ORIGINAL ARTICLE

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

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KEY WORDS

diabetes, gut microbiota, sequencing

ABSTRACT

INTRODUCTION Scientific data indicate a possible influence of gut microbiota on the development of type 1 and type 2 diabetes mellitus (T1DM and T2DM, respectively). Sequence analysis of 16S ribosomal RNA identified several hundred bacterial species of the intestinal ecosystem, most of which cannot be cultured.

OBJECTIVES We aimed to evaluate gut microbiota composition in adult patients with T1DM and T2DM and establish a link between microbiological test results and patients' clinical data.

PATIENTS AND METHODS We examined DNA isolated from fecal samples in 3 groups: healthy volunteers (n = 23), patients with T1DM (n = 22), and patients with T2DM (n = 23). Next-generation sequencing was performed on the MiSeq platform.

RESULTS At the phylum level, the Firmicutes bacteria prevailed (>77%) in all groups. At the taxonomic levels L2 (phylum) and L6 (genus), significant differences were demonstrated in bacterial profiles, particularly in the T2DM group. A negative correlation was observed between several genera of bacteria and the percentage of glycated hemoglobin A_{1c} in the T2DM group, while a positive correlation was revealed between bacteria belonging to the genus *Bifidobacterium* and high-density lipoprotein cholesterol levels in both T1DM and T2DM groups.

CONCLUSIONS Our results provide grounds for conducting research in the field of gut microbiota in order to develop individualized therapy for patients with diabetes based on modifying the microbiota composition, as a new method for controlling glycemia. Next-generation sequencing allows a rapid identification of the DNA of all bacteria present in the sample and their taxonomic classification.

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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.[2-4] In this context, intestinal microflora may be considered as one of the environmental factors involved in the etiopathogenesis of diabetes.[5]

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 highlighted in these patients. Research on animal models indicates a relationship between the gut microbiota and innate immune response in the development of T1DM.[6,7] On the other hand, a significant 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 endotoxemia, which is an increase in the level of bacterial lipopolysaccharide in serum. A reduction in the integrity of enterocytes is also associated with so called metabolic bacteremia due to translocation of live bacteria from the intestinal lumen to the tissues of the host. Both endotoxemia and bacteremia result in low-grade chronic inflammation.[1,8]

It seems plausible that exploring the gut microbial profile in diabetic patients and modifying their individual microbiota could bring about either diabetes reversal or delay of its development.[8-10] However, 20% to 60% of bacteria in the human body cannot be cultured with currently available methods.[11] Therefore, molecular taxonomic and phylogenetic investigations are considered most credible as they are based on nucleotide sequences of marker genes (molecular markers). The application of a high-throughput method based on next-generation sequencing for this purpose allows a simultaneous comprehensive analysis of large quantities of bacterial DNA fragments. The most common molecular marker to determine the species affiliation of a given bacterium is the gene encoding 16S ribosomal RNA (rRNA), or RNA molecule, a component of the small ribosome subunit in prokaryotic 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 selected 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 patients 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 volunteers (control group). The inclusion criteria for the T1DM group were as follows: clinical diagnosis of T1DM, insulin therapy implemented in the 1st year since diagnosis, disease duration of at least 2 years; for T2DM group, clinical diagnosis of T2DM, oral drugs administered for at least 2 years after diagnosis, disease duration of at least 2 years; and for controls, lack of diabetes. The exclusion criteria were: age under 20 and over 65 years, antibiotic therapy within 30 days before drawing fecal samples, use of probiotic therapy within 30 days before drawing fecal samples, confirmed gastrointestinal infections, chronic inflammatory bowel disease (Crohn disease, ulcerative colitis), celiac disease, active cancer (especially gastrointestinal), congenital and acquired immune deficiencies, latent autoimmune diabetes of adults, maturity-onset diabetes of the young, renal failure, cirrhosis, pregnancy, lack of consent to participate in the study or withdrawal of consent during the study.

The study was performed according to the Declaration of Helsinki and was approved by the Bioethical Committee of Jagiellonian University (No. KBET/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 hemoglobin A_{1c} (HbA_{1c}), 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 according to the Modification of Diet in Renal Disease Study Group formula. Age, body mass index (BMI), and disease duration were also recorded.

Bacterial DNA was isolated from 68 fecal samples using Genomic Mini AX Stool Spin (A&A Biotechnology, Gdańsk, Poland) according to the method developed by Gosiewski et al.[13] Amplicon 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 (Illumina, San Diego, California, United States). The sequencing procedure was performed according to the methodology described by Mrozińska et al.[14]

Due to inaccuracy of the method and the possibility of misinterpreting the results obtained, the microbiota composition at the species level (L7) was not assessed. A systematic bacterial profile analysis was carried out at the highest taxonomic level (L2) and the lowest possible in this research method (L6) to obtain a general picture and a detailed analysis of differences in the gut microbiota composition of the samples studied.

Statistical analysis

The statistical analysis was performed using the Statistica software, version 10 (StatSoft, Tulsa, Oklahoma, United States). The results were presented as a mean value (SD) for variables with normal distribution, or as a median (interquartile range) for variables with nonnormal distribution. The distribution of variables was tested with the Shapiro–Wilk test. In the case of the normal distribution, homogeneity of variance was tested using the Levene test. In the case of nonnormal distribution, the Kruskal–Wallis analysis of variance was applied for analysis of variability between the 3 study groups. A post hoc analysis was used to identify significant differences between the groups. The power calculation for

TABLE 1 Clinical data of the study groups

Parameter	Controls (n = 23)	T1DM group (n = 22)	T2DM group (n = 23)	*P* value
Sex, female/male, n	16/7	16/6	8/15	–
Age, y	37 (31–48)	36 (31–47)	60 (57–63)	<0.001[a]
BMI, kg/m^2	23.14 (22.1–24.9)	23.65 (20.96–26.23)	27.51 (25.1–31.6)	<0.001[a]
HbA_{1c}, %	5.4 (5.2–5.5)	7.75 (6.5–9.7)	7.3 (6.41–9.1)	<0.001[b]
Total cholesterol, mmol/l	5.2 (5–5.8)	5 (4.1–5.5)	4.46 (3.86–5.9)	0.17
HDL-C, mmol/l	1.8 (1.5–1.9)	1.6 (1.5 – 2)	1.06 (0.8–1.2)	<0.001[a]
LDL-C, mmol/l	3.2 (2.8–3.6)	2.8 (2.3–3.3)	2.8 (2.33–3.7)	0.29
Triglycerides, mmol/l	0.8 (0.69–1.1)	0.85 (0.7–1.4)	1.74 (1.41–2.25)	<0.001[a]
ALT, U/l	17 (13–20)	15 (12–20)	24 (19–35)	<0.001[a]
Creatinine, µmol/l	59 (56–65)	58 (59–66)	60 (56–64)	0.38
eGFR (MDRD), ml/min/1.73 m^2	89.00 (83.00–95.00)	88.00 (83.5–91.00)	90.00 (82.00–98.5)	0.73
Diabetes duration, y	–	17.5 (9–25)	5 (2–9)	<0.001[c]

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; HbA_{1c}, glycated hemoglobin A_{1c}; 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 (nonparametric *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.

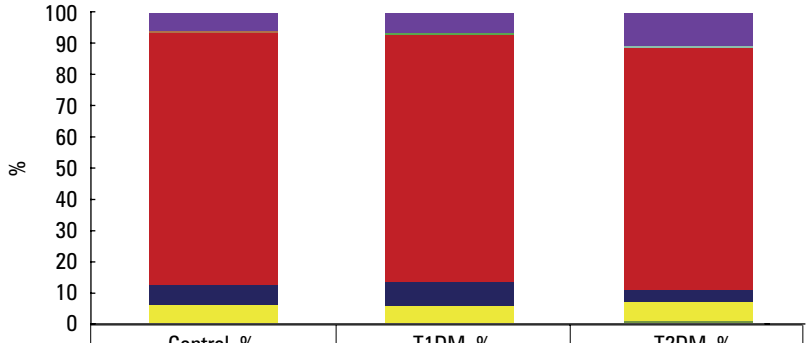
RESULTS Clinical data of the groups are presented in TABLE 1. The 3 groups differed in terms of age, BMI, HbA_{1c}, ALT, HDL-C, and triglyceride levels. We did not observe any differences between the groups in creatinine, eGFR, total cholesterol, and LDL-C levels. The T1DM group was treated with insulin; 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 9031330 paired reads (mean [SD], 132 813.676 [112 510.909] 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 consituted 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

FIGURE 1 Relative percentage distribution of bacteria at the phylum level (L2) in the study groups



	Control, %	T1DM, %	T2DM, %
Verrucomicrobia	5.843	6.450	10.961
Tenericutes	0.072	0.028	0.034
Proteobacteria	0.260	0.579	0.468
Fusobacteria	0.002	0.004	0.001
Firmicutes	81.512	79.297	77.894
Bacteroidetes	6.426	8.222	3.630
Actinobacteria	5.771	5.204	6.542
Euryarchaeota	0.114	0.216	0.469

TABLE 2 Differences in the relative percentages of the microbial types between the study groups at the genus level (L6)

Genus (L6)	Controls (n = 23)	T1DM group (n = 22)	T2DM group (n = 23)	*P* value
g__*Bacteroides*	5.22%	5.96%	2.74%	0.02[a,c]
f__Clostridiaceae; Other	2.29%	2.53%	0.77%	0.03[a] 0.006[b] 0.01[c]
f__Clostridiaceae; g__	2.09%	1.96%	0.88%	0.02[a] 0.04[b]
f__Lachnospiraceae; Other	3.23%	1.63%	1.74%	0.02[a,c]
g__*Ruminococcus*	5.30%	6.66%	10.69%	0.02[a] 0.04[b,c]
g__*Anaerostipes*	0.34%	0.28%	0.21%	0.049[a] 0.04[c]
g__*Roseburia*	0.45%	0.29%	0.13%	0.005[a] 0.003[c]
f__Peptostreptococcaceae;g_	0.12%	0.12%	0.03%	0.001[a] 0.003[b] 0.02[c]
f__Enterobacteriaceae;g__	0.05%	0.53%	0.42%	0.001[a,c]
f__Flavobacteriaceae;g__	0.06%	0.06%	0.02%	0.007[a,c]

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

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 Clostridiaceae (2.09%, 1.96%, and 0.88%, respectively); an isolated, but not yet identified, genus belonging to the family Clostridiaceae (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 3 Correlations between clinical data and the presence of bacteria at the genus level (L6) in patients with type 1 diabetes

Operational taxonomic unit	*R*	*P* value
Age		
g__*Streptococcus*	–0.53	0.01
c_Mollicutes;o_RF39;f_;g_	0.50	0.02
HbA_{1c}		
f__Erysipelotrichaceae;g__	–0.52	0.01
g__*Desulfovibrio*	0.51	0.01
Total cholesterol		
f__Pseudomonadaceae;g__	0.55	0.01
f__Lachnospiraceae;g__[*Ruminococcus*]	0.52	0.01
HDL-C		
g__*Staphylococcus*	0.56	0.005
LDL-C		
f__Pseudomonadaceae;g__	0.64	0.001
Triglycerides		
f__Ruminococcaceae;g__	–0.51	0.01
o__Clostridiales;Other;Other	–0.50	0.02
Diabetes duration		
g__*Atopobium*	0.56	0.003
f__Gemellaceae;g__	0.51	0.01

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

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, HbA_{1c}, 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* ($R = 0.4$, $P = 0.03$ 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,[15,16] while research concerning T1DM was commonly conducted among children[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.[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.[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.[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),[22,23] 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.[9,24-26]

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[27]; as for the latter, it is the opposite: the number of Firmicutes increases.[1,16] A limitation of our study is the lack of nutritional data for participants. Unfortunately,

TABLE 4 Correlations between clinical data and the presence of bacteria at the genus level (L6) in patients with type 2 diabetes

Operational taxonomic unit	R	*P* value
Age		
g__*Akkermansia*	–0.56	0.003
f__Caulobacteraceae;g__	–0.52	0.01
BMI		
f__Ruminococcaceae;g__	–0.51	0.01
g_*Streptococcus*	–0.50	0.01
HbA_{1c}		
g__*Faecalibacterium*	–0.61	0.001
g__*Collinsella*	–0.61	0.001
f__Flavobacteriaceae;g__	0.61	0.002
o__Clostridiales;f__[*Tissierellaceae*];g__*Parvimonas*	–0.60	0.002
c__Mollicutes;o__RF39;f__;g__	–0.57	0.003
g__*Bulleidia*	–0.54	0.006
LDL-C		
g__*Enterococcus*	0.56	0.004
Triglycerides		
g__*Atopobium*	0.56	0.003
f__Gemellaceae;g__	0.51	0.01

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[20] 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,[28] but another study did not confirm such a relationship between the number of *Bacteroides* and age or event reported conflicting results.[19] 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*[29] and *Bacteroides fragilis*,[30] 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.[1,31] 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.[1,5] 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.[1,8] A reduced amount of these microbes was observed particularly in patients with T2DM,[10,26,27] but also in those with T1DM.[32] 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 percetage (but with no statistical significance) of bacteria from the genus *Faecalibacterium* in this group, which is in line with the reports of other authors.[10,26,27]

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.[10,27] However, there are individual reports indicating an inverse relationship.[33] Research on animal model indicated that metformin therapy may affect the growth of the relative percetage of microorganisms from the genus *Akkermansia*.[34,35] The impact of metformin on the human gut microbiota composition was revealed by Forslund et al.[36] 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 HbA_{1c} 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[23] 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[37] 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 α 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,[20,38,39] but also in patients with T2DM, which was confirmed by Mrozińska et al[14] who studied patients with type 2 diabetes and HNF1A-MODY. However, other studies did not corroborate these findings.[26,40] 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/m^2 than in slim patients with diabetes.[41] 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 HbA_{1c} in the T2DM group, which confirms that hyperglycemia and T2DM are associated with a smaller amount of bacteria producing SCFAs, including butyrate.[1,8,27,39]

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.[42,43] 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.[8]

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.[44] 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.

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