Characteristics of gut microbiota in adult patients with type 1 and type 2 diabetes based on next-generation sequencing of the 16S rRNA gene fragment

Dominika Salamon¹, Agnieszka Sroka-Oleksiak¹,², Przemysław Kapusta³, Magdalena Szopa⁴,⁵, Sandra Mrozińska⁴,⁵, Agnieszka H. Ludwig-Słomczyńska², Paweł P. Wołkowski³, Małgorzata Bulanda⁶, Tomasz Klupa⁴,⁵, Maciej T. Malecki³,⁵, Tomasz Gosiewski¹

**INTRODUCTION**

It is believed that a specific composition of the gastrointestinal microflora ensures homeostasis of the human body.¹,² Scientific data suggest that disorders of the microbiota composition, especially in the large intestine, play a vital role in many diseases, such as inflammatory bowel disease, immune disorders, and allergies.²,³ In this context, intestinal microflora may be considered as one of the environmental factors involved in the etiopathogenesis of diabetes.⁴

Studies on patients with type 1 diabetes (T1DM) have shown differences in the composition of their gut microbiota in comparison with healthy individuals. Reduced variety and
decreased bacterial flora stability are highlight-
ed in these patients. Research on animal models
indicates a relationship between the gut micro-
biota and innate immune response in the de-
velopment of T1DM.6,7 On the other hand, a signif-
ificant factor possibly related to the development of
type 2 diabetes (T2DM) is intestinal permeability
caused by a reduced number of intestinal bacteria
producing short-chain fatty acids (SCFAs). This
leads to so called metabolic endotoxia, which is
an increase in the level of bacterial lipopolysac-
charide in serum. A reduction in the integrity of
enterocytes is also associated with so called met-
abolic bacteremia due to translocation of live bac-
teria from the intestinal lumen to the tissues of
the host. Both endotoxia and bacteremia re-
sult in low-grade chronic inflammation.8,9

It seems plausible that exploring the gut mi-
crobial profile in diabetic patients and modify-
ning their individual microbiota could bring about
either diabetes reversal or delay of its develop-
ment.6,10 However, 20% to 60% of bacteria in the
human body cannot be cultured with currently
available methods.11 Therefore, molecular tax-
onomic and phylogenetic investigations are con-
sidered most credible as they are based on nu-
cleotide sequences of marker genes (molecular
markers). The application of a high-throughput
method based on next-generation sequencing
for this purpose allows a simultaneous compre-
hensive analysis of large quantities of bacteri-
a DNA fragments. The most common molecu-
lar marker to determine the species affiliation of
a given bacterium is the gene encoding 16S ribo-
somal RNA (rRNA), or RNA molecule, a compo-
nent of the small ribosome subunit in prokary-
otic organisms.12

The objective of this study was to determine
the quantitative and qualitative composition of
the gut flora in adult patients with T1DM and
T2DM and to assess its associations with select-
ed clinical and biochemical parameters.

PATIENTS AND METHODS The study comprised
an analysis of bacterial DNA isolated from fecal samples of 68 adults (aged 20 to 65 years): 45 pa-
tients with diabetes (T1DM and T2DM groups),
hospitalized in the years 2012 to 2015 at the
Department of Metabolic Diseases, University
Hospital, Kraków, Poland, and 23 healthy vol-
unteers (control group). The inclusion criteria
for the T1DM group were as follows: clinical di-
agnosis of T1DM, insulin therapy implement-
ed in the 1st year since diagnosis, disease dura-
tion of at least 2 years; for T2DM group, clinical
diagnosis of T2DM, oral drugs administered
for at least 2 years after diagnosis, disease dura-
tion of at least 2 years; and for controls, lack of
diabetes. The exclusion criteria were: age under
20 and over 65 years, antibiotic therapy with-
in 30 days before drawing fecal samples, use of
probiotic therapy within 30 days before drawing
fecal samples, confirmed gastrointestinal infec-
tions, chronic inflammatory bowel disease (Crohn
disease, ulcerative colitis), celiac disease, active
cancer (especially gastrointestinal), congenital and acquired immune deficiencies, latent auto-
immune diabetes of adults, maturity-onset dia-
betes of the young, renal failure, cirrhosis, preg-
nancy, lack of consent to participate in the study
or withdrawal of consent during the study.

The study was performed according to the De-
claration of Helsinki and was approved by the Bio-
etical Committee of Jagiellonian University (No.
KBE/81/B/2010). All included patients provided
written informed consent to participate.

Individual stool samples were obtained from all participants and delivered for analysis in deep-
freeze conditions (–70°C). At the same time, all participants underwent routine laboratory testing
including the assessment of glycated hemoglo-
bolin A1c (HbA1c), lipid profile (total cholesterol,
high-density lipoprotein cholesterol [HDL-
-C], low-density lipoprotein cholesterol [LDL-
C], and triglyceride levels), alanine aminotransferase
(ALT) and creatinine levels, as well as estimated
glomerular filtration rate (eGFR) calculated ac-
cording to the Modification of Diet in Renal Dis-
ease Study Group formula. Age, body mass index
(BMI), and disease duration were also recorded.

Bacterial DNA was isolated from 68 fecal sam-
pies using Genomic Mini AX Stool Spin (A&A
Biotechnology, Gdańsk, Poland) according to
the method developed by Gosiewski et al.13 Ampli-
con library was subsequently created. Amplicons
of selected 16S rRNA gene regions for each sample
studied were prepared according to the protocol
for the MiSeq high-throughput sequencer (Illumi-
na, San Diego, California, United States). The se-
quencing procedure was performed according to
the methodology described by Mroziońska et al.14

Due to inaccuracy of the method and the pos-
sibility of misinterpreting the results obtained,
the microbiota composition at the species lev-
el (L7) was not assessed. A systematic bacteri-
al profile analysis was carried out at the high-
est taxonomic level (L2) and the lowest possible
in this research method (L6) to obtain a gener-
al picture and a detailed analysis of differences
in the gut microbiota composition of the sam-
ple studied.

Statistical analysis The statistical analysis was
performed using the Statistica software, ver-
sion 10 (StatSoft, Tulsa, Oklahoma, United
States). The results were presented as a mean
value (SD) for variables with normal distribu-
tion, or as a median (interquartile range) for
variables with nonnormal distribution. The dis-
tribution of variables was tested with the Sha-
piro–Wilk test. In the case of the normal dis-
tribution, homogeneity of variance was tested
using the Levene test. In the case of nonnor-
mal distribution, the Kruskal–Wallis analysis of
variance was applied for analysis of variabili-
ty between the 3 study groups. A post hoc analy-
sis was used to identify significant differences
between the groups. The power calculation for
### Table 1: Clinical data of the study groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls (n = 23)</th>
<th>T1DM group (n = 22)</th>
<th>T2DM group (n = 23)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, female/male, n</td>
<td>16/7</td>
<td>16/6</td>
<td>8/15</td>
<td>–</td>
</tr>
<tr>
<td>Age, y</td>
<td>37 (31–48)</td>
<td>36 (31–47)</td>
<td>60 (57–63)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.14 (22.1–24.9)</td>
<td>23.65 (20.96–26.23)</td>
<td>27.51 (21.5–31.6)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HbA₁c, %</td>
<td>5.4 (5.2–5.5)</td>
<td>7.75 (6.5–9.7)</td>
<td>7.3 (6.41–9.1)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.2 (5–5.8)</td>
<td>5 (4.1–5.5)</td>
<td>4.46 (3.86–5.9)</td>
<td>0.17</td>
</tr>
<tr>
<td>HDL-C, mmol/l</td>
<td>1.8 (1.5–1.9)</td>
<td>1.6 (1.5 – 2)</td>
<td>1.06 (0.8–1.2)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>LDL-C, mmol/l</td>
<td>3.2 (2.8–3.6)</td>
<td>2.8 (2.3–3.3)</td>
<td>2.8 (2.33–3.7)</td>
<td>0.29</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>17 (13–20)</td>
<td>15 (12–20)</td>
<td>24 (19–35)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.8 (0.69–1.1)</td>
<td>0.85 (0.7–1.4)</td>
<td>1.74 (1.41–2.25)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Creatinine, μmol/l</td>
<td>59 (56–65)</td>
<td>58 (59–66)</td>
<td>60 (56–64)</td>
<td>0.38</td>
</tr>
<tr>
<td>eGFR (MDRD), ml/min/1.73 m²</td>
<td>89.00 (83.00–95.00)</td>
<td>88.00 (83.5–91.00)</td>
<td>90.00 (82.00–98.5)</td>
<td>0.73</td>
</tr>
<tr>
<td>Diabetes duration, y</td>
<td>–</td>
<td>17.5 (9–25)</td>
<td>5 (2–9)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range) unless otherwise indicated. A P value of less than 0.05 is considered significant.

**a** T2DM group vs T1DM group and controls (Kruskal–Wallis test with post hoc analysis)

**b** T1DM and T2DM groups vs controls (Kruskal–Wallis test with post hoc analysis)

**c** T1DM group vs T2DM group (Mann–Whitney test)

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA₁c, glycated hemoglobin A₁c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MDRD, Modification of Diet in Renal Disease; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

1-way independent analysis of variance was 0.95. The Mann–Whitney test was applied to evaluate differences in the course of the disease between the T1DM and T2DM groups. Statistical significance of α and β diversity was calculated with the t test (parametric P values) or on the basis of the Monte Carlo permutation method (non-parametric P values). To determine the correlations of clinical data of patients with diabetes with the relative percentage of operational taxonomic units (OTUs), the Spearman rank correlation coefficient R was applied. A P value of less than 0.05 was assumed as significant.

**RESULTS** Clinical data of the groups are presented in Table 1. The 3 groups differed in terms of age, BMI, HbA₁c, ALT, HDL-C, and triglyceride levels. We did not observe any differences between the groups in creatinine, eGFR, total cholesterol, and LDL-C levels. The T1DM group was treated with insulin; the T2DM group, with metformin (all patients), sulfonylurea (13 patients), dipeptidyl peptidase-4 inhibitor (4 patients), long-acting human glucagon-like peptide 1 analogue (2 patients), and acarbose (1 patient).

The sequencing of 68 fecal samples yielded 9031 330 paired reads (mean [SD], 132 813.676 [122 510.909] paired reads per sample). The maximum number of readings per sample was 450 127 and the minimum, 16 715. The median number was 82 638.

The registered number of DNA sequences corresponded to a total of 1021 OTUs at the species level (L7), most of which have not yet been classified taxonomically. At the genus level (L6), 125 OTUs were demonstrated. At the phylum level (L2), the identified OTUs corresponded to 7 phyla belonging to the domain Bacteria and 1 phylum (Euryarchaeota) belonging to the domain Archaea. The Firmicutes bacteria constituted the majority of the microflora in stool samples in all 3 groups and (Figure 1). The remaining 4 phyla constituted only a fraction of the gut microbiota composition in the samples. A comparison of the relative percentages of the above phyla in the study group revealed differences only for Bacteroidetes (P = 0.006; Kruskal–Wallis test with post hoc analysis): T2DM group vs controls (P = 0.01) and T2DM vs T1DM groups (P = 0.02). The ratio of Firmicutes to Bacteroidetes (F/B ratio) was higher in the T2DM group (median [IQR], 72.7 [20.61–429]) than in controls (median [IQR], 15.88 [9.05–50.31]; P = 0.03) or the T1DM group (median [IQR], 16.57 [7.94–50.02]; P = 0.04). The difference was significant (P = 0.01; Kruskal–Wallis test with post hoc analysis).

At L6, the OTUs that were identified corresponded to 124 genera (domain Bacteria) and 1 genus *Methanobrevibacter* (domain Archaea). In all 3 groups, bacteria belonging to an unnamed genus in the family Ruminococcaceae were dominant and constituted the following percentage of bacterial composition: 28.89% in controls, 28.37% in the T1DM group, and 25.6% in the T2DM group. Other genera that constituted the relative percentage of the composition of at least 1% were as follows: an unnamed genus in the family Lachnospiraceae (11.58%, 6.98%, and 7.7% in the control, T1DM, and T2DM groups, respectively); an unnamed genus in an unnamed family of the order...
Next-generation sequencing for gut microbiota analysis in diabetes

Clostridiales (6.43%, 5.98%, and 7.07%, respectively); Akkermansia (5.84%, 6.45%, and 10.96%, respectively); Ruminococcus (5.3%, 6.66%, and 10.69%, respectively); Bacteroides (5.22%, 5.96%, and 2.74%, respectively); Blautia (4.86%, 7.45%, and 5.61%, respectively); an isolated, but not yet identified, genus belonging to the family Lachnospiraceae (3.23%, 1.63%, and 1.74%, respectively); Faecalibacterium (3.14%, 2.71%, and 1.62%, respectively); Bifidobacterium (2.89%, 2.68%, and 2.02%, respectively); Coprococcus (2.76%, 2.42%, and 3.49%, respectively); an unnamed genus in the family Clostridaceae (2.09%, 1.96%, and 0.88%, respectively); an isolated, but not yet identified, genus belonging to the family Clostridaceae (2.29%, 2.53%, and 0.77%, respectively); Collinsella (1.75%, 1.37%, and 3.13%, respectively); Dorea (1.24%, 1.01%, and 1.58%, respectively); and a genus with the suggested name of Ruminococcus, belonging to the family Lachnospiraceae (1.05%, 3.89%, and 1.78%, respectively). The remaining 108 OTUs (corresponding to the genus) constituted a fraction of the gut microbiota composition in the samples examined. A comparison of the relative percentages of the microbial types in the study groups showed significant differences for 10 types (TABLE 2).

An α-diversity analysis showed a slightly lower nonsignificant bacterial richness in the following samples: 1) T1DM group compared with the control group and 2) T2DM group compared with the T1DM group and with the control group. A β-diversity analysis demonstrated a smaller distance between OTUs on the phylogenetic tree, and therefore, a closer phylogenetic relationship of OTUs in the samples obtained from the control group in comparison with the T1DM group and with the T2DM group (P = 0.001; Monte Carlo permutation).

### TABLE 2

**Differences in the relative percentages of the microbial types between the study groups at the genus level (L6)**

<table>
<thead>
<tr>
<th>Genus (L6)</th>
<th>Controls (n = 23)</th>
<th>T1DM group (n = 22)</th>
<th>T2DM group (n = 23)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>g__Bacteroides</em></td>
<td>5.22%</td>
<td>5.96%</td>
<td>2.74%</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>f__Clostridiaceae; Other</em></td>
<td>2.29%</td>
<td>2.53%</td>
<td>0.77%</td>
<td>0.03&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>f__Clostridiaceae; g</em>_</td>
<td>2.09%</td>
<td>1.96%</td>
<td>0.88%</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>f__Lachnospiraceae; Other</em></td>
<td>3.23%</td>
<td>1.63%</td>
<td>1.74%</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>g__Ruminococcus</em></td>
<td>5.30%</td>
<td>6.66%</td>
<td>10.69%</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>g__Anaerostipes</em></td>
<td>0.34%</td>
<td>0.28%</td>
<td>0.21%</td>
<td>0.049&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>g__Roseburia</em></td>
<td>0.45%</td>
<td>0.29%</td>
<td>0.13%</td>
<td>0.005&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>f__Peptostreptococcaceae; g</em>_</td>
<td>0.12%</td>
<td>0.12%</td>
<td>0.03%</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>f__Enterobacteriaceae; g</em>_</td>
<td>0.05%</td>
<td>0.53%</td>
<td>0.42%</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>f__Flavobacteriaceae; g</em>_</td>
<td>0.06%</td>
<td>0.06%</td>
<td>0.02%</td>
<td>0.007&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

A P value of less than 0.05 is considered significant.

a Control group vs T1DM group vs T2DM group
b T2DM group vs T1DM group (Kruskal–Wallis test with post hoc analysis)
c T2DM group vs control group (Kruskal–Wallis test with post hoc analysis)

“Other” denotes a taxonomic unit isolated but not yet identified; no name after the character “__” denotes a taxonomic unit identified but still unnamed.

Abbreviations: f__, family; g__, genus; others, see TABLE 1

Abbreviations: f__, family; g__, genus; others, see TABLE 1
The F/B ratio analysis did not show any significant correlations with clinical data in any of the groups. In the T1DM group, correlations were observed at the genus level (L6) between selected bacteria and age, HbA1c, total cholesterol, and HDL-C levels, as well as diabetes duration (TABLE 3). No correlations were observed between the presence of bacteria and BMI or ALT. In the T2DM group, correlations were observed at the genus level (L6) between selected bacteria and age, BMI, LDL-C, and triglycerides (TABLE 4). There was no correlation between the presence of bacteria and total cholesterol, HDL-C, and ALT levels as well as diabetes duration.

Both T1DM and T2DM groups demonstrated a positive correlation between HDL-C levels and bacteria at the level of the genus Bifidobacterium (R = 0.43, P = 0.04 for T1DM and R = 0.43, P = 0.04 for T2DM).

**DISCUSSION**

Numerous studies on gut microbiota in patients with diabetes focused on individuals with only one type of the disease: the population with T2DM was most frequently represented by patients with newly diagnosed diabetes,\textsuperscript{15,16} while research concerning T1DM was commonly conducted among children\textsuperscript{17,18} whose gut microbiota is still not yet formed or stable. The current study compared bacterial profiles in the large intestine in adult patients with the diagnosis of T1DM or T2DM who underwent treatment and follow-up of at least several months in duration. We used methods allowing a detection of nonculturable microorganisms, which were previously unknown, in the human colon. It is one of the first studies in Poland presenting results on the microflora of the human gastrointestinal tract, obtained by next-generation sequencing.

The composition of the intestinal microbiota is affected by many factors, such as the genetic status, place of residence (continent, climate), age, or diet.\textsuperscript{1,16,19,20} Our patients were unrelated to one another but they all came from the same geographical region (the south of Poland). Our study groups differed in terms of age, but there is no conclusive evidence pointing to age as an independent factor influencing the composition of the gut microbiota. However, there are studies confirming such a relationship when comparing young adults with centenarians.\textsuperscript{19} American studies on 37 adults followed for up to 296 weeks indicated stable intestinal microbiota composition in this population, which proves that time is probably not a crucial factor affecting the gut microflora.\textsuperscript{21} Fecal samples in our study came from patients with T2DM, who were no older than 65 years, and as this disease type is diagnosed in middle-aged patients (more commonly aged over 45 years), the youngest participant in the study, who at the same time did not meet any exclusion criteria, already reached the age of 40. It was difficult to recruit controls at a similar age because the candidates frequently met the exclusion criteria and could not be enrolled in the study.

The results of microbiological testing of fecal samples revealed quantitative and qualitative differences in the composition of the gut microbiota between the 3 groups studied. This was especially visible in the bacterial profile analysis for T2DM patients (FIGURE 1). The predominant phylum of bacteria (L2 level) in all 3 groups was Firmicutes. It is a large group of Gram-positive bacteria, which includes both anaerobic bacilli (eg, Clostridium) as well as aerobic, or relatively anaerobic, cocci (eg, Staphylococcus). Other phyla were: Bacteroidetes (eg, Gram-negative anaerobic bacilli Bacteroides), Verrucomicrobia (eg, Gram-negative anaerobic oval-shaped bacteria Akkermansia), and Actinobacteria (eg, Gram-positive anaerobic bacilli Bifidobacterium). Our results are consistent with observations reported by other authors.\textsuperscript{3,24-26}

The F/B ratio in T2DM patients was significantly higher than in the other groups. There are reports suggesting that a change in the F/B ratio is associated with either an increase in glycemia or calorie intake and weight gain. As for the former, there is a decrease in the number of Firmicutes in favor of Bacteroidetes;\textsuperscript{27} as for the latter, it is the opposite: the number of Firmicutes increases.\textsuperscript{1,16} A limitation of our study is the lack of nutritional data for participants. Unfortunately,

**TABLE 3** Correlations between clinical data and the presence of bacteria at the genus level (L6) in patients with type 1 diabetes

<table>
<thead>
<tr>
<th>Operational taxonomic unit</th>
<th>R</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g__Streptococcus</td>
<td>-0.53</td>
<td>0.01</td>
</tr>
<tr>
<td>c__Mollicutes:o_RF39:f__:g__</td>
<td>0.50</td>
<td>0.02</td>
</tr>
<tr>
<td>HbA1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f__Erysipelotrichaceae;g__</td>
<td>-0.52</td>
<td>0.01</td>
</tr>
<tr>
<td>g__Desulfovibrio</td>
<td>0.51</td>
<td>0.01</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f__Pseudomonadaceae;g__</td>
<td>0.55</td>
<td>0.01</td>
</tr>
<tr>
<td>f__Lachnospiraceae;g__ [Ruminococcus]</td>
<td>0.52</td>
<td>0.01</td>
</tr>
<tr>
<td>HDL-C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f__Pseudomonadaceae;g__</td>
<td>0.64</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f__Ruminococcaceae;g__</td>
<td>-0.51</td>
<td>0.01</td>
</tr>
<tr>
<td>o__Clostridiales;Other;Other</td>
<td>-0.50</td>
<td>0.02</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g__Atopobium</td>
<td>0.56</td>
<td>0.003</td>
</tr>
<tr>
<td>f__Gemellaceae;g__</td>
<td>0.51</td>
<td>0.01</td>
</tr>
</tbody>
</table>

A P value of less than 0.05 is considered significant.

“Other” denotes a taxonomic unit isolated but not yet identified; no name after the character “_” denotes a taxonomic unit identified but still unnamed; the name in square brackets denotes suggested name (based on the analysis of the phylogenetic tree), but not yet verified, for an already identified taxonomic unit.

Abbreviations: c__ class; o__ order; others, see TABLES 1 and 2.
TABLE 4 Correlations between clinical data and the presence of bacteria at the genus level (L6) in patients with type 2 diabetes

<table>
<thead>
<tr>
<th>Operational taxonomic unit</th>
<th>R</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g__ Akkermansia</td>
<td>−0.56</td>
<td>0.003</td>
</tr>
<tr>
<td>f__ Caulobacteraceae;g__</td>
<td>−0.52</td>
<td>0.01</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f__ Ruminococcaceae;g__</td>
<td>−0.51</td>
<td>0.01</td>
</tr>
<tr>
<td>g__ Streptococcus</td>
<td>−0.50</td>
<td>0.01</td>
</tr>
<tr>
<td>HbA1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g__ Faecalibacterium</td>
<td>−0.61</td>
<td>0.001</td>
</tr>
<tr>
<td>g__ Collinsella</td>
<td>−0.61</td>
<td>0.001</td>
</tr>
<tr>
<td>f__ Flavobacteriaceae;g__</td>
<td>0.61</td>
<td>0.002</td>
</tr>
<tr>
<td>o__ Clostridales;f__ [Tissierellaceae];g__ Parvimonas</td>
<td>−0.60</td>
<td>0.002</td>
</tr>
<tr>
<td>c__ Mollicutes;o_RF39;f__g__</td>
<td>−0.57</td>
<td>0.003</td>
</tr>
<tr>
<td>g__ Bulleidia</td>
<td>−0.54</td>
<td>0.006</td>
</tr>
<tr>
<td>LDL-C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g__ Enterococcus</td>
<td>0.56</td>
<td>0.004</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g__ Atopobium</td>
<td>0.56</td>
<td>0.003</td>
</tr>
<tr>
<td>f__ Gemellaceae;g__</td>
<td>0.51</td>
<td>0.01</td>
</tr>
</tbody>
</table>

A P value of less than 0.05 is considered significant.

“Other” denotes a taxonomic unit isolated but not yet identified; no name after the character “__” denotes a taxonomic unit identified but still unnamed; the name in square brackets denotes suggested name (based on the analysis of the phylogenetic tree), but not yet verified, for an already identified taxonomic unit.

Abbreviations: see TABLES 1, 2, and 3

During sample collection, it was not possible to perform objective tests, and a detailed diet questionnaire turned out to be subjective. However, on the basis of geographic and cultural homogeneity of the population, we could assume that their nutrition followed the so called Western pattern diet. Considering the clinical data available in our study, including BMI in the T2DM group (indicating overweight or obesity), we can hypothesize that it was weight gain, and not diabetes itself, that contributed to the decrease in the number of Bacteroidetes and a high F/B ratio. This is in line with a study by Turnbaugh et al. who found a similar tendency for the intestinal microbiota in slim and obese twins.

At L6, out of the 10 genera of bacteria whose relative percentages differed between our study groups, Bacteroides and Roseburia are particularly interesting. A positive correlation between the number of bacteria of the genus Bacteroides and a negative relationship between the F/B ratio and patient age were reported, but another study did not confirm such a relationship between the number of Bacteroides and age or event reported conflicting results. In our study, stool samples classified into T2DM came from patients older in comparison with the control and T1DM groups. But the number of Bacteroides in this group was lower vs control and T1DM groups, which might have been caused by a higher BMI in T2DM. Studies on species representing the genus Bacteroides, Bacteroides thetaiotaomicron and Bacteroides fragilis, emphasize their important regulatory and anti-inflammatory role. Smaller numbers of bacteria from this genus (as T2DM in our study) probably contribute to the development of metabolic endotoxemia and chronic inflammation, which in turn can lead to obesity, de novo triglyceride synthesis, and insulin resistance. On the other hand, obesity itself is associated with plasma lipopolysaccharide, the major component of the outer membrane of Gram-negative bacteria. This contributes to the development of low-grade chronic inflammation as well as intestinal permeability. These phenomena were shown to be facilitated by a decrease in the number of bacteria of the genus Bifidobacterium in the human large intestine, which participate in maintaining the appropriate intestinal wall permeability and they also have the ability to neutralize some Gram-negative bacteria by disturbing the continuity of their outer membrane. T2DM patients in our study were overweight and had hyperglycemia and dyslipidemia. Their relative percentage of Gram-negative bacteria belonging to the family Enterobacteriaceae was higher in comparison with the control group. Many bacterial species from this family are pathogenic to humans. Hence, our results seem to confirm the relationship of obesity, low-grade chronic inflammation, and insulin resistance with the composition of the gut microbiota.

Some studies reported the special role of bacteria from the genus Roseburia (especially Roseburia intestinalis) or from the genus Faecalibacterium (especially Faecalibacterium prausnitzii) in maintaining intestinal wall integrity. These bacteria produce SCFAs, including butyrate. A reduced amount of these microbes was observed particularly in patients with T2DM, but also in those with T1DM. We found a lower relative percentage of bacteria from the genus Roseburia in samples from patients with T2DM than in controls and also the lowest relative percentage (but with no statistical significance) of bacteria from the genus Faecalibacterium in this group, which is in line with the reports of other authors.

Recent animal and human studies on the genus Akkermansia, belonging to the phylum Verrucomicrobia, especially the species Akkermansia muciniphila, which degrades mucin in the mucous membrane of the intestinal wall, have reported a negative correlation between the presence of this Gram-negative bacterium and overweight, T2DM, and T1DM. However, there are individual reports indicating an inverse relationship. Research on animal model indicated that metformin therapy may affect the growth of the relative percentage of microorganisms from the genus Akkermansia. The impact of metformin on the human gut microbiota composition was revealed by Forslund et al. In our study, the relative proportion of the genus Akkermansia was...
the highest in the T2DM group, in which all patients were treated with metformin.

We determined that the microflora composition of fecal samples in T1DM did not differ significantly from the microbiota composition in controls. The age difference between the control and T1DM groups vs the T2DM group probably did not have a decisive influence on the bacterial profile, as was demonstrated above. Nonetheless, the percentage of HbA1c was higher in T1DM and T2DM groups than in controls, and also the disease duration, which was longer in T1DM than T2DM, allowed us to expect bacterial profile similarities in the T1DM and T2DM groups. The reason behind the similarity between T1DM and control groups might have been the treatment for this type of diabetes. All patients started getting insulin as soon as they were diagnosed, which in most cases was in childhood and that could have facilitated the adoption of the necessary habits, including glycemic control and efficient modification of insulin dosage. This hypothesis is corroborated by Stewart et al who investigated gut microbiota in adult patients with T1DM with a 5-year and over 12-year disease duration. Good glycemic control and increased physical fitness of T1DM patients contributed to no differences in the gut microbiota between the study and control groups.

We established that a diversity (variety of microorganisms within a single sample) did not differ between our study groups. A number of studies indicated a lower α diversity in obese patients, but also in patients with T2DM, which was confirmed by Mrozińska et al who studied patients with type 2 diabetes and HNF1A-MODY. However, other studies did not corroborate these findings. Furthermore, a Danish study on men revealed that this diversity was lower in people with T2DM than in controls, but among them, it was slightly higher in men with a body mass index higher than 31 kg/m² than in slim patients with diabetes. However, the analysis of β diversity (diversity of microorganisms between individual samples in all 3 groups) was lower in the control group in our study, which meant a smaller distance between individual microbes and made this group more homogeneous.

We observed several correlations between microbiological test results for fecal samples and clinical data of patients with T1DM and T2DM, particularly at L6. The most interesting is the negative correlation between the number of bacteria of the genus Faecalibacterium with HbA1c in the T2DM group, which confirms that hyperglycemia and T2DM are associated with a smaller amount of bacteria producing SCFAs, including butyrate.

We also observed a positive correlation between bacteria from the genus Bifidobacterium with the HDL-C level in both T1DM and T2DM groups. Research on animal model (rodents on a high-fat diet) showed that the use of chosen bacterial strains from the genus Bifidobacterium resulted in reduced serum total cholesterol and LDL-C levels, and a simultaneous increase in HDL-C levels. The positive correlation of the genus Bifidobacterium with HDL-C levels in our T1DM and T2DM groups appears to confirm the above observations. Perhaps the use of probiotic preparations with selected species of the genus Bifidobacterium would result in an improvement of the lipid profile in patients with diabetes.

In our study, we used molecular testing for microbial identification; the lowest taxonomic level allowing to obtain reliable results is the genus level (L6). It is possible that the analysis of entire microbial genomes would enable a microbiome assessment at L7 (species) and would yield more reliable and comprehensive data on correlations. Nonetheless, the results of our observations confirm the fact that the bacteria making up the human intestinal microbiota play a vital role and could possibly prove beneficial in long-term glycemic control in patients with diabetes.

The development of a method for quick and repetitive bacterial intestinal profile marking could also help in designing therapy using probiotics, the bacterial profile of which could be tailored to the individual patient’s needs. Such a possibility is indicated by the concept of next-generation probiotics, which assumes the application not only of the bacteria of the genus Lactobacillus and Bifidobacterium, but above all, of other microorganisms (such as Faecalibacterium or Akkermansia), which until now have not been classified as probiotics. Their properties could be used for individualized therapy modifying the composition of the intestinal microbiota. It would constitute a new method for preventing or treating the complications of diabetes.

ACKNOWLEDGMENTS We would like to thank participants in this study. The study was carried out as part of the project entitled “Evaluation of the microbiota in the gastrointestinal tract of the patients with diabetes type 1 and 2 and with morbid or pathological obesity undergoing laparoscopic sleeve gastrectomy”, supported by the National Science Center in Poland: “SONATA” (No. DEC-2011/03/D/NZ5/00 551; to TG).

CONTRIBUTION STATEMENT DS contributed to study design, acquisition and interpretation of data, drafting the article, and writing the manuscript. AS-O contributed to study design, acquisition and interpretation of data. PK performed statistical analysis. MS contributed to acquisition and interpretation of data. SM was involved in data collection. AHL-S, PPW, MB, and TK interpreted the data. MTM contributed to study design, revising the manuscript for important intellectual content, interpretation of data, and approval of the final version of the manuscript. TG contributed to study design, project coordination, interpretation of data, writing the manuscript, and approval of the final version of the manuscript.
OPEN ACCESS This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License (CC BY-NC-SA 4.0), allowing third parties to copy and redistribute the material in any medium or format and to remix, transform, and build upon the material, provided the original work is properly cited, distributed under the same license, and used for non-commercial purposes only. For commercial use, please contact the journal office at pmw@mp.pl.

REFERENCES