmolans* (−)	0.15	0.058
**Acetanaerobacterium elongatum* (−)	0.06	0.043
Bifidobacterium animalis (+)	0.18	0.028
Lactobacillus acidophilus (+)	0.03	0.037
Clostridium perfringens (+)	0.03	0.018
Bacteroides genus (+)	3.1	0.031
Bacteroides genus males (+)	3.6	0.005
Bacteroides genus females (+)	2.3	0.655
B. vulgatus males (+)	1.3	0.023
B. ovatus males (+)	0.21	0.049
B. fragilis females (+)	0.16	0.017
B. thetaiota. females (−)	0.015	0.025
B. ovatus females (−)	0.31	0.178

*Butyrate-producing bacteria.

In children from the FINDIA study, it is furthermore noteworthy that *Eubacterium hallii* (6.0%) was strongly inversely related to the number of β-cell autoantibodies ($P = 0.002$; Spearman ρ correlation test).

IgA and calprotectin levels. Calprotectin levels, a marker of intestinal inflammation, were not observed to be correlated with autoantibody positivity. *Clostridium orbiscindens* (0.47%) correlated inversely with calprotectin levels ($P = 0.004$; Spearman ρ test). Lower levels of calprotectin were seen in children with the lower-risk HLA genotype, i.e., (DR3)DQB1*02-DQA1*05, when compared with the children with moderate-risk or high-risk HLA risk genotypes ($P = 0.001$ and 0.005; Mann-Whitney *U* test). Fecal IgA levels did not differ between autoantibody-positive and autoantibody-negative children but were inversely correlated with age ($P = 0.007$; Mann-Whitney *U* test).

Diversity analyses. The analysis of the 90, 95, and 97% operational taxonomic similarity levels showed that the diversity was significantly higher in the children in the TRIGR pilot cohort than in the children in the FINDIA pilot cohort ($P < 0.001$; Mann-Whitney *U* test). Furthermore, there was a trend that the diversity per sample was higher in autoantibody-negative children than in the autoantibody-positive children (Supplementary Table 2). This difference in diversity, measured in the number of observed operational taxonomic units and the Chao index, was significantly different at the 95% taxonomic similarity level when comparing the autoantibody-positive children and their corresponding control subjects in the TRIGR cohort ($P = 0.028$ and 0.034, respectively; Mann-Whitney *U* test).

DISCUSSION

We observed significant differences in the composition of fecal microbiota between children positive for at least two diabetes-associated autoantibodies and autoantibody-negative children. Because the major histocompatability complex genotype may affect the intestinal microbiota composition (29), we matched the case and control subjects not only for age, sex, and early feeding history but also for their HLA class II risk genotype of T1D. Our results thus should not be secondary to HLA-related differences in gut microbiota.

We found that the score reflecting the abundance of several lactate- and butyrate-producing bacteria, i.e., PC1, was inversely related to the number of β-cell autoantibodies in children; the lowest levels were observed in children positive for three or four autoantibodies. The bacteria, which correlated with PC1 included *B. adolescentis*, an acetate- and lactate-producing bacterium, *R. faecis*, a member from *Clostridium* cluster XIVa, which produces butyrate using acetate (30), and *F. prausnitzii*, a member from *Clostridium* cluster IV, an acetate utilizing butyrate-producing bacterium (30,31) with anti-inflammatory properties (32). In addition to this, we found that in children from the FINDIA study, *E. hallii*, an acetate- and lactate-utilizing and butyrate-producing bacterium from *Clostridium* cluster XIVa (31), was inversely related to the number of β-cell autoantibodies. A recent study that included four pairs of cases with development of T1D and autoantibody-negative control subjects suggested that higher proportions of butyrate-producing and mucin-degrading bacteria were observed in control subjects compared with case subjects (33). Because lactate can be further metabolized to butyrate, the producers of lactate may contribute to the net production of butyrate; bifidobacteria represents a prime example of this (31). PCA analysis revealed that a low abundance (<12%) of the sum of the two most common bifidobacteria, *B. adolescentis* and *B. pseudocatenulatum*, was associated with β-cell autoimmunity.

Butyrate is thought to be beneficial because it is the main energy source for colonic epithelial cells (34). Furthermore, butyrate has been shown to regulate the assembly of tight junctions and gut permeability (35). In animal models of autoimmune diabetes, increased gut permeability precedes the development of diabetes, and environmental factors, which modulate the permeability, modulate the disease incidence. Although butyrate treatment during the weaning period in BB-DP rats did not prevent autoimmune diabetes, it modulated the gut inflammatory response (36). Both gut permeability and inflammation have been linked to the development of T1D in humans (37). In children with T1D, subclinical small intestinal inflammation with T-cell activation has been reported previously (37), but there are no studies of children at risk for T1D, i.e., autoantibody-positive children. We did not find increased fecal calprotectin or IgA levels in β-cell autoantibody–positive children, which does not support the presence of significant intestinal inflammation in preclinical T1D, although differences in the composition of fecal microbiota were observed.

Our results suggest that the changes characterized by low numbers of butyrate producers are related to the late phase of prediabetes, i.e., positivity for multiple autoantibodies and IA-2A. The correlation of certain bacterial findings with the number of positive autoantibodies could indicate a role of dysbiosis as a regulator of β-cell autoimmunity in the progression of the autoimmune process toward β-cell destruction and clinical disease. It should be emphasized that our findings demonstrate only microbial changes and functional studies are needed to prove causality between these kinds of changes and β-cell autoimmunity. The results thus are tentative and support the animal studies in which causality has been demonstrated.

There is increasing evidence that the *Bifidobacterium* genus in the human gut plays an important role in maintaining health, both within the gastrointestinal tract and in the rest of the body (38), and this also is supported by the current observations, although we did not provide any data on functional changes related to the observed bacterial diversity. It has been shown that besides contributing to the production of butyrate, bifidobacteria inhibit bacterial translocation (39–41). In contrast, bacterial translocation was found to be enhanced by the *Bacteroides* genus and, in particular, the *Bacteroides fragilis* group, which includes *B. fragilis*, *B. ovatus*, and *B. vulgatus* (40). A role of the *Bacteroides* genus in the development of autoimmune diabetes has been implicated in animal models and in humans (11,12,14,33). In our study, the *Bacteroides* genus and *C. perfringens*, which is known to be associated with increased gut permeability and inflammation via its production of several toxins (42), were positively associated with β-cell autoimmunity. Bifidobacteria, however, might inhibit the translocation or the growth of the *B. fragilis* group and *C. perfringens* as they compete for space/adherence (43) and nutrients (44), and they enhance the intestinal epithelial barrier function (45) by increasing the thickness of the mucus layer (46,47). Accordingly, our findings actually may be interrelated because a low abundance of bifidobacteria might favor the growth of *Bacteroides* (40).

Concerning the biological importance of the *Bacteroides* genus, it should be noted that its abundance as reported here obtained via pyrosequencing (~3%) greatly underestimates its actual predominance within the gut of these children. The reason for this is that the primer pair used in this study, which was chosen because it provides a more complete assessment of actinobacterial abundance (20), very likely caused an overestimation of the amount of the *Bifidobacterium* genus and possibly caused an underestimation of the *Bacteroides* genus. We confirmed our suspicions using fluorescent in situ hybridization (data not shown); the *Bifidobacterium* genus had been overestimated by a factor of two and the *Bacteroides* genus was underestimated by a factor of six.

In agreement with a previous study in which the diversity decreased with increasing age in the four children with development of T1D (14), the microbial diversity was lower in autoantibody-positive children when compared with autoantibody-negative children, especially in the children aged 12 to 14 years from the TRIGR pilot study. The samples from the children of the TRIGR and FINDIA studies were collected at the same time using the same protocol for transportation and storage. Thus, the findings are not influenced by differences in the storage time or transportation history of the samples.

In conclusion, our study emphasizes the importance of bifidobacteria and lactate- or butyrate-producing species in general in relation to the development of β-cell autoimmunity. Bifidobacteria not only supply butyrate-producing species with lactate and acetate but also enhance the intestinal epithelial barrier function by modulating the gut

mucosa, thereby possibly preventing the translocation of for example *Bacteroides*, which were, in turn, confirmed to be more highly abundant in children with β-cell autoimmunity. Because only children with at least two autoantibodies were included in this study, it was not possible to demonstrate that dysbiosis has a role in the initiation of β-cell autoimmunity. The pyrosequencing method does have its limitations because it does not give direct data on the functionally important changes of the microbiota, which should be demonstrated if pathogenetic importance is considered. One also should be careful with making conclusions based on PCA of the data on the bacterial diversity. Our results only indicate that alterations in the intestinal bacterial diversity are seen at the prediabetes stage of the disease, and these changes precede the development of T1D. Large birth cohort studies that include time series of samples and methodology to study the functional changes in the intestinal microbiota are needed for the evaluation of the role of gut microbiota in the development of β-cell autoimmunity and T1D.

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M.C.d.G. and K.L. analyzed the data. M.C.d.G., K.L., and O.V. wrote the manuscript. M.C.d.G., G.W.W., and H.J.H. were responsible for the microbiological studies. K.L. and T.R. coordinated the study recruitment and sample collection. M.K. contributed to the study recruitment and edited the manuscript. M.K. and T.H. were responsible for the autoantibody analyses. J.I. was responsible for HLA typing. L.O. performed the fecal IgA determinations. S.H. performed the fecal calprotectin tests. O.V. was responsible for the study design. O.V. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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