Gut Microbiota Differs in Composition and Functionality Between Children With Type 1 Diabetes and MODY2 and Healthy Control Subjects: A Case-Control Study

Diabetes Care 2018;41:2385–2395 | https://doi.org/10.2337/dc18-0253

OBJECTIVE

Type 1 diabetes is associated with compositional differences in gut microbiota. To date, no microbiome studies have been performed in maturity-onset diabetes of the young 2 (MODY2), a monogenic cause of diabetes. Gut microbiota of type 1 diabetes, MODY2, and healthy control subjects was compared.

RESEARCH DESIGN AND METHODS

This was a case-control study in 15 children with type 1 diabetes, 15 children with MODY2, and 13 healthy children. Metabolic control and potential factors modifying gut microbiota were controlled. Microbiome composition was determined by 16S rRNA pyrosequencing.

RESULTS

Compared with healthy control subjects, type 1 diabetes was associated with a significantly lower microbiota diversity, a significantly higher relative abundance of Bacteroides, Ruminococcus, Veillonella, Blautia, and Streptococcus genera, and a lower relative abundance of Bifidobacterium, Roseburia, Faecalibacterium, and Lachnospira. Children with MODY2 showed a significantly higher Prevotella abundance and a lower Ruminococcus and Bacteroides abundance. Proinflammatory cytokines and lipopolysaccharides were increased in type 1 diabetes, and gut permeability (determined by zonulin levels) was significantly increased in type 1 diabetes and MODY2. The PICRUSt analysis found an increment of genes related to lipid and amino acid metabolism, ABC transport, lipopolysaccharide biosynthesis, arachidonic acid metabolism, antigen processing and presentation, and chemokine signaling pathways in type 1 diabetes.

CONCLUSIONS

Gut microbiota in type 1 diabetes differs at taxonomic and functional levels not only in comparison with healthy subjects but fundamentally with regard to a model of nonautoimmune diabetes. Future longitudinal studies should be aimed at evaluating if the modulation of gut microbiota in patients with a high risk of type 1 diabetes could modify the natural history of this autoimmune disease.

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Received 5 February 2018 and accepted 26 August 2018.

This article contains Supplementary Data online at http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc18-0253/-/DC1.

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Type 1 diabetes is a multifactorial immune-mediated disease characterized by the progressive loss of insulin-producing β-cells in the islets of Langerhans in the pancreas. The causes that lead to the appearance of type 1 diabetes have not yet been fully identified, with genetic factors playing a major role; however, environmental factors are also closely linked, such as birth delivery mode (1), diet in early life (cow milk proteins or gluten-containing cereals) (2), and widespread usage of antibiotics (3), all factors closely related to gut microbiota.

Recent studies have associated the microbiome with the development of type 1 diabetes; animal studies have demonstrated a close link between intestinal microbiota and type 1 diabetes in BioBreeding diabetes-prone rats (4) and in nonobese diabetic mice (5). Also, in children with type 1 diabetes, auto-immune positivity has been related to changes in microbiota composition (6,7).

In a previous study, we found that in comparison with healthy control subjects, children with type 1 diabetes presented large significant differences in the relative abundance of predominant phyla, families, and genera (8). Potentially, the altered microbiota profile in type 1 diabetes may be associated with alterations in the gut immune system, such as increased gut permeability (9). Recent studies have shown that commensal bacteria are crucial for the maturing and functioning of the mucosal immune system. Moreover, an impaired integrity of the intestinal barrier with an increase in permeability has been described in both animal models and human type 1 diabetes studies (10).

Therefore, given that intestinal microbes may affect intestinal permeability, intestinal ecology could also play a crucial role in the development of type 1 diabetes (11). On the other hand, zonulin, a physiological modulator of intercellular tight junctions, increases gut permeability and macromolecule absorption, and previous studies claim a role for zonulin as a capital modulator of intercellular tight junctions, increases gut permeability and physiological modulator of intercellular tight junctions (12). Maturity-onset diabetes of the young (MODY) is a genetic form of diabetes that accounts for 1–2% of all diabetes cases in Europe and is associated with specific loss-of-function mutations with characteristic phenotypes (13). The most common presentation of MODY is MODY2, caused by a heterozygous inactivating mutation in the glucokinase (GCK) gene (14). To date, no studies have evaluated the gut microbiome structure in MODY2 patients. Nevertheless, MODY2 is a highly attractive model to assess the relation of gut microbiota with type 1 diabetes, as MODY2 is not normally associated with obesity, a glycemic control similar to type 1 diabetes can be achieved, and, importantly, its cause is not of autoimmunity origin (15).

We hypothesize that if the fecal microbiota in type 1 diabetes differs from that of MODY2, the gut microbiota profile could constitute a novel associated risk factor for the type 1 diabetes autoimmune process. On the contrary, if the fecal microbiota were similar and different from the microbiota of healthy control subjects, this would indicate that differences in intestinal microbiota could be attributed to hyperglycemia per se.

Therefore, the aim of this case-control study is to evaluate the gut microbiota profile, functional capacity, low-grade inflammation, and gut permeability between patients with type 1 diabetes and MODY2 and healthy control subjects.

**RESEARCH DESIGN AND METHODS**

This study was a case-control study, including 15 children with type 1 diabetes, 15 children with MODY2, and 13 healthy control children, all under 18 years old, of Caucasian origin and with the same geographical location.

Type 1 diabetes was diagnosed according to the criteria of the American Diabetes Association (16) and the positivity of at least two persistent, confirmed anti-islet autoantibodies (anti-insulin autoantibodies, GAD autoantibodies, or tyrosine phosphatase autoantibodies). MODY2 children were diagnosed by suggestive clinical history, negative anti-islet autoantibodies, and positive genetic testing. Healthy control subjects were children with negative anti-islet autoantibodies, matched to children with type 1 diabetes and MODY2 for age, sex, race, BMI, mode of delivery, and duration of breastfeeding. In addition, patients with type 1 diabetes and MODY2 were controlled by HbA1c levels. Patients with type 1 diabetes were undergoing treatment with multiple doses of insulin, whereas MODY2 patients were drug naïve. Exclusion criteria to participate in this study included acute or chronic inflammatory diseases or infectious diseases or undergoing treatment with antibiotics, prebiotics, or probiotics or any other medical treatment that could potentially influence intestinal microbiota 3 months before inclusion.

The parents of all the participants completed a structured interview to obtain health status, lifestyle aspects, and dietary habits. Patients with type 1 diabetes and MODY2 were instructed to follow a standard diabetic diet, containing 40–50% of calories from carbohydrates, 20–30% from fat, and 20% from protein. Dietary intake patterns were determined from a food frequency questionnaire.

The written informed consents of the children’s guardians or parents were obtained. The sampling and experimental processes were performed with the approval of the local Ethics Committee of the Regional Hospital of Málaga.

**Laboratory Measurements**

Serum glucose, cholesterol, triglycerides, and interleukin-1β (IL-1β) and IL-10 cytokines were measured by ELISA as previously described (17). Concentrations of IL-6, IL-13, and tumor necrosis factor (TNF-α) were quantified by ELISA assay kits (Thermo Fisher Scientific) in serum samples according to the instructions of the manufacturer. The detection limits were as follows: 7.8–500 pg/mL for IL-6, 1.6–100 pg/mL for IL-13, and 15.6–1,000 pg/mL for TNF-α.

**DNA Extraction, Pyrosequencing of 16S rRNA Sequences, and Bioinformatic Analysis**

Study participants collected their fecal samples in a sterile and hermetically sealed receptacle provided by the research team. Fecal samples were collected in the morning of the day of sample receipt and were immediately refrigerated in household freezers and transported to the laboratory during the following 4 h. Frozen fecal samples were transported with ice to avoid important changes of temperature that might cause bacterial DNA degradation and were subsequently stored at −80°C in the laboratory until analysis. No DNA stabilizers were added to the fecal samples.

DNA was extracted from the fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. Amplification of genomic DNA was
performed using bar-coded primers that targeted the V2–V3 regions of the bacterial 16S rRNA gene. Amplification, sequencing, and basic analysis were performed using a GS Junior 454 platform according to the protocols of the manufacturer and a Titanium chemistry apparatus (Roche Applied Science, Indianapolis, IN). The 454 pyrosequencing data sets were analyzed by Quantitative Insights into Microbial Ecology (QIIME) 1.8.0 software as previously described (17). PICRUSt analysis was used to predict metagenome function by picking operational taxonomic units (OTUs) against described (17). PICRUSt analysis was used to identify which bacteria taxa were independent predictors for serum zonulin, LPS, inflammatory mediators, and HbA1c levels in each study group. Values were considered to be statistically significant when \( P \leq 0.05 \).

**RESULTS**

**Diet and Anthropometric and Biochemical Measurements**

All study participants had a similar physical activity and dietary profile. No significant differences in the consumption patterns of wheat, rice, vegetables, fish, or meat were found between study groups.

**Statistical Analysis**

The Chao index (community richness) of gut microbiota between type 1 diabetes, MODY2, and healthy control subjects, a priori sample size estimation was not performed.

The relative abundances of each OTU (taxa) were compared by a Wilcoxon signed rank test with a continuity correction using the Explicet software package specifically addressed to analyze microbiome data. All the resulting \( P \) values were then adjusted for multiple comparisons via the Benjamini-Hochberg false discovery rate (FDR) correction (FDR-corrected \( P \) value of <0.05). \( \alpha \) - and \( \beta \)-diversities were achieved by QIIME, \( \beta \)-diversity using a nonparametric Student \( t \) test using a default number of Monte Carlo permutations of 999, and \( \beta \)-diversity with the analysis of similarities statistical method with 99 permutations. Differences in the clinical characteristics between two groups were analyzed using Mann-Whitney \( U \) test, and differences among the three groups were analyzed using the Kruskal-Wallis test with Bonferroni post hoc test. The Spearman correlation coefficient was calculated to estimate the linear correlations between variables. A multiple linear regression analysis was performed to identify which bacteria taxa were independent predictors for serum zonulin, LPS, inflammatory mediators, and HbA1c levels in each study group. Values were considered to be statistically significant when \( P \leq 0.05 \).
diabetes, 7.01% MODY2, 11.19% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P < 0.001 \). Prevotellaceae (2.65% type 1 diabetes, 15.41% MODY2, 1.25% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.003 \)) was significantly higher in type 1 diabetes than in healthy control subjects, and Prevotellaceae was significantly higher in MODY2 than in type 1 diabetes \( (P < 0.001, \text{FDR-adjusted } P < 0.001) \). In the Firmicutes, another three families were significantly higher in type 1 diabetics compared with MODY2 and healthy control subjects: Ruminococcaceae (38.19% type 1 diabetes, 25.74% MODY2, 30.84% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P < 0.001 \)), Veillonellaceae (31.94% type 1 diabetes, 20.33% MODY2, 18.03% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.003 \), and Streptococcaceae (1.93% type 1 diabetes, 0.96% MODY2, 0.56% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.004 \)). No significant differences at the Firmicutes family level were found between MODY2 and healthy control subjects except in the abundance of Ruminococcaceae \( (P < 0.001, \text{FDR-adjusted } P < 0.001) \). In healthy children, only Lachnospiraceae (22.1% type 1 diabetes, 27.95% MODY2, 42.0% healthy control subjects; \( P = 0.002 \), FDR-adjusted \( P = 0.015 \)) was significantly higher in comparison with type 1 diabetes and MODY2. In Actinobacteria, a significant enrichment of Bifidobacteriaceae (2.71% type 1 diabetes, 4.50% MODY2, 5.68% healthy control subjects; \( P = 0.004 \), FDR-adjusted \( P = 0.017 \)) was found in healthy control subjects when compared with MODY2 and type 1 diabetes. Finally, for the Proteobacteria families, a significant increase of Enterobacteriaceae (25.20% type 1 diabetes, 15.03% MODY2, 13.04% healthy control subjects; \( P = 0.006 \), FDR-adjusted \( P = 0.03 \)) was found in type 1 diabetes when compared with MODY2 and healthy control subjects, but no significant differences were found for Alcaligenaceae (62.18% type 1 diabetes, 49.03% MODY2, 57.07% healthy control subjects; \( P = 0.019 \), FDR-adjusted \( P = 0.267 \)) (Supplementary Fig. 2 and Supplementary Data).

Twelve genera were differentially abundant between study groups. For the Bacteroidetes genera, the type 1 diabetes group was significantly enriched with sequences attributed to the genus Bacteroides (72.21% type 1 diabetes, 52.41% MODY2, 58.45% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P < 0.001 \)). Prevotella was significantly increased in MODY2 and type 1 diabetes when compared with healthy control subjects (1.95% type 1 diabetes, 8.32% MODY2, 1.42% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.005 \)). Regarding Firmicutes, the relative abundances of four genera were significantly higher in type 1 diabetes than in MODY2 and healthy control subjects: Ruminococcus (17.19% type 1 diabetes, 5.74% MODY2, 8.85% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.002 \)), Blautia (15.50% type 1 diabetes, 5.73% MODY2, 3.74% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.003 \), Veillonella (21.59% type 1 diabetes, 12.33% MODY2, 7.20% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.006 \), and Streptococcus (4.86% type 1 diabetes, 2.64% MODY2, 1.47% healthy control subjects; \( P = 0.003 \), FDR-adjusted \( P = 0.028 \)). In addition, four genera were significantly lower in type 1 diabetes and MODY2 than in healthy control subjects: Lachnospira (5.34% type 1 diabetes, 7.15% MODY2, 15.25% healthy control subjects; \( P < 0.001 \), FDR-adjusted \( P = 0.012 \), Roseburia (1.35% type 1 diabetes, 4.16% MODY2, 6.99% healthy control subjects; \( P < 0.001 \), FDR-adjusted

### Table 1—Baseline anthropometric and biochemical variables

<table>
<thead>
<tr>
<th></th>
<th>Healthy control subjects ( n = 13 )</th>
<th>Type 1 diabetes ( n = 15 )</th>
<th>MODY2 ( n = 15 )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female, n</td>
<td>7/6</td>
<td>7/8</td>
<td>7/8</td>
<td></td>
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<tr>
<td>Vaginal delivery/cesarean section, n</td>
<td>8/5</td>
<td>10/5</td>
<td>10/5</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>12.25 ± 2.92</td>
<td>12.56 ± 3.59</td>
<td>13.06 ± 3.20</td>
<td>0.654</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>17.35 ± 1.82</td>
<td>17.89 ± 2.01</td>
<td>18.23 ± 1.90</td>
<td>0.430</td>
</tr>
<tr>
<td>Age of onset of diabetes (years)</td>
<td>7.35 ± 1.76</td>
<td>6.91 ± 1.40</td>
<td>0.455</td>
<td></td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>5.68 ± 1.84</td>
<td>6.10 ± 1.97</td>
<td>0.551</td>
<td></td>
</tr>
<tr>
<td>Breastfeeding time (months)</td>
<td>6.58 ± 2.32</td>
<td>6.41 ± 2.81</td>
<td>6.54 ± 3.2</td>
<td>0.911</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.19 ± 0.45</td>
<td>3.28 ± 0.38</td>
<td>3.22 ± 0.55</td>
<td>0.249</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>37.35 ± 9.0</td>
<td>38.32 ± 8.92</td>
<td>36.91 ± 7.72</td>
<td>0.765</td>
</tr>
<tr>
<td>HbaA[1c] (%)</td>
<td>4.47 ± 0.21</td>
<td>6.26 ± 0.38</td>
<td>6.11 ± 0.33</td>
<td>0.001</td>
</tr>
<tr>
<td>HbaA[1c] (mmol/mol)</td>
<td>25.3 ± 2.3</td>
<td>44.9 ± 4.2</td>
<td>43.3 ± 3.6</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>52.67 ± 9.43</td>
<td>53.50 ± 10.15</td>
<td>53.88 ± 9.88</td>
<td>0.843</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>153.88 ± 14.64</td>
<td>153.62 ± 16.87</td>
<td>154.5 ± 17.9</td>
<td>0.920</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>83.21 ± 28.25b</td>
<td>119.41 ± 27.12a</td>
<td>89.45 ± 25.31b</td>
<td>0.004</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>126.67 ± 9.87b</td>
<td>81.03 ± 9.97a</td>
<td>121.28 ± 5.46b</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>85.71 ± 6.28a</td>
<td>109.89 ± 6.50b</td>
<td>88.98 ± 7.49b</td>
<td>0.001</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>164.31 ± 16.78b</td>
<td>373.46 ± 90.65a</td>
<td>169.54 ± 18.3b</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-13 (pg/mL)</td>
<td>50.15 ± 8.37b</td>
<td>22.83 ± 5.47a</td>
<td>45.46 ± 7.31b</td>
<td>0.001</td>
</tr>
<tr>
<td>LPS (EU/mL)</td>
<td>0.49 ± 0.10a</td>
<td>1.10 ± 0.40a</td>
<td>0.56 ± 0.38b</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD unless otherwise specified. \( P \) value was based on Kruskal-Wallis test. Mann-Whitney U test was used to compare the clinical characteristics between two groups. Different superscript letters (a,b) next to values in a row indicate that the means of the different groups are significantly different \( (P < 0.05, \text{Bonferroni post hoc test}) \).
Anaerostipes (2.15% type 1 diabetes, 2.97% MODY2, 5.79% healthy control subjects; \( P < 0.001, \text{FDR-adjusted } P = 0.023 \)), and Faecalibacterium (4.21% type 1 diabetes, 8.08% MODY2, 13.26% healthy control subjects; \( P < 0.001, \text{FDR-adjusted } P = 0.004 \)). In Actinobacteria, only the Bifidobacterium was significantly increased in healthy control subjects when compared with type 1 diabetes (1.93% type 1 diabetes, 6.75% healthy control subjects; \( P < 0.001, \text{FDR-adjusted } P = 0.017 \)), but not regarding MODY2 (6.07% MODY2, 6.75% healthy control subjects; \( P = 0.503, \text{FDR-adjusted } P > 0.05 \)). Finally, in the Proteobacteria genera, Sutterella and Enterobacter were significantly increased in type 1 diabetes when compared with MODY2 and healthy control subjects (61.30% type 1 diabetes, 49.32% MODY2, 57.07% healthy control subjects [\( P = 0.002, \text{FDR-adjusted } P = 0.027 \)] and 16.18% type 1 diabetes, 8.25% MODY2, 6.99% healthy control subjects [\( P < 0.001, \text{FDR-adjusted } P = 0.003 \)], respectively).

No significant differences between study groups were found in the relative abundance of Desulfovibrio, Haemophilus, and Bilophila (FDR-adjusted \( P > 0.05 \)) (Supplementary Fig. 3 and Supplementary Data). A cladogram showing differences in fecal microbiota between study groups is shown in Fig. 2.

**Serum Zonulin Levels in Type 1 Diabetes, MODY2, and Healthy Control Subjects**

Patients with MODY2 showed higher serum zonulin levels (4.80 ± 1.41 ng/mg protein) when compared with patients with type 1 diabetes (3.94 ± 1.44 ng/mg protein, \( P = 0.02 \)) and healthy control subjects (3.21 ± 1.24 ng/mg protein, \( P < 0.001 \)). Significant differences in serum
zonulin levels were also found between type 1 diabetes and control subjects ($P < 0.05$).

The Relationship Between Gut Microbiota Composition, Serum Zonulin, HbA1c, Inflammatory Mediators, and LPS

Several significant correlations between the relative abundance of specific bacteria at different taxa levels and serum zonulin levels and HbA1c were found in children with type 1 diabetes and MODY2, but not in healthy control subjects (Table 2). In addition, significant correlations between serum levels of IL-1β, IL-6, TNF-α, IL-10, IL-13, and LPS and gut microbiota composition were found in type 1 diabetes (Supplementary Table 1).

The linear regression analysis including all the bacterial groups showed that the increase in Bacteroides ($P = 0.002$, $\beta = 0.995$, $r^2 = 0.94$) and Veillonella ($P = 0.021$, $\beta = 0.636$, $r^2 = 0.93$) and the decrease in Faecalibacterium ($P = 0.041$, $\beta = 0.671$, $r^2 = 0.94$) and Roseburia ($P = 0.038$, $\beta = 0.694$, $r^2 = 0.93$) in type 1 diabetes and the rise in Prevotella in MODY2 ($P = 0.002$, $\beta = 0.682$, $r^2 = 0.90$) were associated with the increment in serum zonulin levels. On the other hand, the increase in the abundance of Blautia ($P = 0.043$, $\beta = 0.995$, $r^2 = 0.94$) and Veillonella ($P = 0.021$, $\beta = 0.636$, $r^2 = 0.93$) and the decrease in Faecalibacterium ($P = 0.041$, $\beta = 0.671$, $r^2 = 0.94$) and Roseburia ($P = 0.038$, $\beta = 0.694$, $r^2 = 0.93$) in type 1 diabetes and the rise in Prevotella in MODY2 ($P = 0.002$, $\beta = 0.682$, $r^2 = 0.90$) were associated with the increment in serum zonulin levels.
β = 0.469, \( r^2 = 0.93 \) and the decrease in the Fimbicutes-to-Bacteroidetes ratio (\( P = 0.001, \beta = -0.947, r^2 = 0.91 \)) in patients with type 1 diabetes were associated with HbaA1c, whereas in MODY2 patients, only the decrease in Ruminococcus (\( P = 0.003, \beta = -0.877, r^2 = 0.92 \)) was associated with HbaA1c levels. In addition, the increase in the relative abundance of Bacteroides (\( P = 0.006, \beta = 0.991, r^2 = 0.95 \)) and Veillonella (\( P = 0.012, \beta = 0.825, r^2 = 0.95 \)) and the decrease in Bifidobacterium (\( P = 0.039, \beta = -0.654, r^2 = 0.94 \)), Roseburia (\( P = 0.032, \beta = -0.675, r^2 = 0.95 \)), and Faecalibacterium (\( P = 0.023, \beta = -0.678, r^2 = 0.94 \)) in type 1 diabetes were associated with serum IL-1β levels. Finally, in type 1 diabetes, the increase in the abundance of Bacteroides (\( P = 0.007, \beta = 0.632, r^2 = 0.85 \)) and the decrease in Roseburia (\( P = 0.029, \beta = -0.518, r^2 = 0.85 \)) were associated with serum IL-6 and TNF-α levels, and the increase in Streptococcus (\( P = 0.014, \beta = 0.616, r^2 = 0.82 \)) and the decrease in Bifidobacterium (\( P = 0.009, \beta = -0.904, r^2 = 0.82 \)) were associated with serum IL-10 and IL-13 levels. Regarding the serum levels of LPS, only the increase in the abundance of Veillonella (\( P = 0.006, \beta = 0.887, r^2 = 0.83 \)) was associated with the levels in type 1 diabetes.

**Functional Differences in the Gut Microbiota of Study Groups**

The PICRUSt analysis indicated that genes for energy (\( P = 0.015 \)) and carbohydrate metabolism (\( P = 0.014 \)) were significantly depleted in type 1 diabetes in comparison with MODY2 and healthy control subjects. Nevertheless, lipid metabolism functions (\( P = 0.008 \)), together with amino acid metabolism functions (\( P = 0.013 \)), were overrepresented in type 1 diabetes when compared with the other groups. In addition, pathways of lipid and carbohydrate metabolism in type 1 diabetes had a significant enrichment in the proportion of genes related to arachidonic acid and propanoate metabolism (\( P = 0.002 \) and \( P = 0.003 \), respectively), in comparison with MODY2 and healthy subjects (Fig. 3).

Metagenomic comparison between study groups showed that gene families linked to amino acid metabolism, such as amino acid–related enzymes (\( P = 0.010 \)), arginine and proline metabolism (\( P = 0.006 \)), and valine, leucine, and isoleucine biosynthesis (\( P = 0.020 \)) were significantly increased in type 1 diabetes, whereas genes for glutination metabolism (\( P = 0.005 \)) were significantly depleted. Conversely, in MODY2 and healthy control subjects, in comparison with type 1 diabetes, a significant increase in genes related to the autoimmune process. In a previous study evaluating type 1 diabetes markers, a lower microbial diversity was found in fecal samples of children with at least two positive disease-associated autoantibodies than in samples from autoantibody-negative children matched for age, sex, early feeding history, and HLA genotyping (19). Also, in longitudinal studies from birth with children at risk for type 1 diabetes, a decrease of microbial diversity occurred just before the appearance of anti-islet cell antibodies and subsequent onset of type 1 diabetes (20).

We demonstrate that there are clear differences in the gut microbiota profile of type 1 diabetes, MODY2, and healthy control subjects, given that in the OTU-based PCoA plot analysis, patients with type 1 diabetes clustered separately when compared with MODY2 patients and healthy control subjects. Accordingly, children with type 1 diabetes showed a significant increase in the relative abundance of Bacteroides, Ruminococcus, Veillonella, Blautia, Enterobacter, and Streptococcus genera and a decrease in the relative abundance of Bifidobacterium, Roseburia, Faecalibacterium, and Lachnospira, when compared with MODY2 and healthy control subjects. On the other hand, MODY2 was related to a significant increase in

**Table 2—Correlation between gut microflora composition and serum zonulin and HbaA1c levels in children with type 1 diabetes and MODY2**

<table>
<thead>
<tr>
<th>Zonulin</th>
<th>Type 1 diabetes</th>
<th>MODY2</th>
<th>HbaA1c</th>
<th>Type 1 diabetes</th>
<th>MODY2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruminococcus</td>
<td>-0.162 (( P = 0.55 ))</td>
<td>-0.540 (( P = 0.036 ))</td>
<td>-0.362 (( P = 0.18 ))</td>
<td>-0.584 (( P = 0.025 ))</td>
<td></td>
</tr>
<tr>
<td>Roseburia</td>
<td>-0.320 (( P = 0.031 ))</td>
<td>-0.732 (( P = 0.48 ))</td>
<td>-0.561 (( P = 0.029 ))</td>
<td>-0.332 (( P = 0.45 ))</td>
<td></td>
</tr>
<tr>
<td>Prevotella</td>
<td>-0.560 (( P = 0.37 ))</td>
<td>0.798 (( P = 0.037 ))</td>
<td>Blautia</td>
<td>0.559 (( P = 0.038 ))</td>
<td>0.740 (( P = 0.79 ))</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>-0.703 (( P = 0.027 ))</td>
<td>-0.547 (( P = 0.49 ))</td>
<td>Streptococcus</td>
<td>0.068 (( P = 0.018 ))</td>
<td>0.441 (( P = 0.12 ))</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>0.739 (( P = 0.004 ))</td>
<td>0.350 (( P = 0.090 ))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veillonella</td>
<td>0.570 (( P = 0.033 ))</td>
<td>0.704 (( P = 0.04 ))</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correlations are reported as Spearman \( \rho \) (\( r \)), and \( P \) values are given in parentheses. Statistical significance was set at a \( P \) value of <0.05.
Prevotella abundance and a significant decrease in Ruminococcus and Bacteroides, when compared with type 1 diabetes and healthy control subjects. Despite the great variability of intestinal microbiota in type 1 diabetes (regardless of ethnicity, age, and geography), most published studies (including the present one) identify Bacteroides (acetate- and propionate-producing bacteria) as the main genus leading to type 1 diabetes–associated dysbiosis (21,22).

Bacteroides, a Gram-negative bacterium, could contribute to chronic inflammation by the impairment of the barrier...
function of the epithelial cell layer (23). In our study, subjects with type 1 diabetes, when compared with MODY2 and healthy control subjects, presented significantly higher levels of LPS and proinflammatory cytokines IL-1β, IL-6, and TNF-α along with a significant decrease in anti-inflammatory cytokines IL-10 and IL-13. Moreover, the significant correlations found between gut microbiota and host serum levels of LPS and cytokines indicate that the significant depletion of Faecalibacterium, Roseburia, and Bifidobacterium, reported to exert anti-inflammatory effects and strengthen gut barrier function (through cytokine production modulation and butyrate production, respectively) (24,25), together with the significant increase of Veillonella (a lactate-utilizing and propionate-producing bacteria with proinflammatory capacity) and Bacteroides, could raise paracellular permeability and low-grade inflammation in type 1 diabetes (26,27). This situation could allow luminal antigens to escape from the gut and promote islet-directed autoimmune responses. Therefore, the lower abundance of anti-inflammatory bacteria unable to regulate epithelial integrity could be associated with the intestinal immune activation in type 1 diabetes (28,29).

On the other hand, we have found a significant increase in the relative abundance of Prevotella in MODY2 when compared with type 1 diabetes and healthy control subjects. Given that Prevotella is an important succinate producer and mucin degrader bacteria, this could suggest a lack of mucin on the intestinal epithelial layer of MODY2 subjects, disturbing the protection of the host mucosal surfaces. Moreover, Prevotella-produced succinate is a bacterial metabolite that leads to inhibition of hepatic glucose output and improves glycemic control and energy metabolism through the activation of intestinal gluconeogenesis (30). Accordingly, Spiegel et al. (13) suggested that in MODY2, despite the shift in insulin secretion, metabolic control remains intact probably due to the existence of compensatory mechanisms external to β-cells. Therefore, we suggest that the significant increase in the abundance of Prevotella found in MODY2 patients could be related to the maintenance of glycemic control.

In addition, we have found in type 1 diabetes a significant positive correlation between zonulin levels and the relative abundance of Bacteroides and Veillonella, and a significant negative correlation with Faecalibacterium and Roseburia, as well as a significant positive correlation between zonulin and Prevotella in patients with MODY2. Zonulin regulates intestinal permeability by modulating intercellular tight junctions. A possible mechanism is based on bacterial antigens and microorganism toxins that may be sensed by molecules related to epithelial cell tight junctions like zonulin, altering their activity and consequently increasing gut permeability and bacterial translocation (21,31). However, zonulin levels alone do not show impaired epithelial integrity, and in accordance with other authors, the impaired epithelial integrity found in the patients with type 1 diabetes might be caused by the binding of Veillonella (a lactate-utilizing bacteria) to immature cells in colonic crypts able to ferment glucose to lactate, which are pushed to the luminal surface and form poor tight junctions (32). Also, in type 1 diabetes, there is an increased intestinal permeability that may affect absorption of antigens capable of attacking and damaging pancreatic β-cells (33–35). Thus, the significant increase in gut permeability may be an important player in the development of type 1 diabetes. Moreover, some authors have stated that a leaky gut could be involved in the development of type 1 diabetes complications, as high serum LPS activity has been associated with features of metabolic syndrome, visceral fat mass, and the progression of kidney disease in type 1 diabetes (36–38).

On the other hand, we have shown that the relative abundance of Blautia and the Firmicutes-to-Bacteroidetes ratio were positively correlated with HbA1c in type 1 diabetes, and in MODY2, HbA1c levels were negatively correlated with the abundance of Ruminococcus. Butyrate-producing intestinal bacteria such as Ruminococcus and Blautia could play an important role in blood glucose regulation and lipid metabolism, as shown by fecal transplantation studies (39). Some authors have reported that Blautia is positively correlated with serum glucose, HbA1c levels, and the number of type 1 diabetes autoantibodies, suggesting that Blautia might influence the development of type 1 diabetes through the regulation of T-cell differentiation (20,40).

Also, we report a significantly lower Firmicutes-to-Bacteroidetes ratio in type 1 diabetes and a negative correlation between this ratio and HbA1c. This has been previously demonstrated by our group (8) and by other authors who have reported a decline in Firmicutes and an increase in Bacteroidetes abundance over time in the gut microbiome until the development of type 1 diabetes (41).

The PICRUSt analysis demonstrated that several microbial functions were significantly over- or underrepresented between study groups, due to important differences in bacteria composition. Thus, when compared with healthy control subjects and MODY2, gut microbiota in type 1 diabetes showed a depleted abundance of genes involved in metabolic pathways such as carbohydrate and energy metabolism. Conversely, there was an increase in genes related to lipid and amino acid metabolism, ABC transport, LPS biosynthesis, arachidonic acid metabolism, antigen processing and presentation, and chemokine signaling pathways related to inflammation and immune response. The relative abundance of genes associated with a given pathway may indicate an increased metabolic capacity of the gut microbiota with regard to this pathway. Interestingly, a higher level of arachidonic acid metabolism (inflammatory intermediate) in type 1 diabetes gut microbiota might be the result of a growth of proinflammatory pathobionts in the gut (42).

Our study has certain limitations but also some important strengths. The limitations include the relatively small sample size (mainly caused by the very low prevalence of MODY2 in children), which may not be enough for detecting differences between low-abundance microbes that may be of relevance or to assess overall differences between type 1 diabetes and MODY2. Another limitation is the inherent nature of the study, a cross-sectional design, where only an association and not a cause can be inferred, and where potential changes of the gut microbiota from a healthy state to a gut pattern potentially boosting autoimmunity in type 1 diabetes were not evaluated. Finally, although PICRUSt gives functional information of potential importance, it is a limitation compared with shotgun metagenomics analysis. On the other hand, the strengths of our study lie in the careful design, the inclusion of patients...
Author Contributions. I.L.-G. conceived the study, developed the experimental design, acquired and selected all samples used in this study, compiled the database, performed statistical analysis and data interpretation, and wrote the manuscript and provided critical revision. L.S.-A. performed laboratory assays and wrote the manuscript. B.M.-T. acquired and selected all samples used in this study, compiled the database, performed statistical analysis and data interpretation, and wrote the manuscript and provided critical revision. F.I.T. conceived the study, developed the experimental design, and wrote the manuscript and provided critical revision. J.C.F.-G. conceived the study, developed the experimental design, compiled the database, performed statistical analysis and data interpretation, and wrote the manuscript. A.U.C. acquired and selected all samples used in this study, compiled the database, and performed statistical analysis and data interpretation. M.I.-Q.-O. conceived the study, developed the experimental design, acquired and selected all samples used in this study, performed laboratory assays, compiled the database, performed statistical analysis and data interpretation, and wrote the manuscript and provided critical revision. A.U.C. conceived the study, developed the experimental design, and wrote the manuscript. A.U.C. conceived the study, developed the experimental design, and wrote the manuscript. A.U.C. conceived the study, developed the experimental design, and wrote the manuscript.

Acknowledgments. The research group belongs to the Centro de Investigación Biomédica en Red (CIBER, CB06/0018) of the Instituto de Salud Carlos III. Funding. This study was supported by the Ministerio de Educación, Cultura y Deporte (FP13/04211 to D.C.-C.), the Miguel Servet Type I program from the Instituto de Salud Carlos III (cofounded by the Fondo Europeo de Desarrollo Regional) (CP16/00163 to I.M.-I. and CP13/00065 to M.I.Q.-O.), and the Servicio Andaluz de Salud Regional (CP16/00163 to I.M.-I. and CP13/00065 to M.I.Q.-O.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

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In conclusion, our data suggest that gut microbiota in type 1 diabetes not only differs at the taxonomic level regarding MODY2 and healthy subjects but also at the functional level, involving different metabolic pathways.

Type 1 diabetes was characterized by a less diverse gut microbiota profile, with Bacteroides dominating at the phylum level and an increased proportion of proinflammatory bacteria. Therefore, the type 1 diabetes gut microbiota profile was associated with impaired epithelial integrity, low-grade inflammation, and autoimmune response. On the other hand, gut microbiota in MODY2 was characterized by a dominance of succinate-producer and mucin-degrading bacteria, potentially modulating glucose metabolism in the intestine of the host and influencing systemic energy homeostasis.

Our results provide evidence of a different microbiota profile and functionality in children with type 1 diabetes, not only in comparison with healthy subjects but fundamentally with regard to a model of nonautoimmune diabetes, suggesting a potential role of gut microbiota in the autoimmune process involved in type 1 diabetes. Given that most studies to date have shown that intestinal microbiota, rather than being involved in the initiation of the disease process of type 1 diabetes, might be involved in the progression from β-cell autoimmunity to the clinical disease (43), future longitudinal studies should be aimed at evaluating if the modulation of gut microbiota in patients with a high risk of type 1 diabetes could modify the natural history of this autoimmune disease.
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