Quantitative evaluation of the genus *Bifidobacterium* in stool samples of patients with type 1 and 2 diabetes

**Introduction.** There is evidence of the existence of quantitative changes in the microbiome, including *Bifidobacterium* spp., due to some chronic diseases, such as liver cirrhosis, inflammatory bowel diseases, obesity, or celiac disease.

**Materials and Methods.** We aimed to examine the number of *Bifidobacterium* and total bacteria present in the colon of patients with type 1 diabetes (T1DM) and type 2 diabetes (T2DM), as well as in healthy subjects. DNA was extracted from patients’ fecal samples and then amplified by real-time PCR to determine the number of *Bifidobacterium* and total bacteria. Statistical association with selected clinical and biochemical features was examined.

**Results.** The mean numbers of bacteria belonging to the genus *Bifidobacterium* in T1DM and T2DM were lower compared to the control group (p = 0.006, p < 0.001 respectively). There were no statistical differences in the total number of bacteria between all groups (p = 0.397). In the T1DM group, a significant correlation was detected between the number of bifidobacteria and age (r = 0.441, p = 0.010), as well as bifidobacteria and alanine aminotransferase (p = 0.022, r = -0.11). In the group T2DM, a correlation was observed between triglycerides and bifidobacteria (p < 0.001, r = -0.61). Moreover, we have found a negative correlation between HBA1, glucose level, and bifidobacteria (r = -0.35, p < 0.001 and r = -0.024, p = 0.019, respectively).

**Conclusions.** The quantitative composition of *Bifidobacterium* is lower in T1DM and T2DM patients compared to the healthy controls. Further studies are needed to clarify the relationship between the number of these bacteria and elements of the clinical picture of T1DM.

**Keywords**

*bifidobacteria* • biochemical parameters • diabetes • intestinal microbiota • quantitative composition • real-time PCR

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**1. Introduction**

Some research studies have revealed that abnormalities in *Bifidobacterium* spp. levels, including their reduction or disappearance, are observed in some chronic diseases, such as chronic hepatitis B, liver cirrhosis [1], inflammatory bowel disease [2, 3] celiac disease [4], autism [5], or obesity [6, 7]. These fluctuations might be observed in the beginning of a disease or later in the course of a disease, but it is still not clear whether they are a cause or consequence of these diseases. Based on these observations, the number of *Bifidobacterium* cells per gram of feces seems to be a biomarker, which could also be useful in the course of other diseases associated with changes in the microbiome. Diabetes and obesity are currently widespread public health problems. Multiple environmental and genetic factors play an important role in the development and progression of type 1 (T1DM) and type 2 diabetes (T2DM) (Figure 1).

Interestingly, a number of studies have demonstrated that the microbiota of the gastrointestinal tract may be one of these key contributors [8–10]. Due to the beneficial role of *Bifidobacterium* (which contributes to modulation of the intestinal microbiota, cholesterol reduction, and alleviation of the clinical symptoms of lactose intolerance), it is important to assess if the imbalance in the fecal concentration of these bacteria is also present in diabetic patients, and how intestinal bifidobacteria differ quantitatively between patients with different types of diabetes and non-diabetic individuals. In this study, we carried out an investigation intended to help prove our above assumptions. We aimed to assess the number of *Bifidobacterium* spp.,
the genus most often described in the literature, as well as total bacteria present in the colon of patients with T1DM and T2DM as well as in healthy subjects.

2. Materials and Methods

2.1. Patients and samples
The study included 64 patients admitted to the Department of Metabolic Diseases, University Hospital, Krakow, Poland (33 with T1DM, 31 with T2DM), according to the inclusion criteria: age 30–65 years, clinical diagnosis of T1DM or T2DM, and disease duration of at least 2 years. The exclusion criteria included: antibiotic therapy up to 30 days prior to entering the study, and the use of probiotic preparations up to 30 days before entering the study. The control group consisted of 33 healthy individuals, who had not been treated with antibiotics in the period of at least 30 days before collecting the fecal sample, had not taken probiotic preparations, and did not have reported or clinically confirmed gastrointestinal symptoms. All study participants gave written consent to use the data obtained from the biological materials.

The tested materials were fecal samples. Additionally, blood samples were collected in order to evaluate the following parameters: glycated hemoglobin (HbA1c), serum fasting glucose levels, lipid profile (HDL, LDL, total cholesterol, triglycerides [TGs]), and alanine transaminase (ALT). All analysis from blood samples was performed in specialized laboratories of the University Children’s Hospital in Krakow according to routine laboratory procedures designed for medical diagnosis in accordance with GPL and IVD/FDA standards. Fecal samples were transported under deep-freeze conditions to the laboratory at the Department of
Microbiology, Jagiellonian University Medical College. Bacterial DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN, GmbH, Germany) with our modified preliminary procedure [11, 12], involving mechanical lysis using the FastPrep homogenizer (MP Biomedicals, California, USA) and enzymatic lysis with mutanolysin (A&A Biotechnology, Gdansk, Poland), lysozyme (Sigma, Saint Louis, USA), and lysostaphin (A&A Biotechnology, Gdansk, Poland). Obtained DNA isolates were used for quantitative real-time PCR amplification (qPCR) to estimate the number of bifidobacteria and the total number of bacterial cells in 1 gram of the fecal samples.

3. Quantitative real-time PCR

Specific primers targeting *Bifidobacterium* spp. [13] and total bacteria [14] as well as the reaction mixture and the amplification program are shown in Table 1. The bacterial number was calculated by comparing the C_\text{T} values to standard curves, prepared by a serial dilution of *Bifidobacterium longum* DSM 20104 and *Escherichia coli* ATCC 25922. *E. coli* reference strain was selected as an example of representative species for total bacteria. We relied on the assumption that primers directed to the universal 16S rRNA sequence (also present in *E. coli*) would allow the detection of all bacteria in the sample. *E. coli* was used as a model microorganism to prepare the calibration curve for qPCR.

The reactions were conducted with a negative control containing water instead of DNA and a positive control containing the DNA of the appropriate reference strain of the species. Each sample was tested in duplicate.

3.1. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 28. Differences between the studied groups were analyzed with a one-way ANOVA test. In the case of the ANOVA test, the assumption regarding the homogeneity of variance was not met; therefore, Welch’s F correction was applied. Correlations between biochemical parameters in diabetic patients and the logarithm of number of bacteria in each group was calculated with a Spearman correlation test. In every case, p-values < 0.05 were considered statistically significant. All data shown are mean ± standard deviation.

4. Results

Anthropometric and clinical characterizations of the examined study groups, and also the mean number of bacteria belonging to *Bifidobacterium* spp., and total bacteria in the research groups, are presented in Table 2.

The number of *Bifidobacterium* was significantly lower in patients with type 1 and type 2 diabetes compared to the control group (p = 0.006, p < 0.001 respectively). There was no statistical significance when comparing the total number of bacteria between the studied patient groups and the control group.

In the T1DM group, a positive correlation was found between the number of *Bifidobacterium* spp. cells and age (r = 0.441, p = 0.010), and a negative correlation was found between bifidobacteria and ALT (p = 0.022, r = -0.11). In the T2DM group, a negative correlation was observed between triglycerides and bifidobacteria (p < 0.001, r = -0.61). Moreover, we found a negative correlation between HBA1, serum fasting glucose level, and bifidobacteria (r = -0.35; p < 0.001; r = -0.024, p = 0.019 respectively) for all patients in total.

5. Discussion

In this study, we carried out an assessment of the number of bacteria belonging to the genus *Bifidobacterium* and total bacteria cluster in fecal samples of T1DM and T2DM patients, as well as of healthy non-diabetic individuals. Recruited patients came from the same geographical region (the south of Poland) and were not related. Our study groups differed in age; however, type 1 diabetes usually develops in younger people than type 2 diabetes. Therefore, it was difficult to recruit similar groups in terms of this parameter. Moreover, the groups were different in the mean value of biochemical parameters (Table 2); however, this is due to the fact that we studied two different disease entities and a control group. It is obvious that patients suffering from a given

<table>
<thead>
<tr>
<th>Table 1. Sequences of primers, reaction mixture and amplification program used in the study</th>
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<tbody>
<tr>
<td><strong>Bifidobacterium spp.</strong></td>
</tr>
<tr>
<td>Oligonucleotide sequence (5’ -&gt; 3’)</td>
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<tr>
<td><strong>Reaction mixture</strong></td>
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<td><strong>Amplification program</strong></td>
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</table>
disease entity have disturbed values of morphological or biochemical parameters, which is reflected in the statistical analysis. We tested the hypothesis concerning the existence of abnormalities in the number of *Bifidobacterium* spp. and its relationships to the course of T1DM and T2DM [15, 16]. Our research has shown that the number of *Bifidobacterium* is significantly lower in patients with type 1 and type 2 diabetes compared to the control group (Table 2), which is consistent with previous reports [15–17]. The exact role of bifidobacteria in diabetes is unknown, but their presence has been suggested to improve glucose tolerance and reduce low-grade inflammation that contributes to the development of insulin resistance and the development of diabetes [18–20].

In our study, the numbers of bacteria belonging to *Bifidobacterium* spp. in the T2DM and control groups (Table 2) were approximately 10 times greater than those reported by a Brazilian research team [17]. On the other hand, other scientists indicated similar values in their T2DM group and almost 10 thousand times greater in the control group [15]. Differences in the number of bifidobacteria in various populations are probably due to different diets and lifestyles.

When comparing total levels of bacteria, our work and earlier studies showed no significant differences between the T2DM and control groups [17]. It is possible that the total content of bacteria in the colon, even if its qualitative composition was changed, was similar in both groups. This conclusion has also been drawn by other research [21, 22].

There were no such quantitative sets of published data for adults for us to draw comparisons with the T1DM patients from our study. Most studies are based on comparisons of microbiota between healthy children and those with T1DM [23, 24]. However, it is difficult to compare these results with our data, since young individuals have a physiologically larger number of these bacteria.

We also compared the number of *Bifidobacterium* cells and total bacteria clusters within each of the investigated groups to biochemical parameters, age, and BMI. We observed a negative correlation between glucose level and number of bifidobacteria, as well as between HbA1c and bifidobacteria. Many authors have documented the beneficial effect of supplementation with probiotics containing strains of the genus *Bifidobacterium* and *Lactobacillus* on glucose metabolism, inflammatory markers, plasma and hepatic lipids, and plasma cholesterol levels [8, 25–27].

In their meta-analysis, Akbari et al. concluded that supplementation of probiotics (including, among others, *Bifidobacterium*) significantly (p < 0.05) decreased fasting blood glucose and hemoglobin A1c in diabetic patients, which is consistent with our observations [28].

Another relationship observed in the T1DM group was a positive correlation between age and the number of *Bifidobacterium*. It is generally known that the *Bifidobacterium* population in the healthy human colon decreases with age [4]. This genus dominates the gut of healthy breast-fed infants; in turn, its levels are lower but relatively stable in adults. The presence of different

<table>
<thead>
<tr>
<th></th>
<th>T1DM n = 33</th>
<th>T2DM n = 31</th>
<th>CONTROL n = 33</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex: male: female (n)</strong></td>
<td>9 : 24</td>
<td>13 : 18</td>
<td>10 : 23</td>
<td>0.424</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>39.36 (± 13.27)</td>
<td>62.68 (± 8.87)</td>
<td>41.21 (± 13.96)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>BMI [kg/m²]</strong></td>
<td>24.27 (± 4.80)</td>
<td>29.28 (± 4.95)</td>
<td>23.62 (± 1.74)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Glucose [mmol/l]</strong></td>
<td>7.26 (± 1.63)</td>
<td>7.85 (± 2.62)</td>
<td>4.67 (± 0.56)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>Total cholesterol [mmol/l]</strong></td>
<td>4.66 (± 0.89)</td>
<td>4.71 (± 1.19)</td>
<td>5.17 (± 0.70)</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>HDL [mmol/l]</strong></td>
<td>1.68 (± 0.40)</td>
<td>1.12 (± 0.37)</td>
<td>1.65 (± 0.33)</td>
<td>&lt;0.001</td>
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<tr>
<td><strong>LDL [mmol/l]</strong></td>
<td>2.60 (± 0.75)</td>
<td>2.70 (± 1.06)</td>
<td>3.09 (± 0.54)</td>
<td>0.009</td>
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<tr>
<td><strong>Triglycerides (TG) [mmol/l]</strong></td>
<td>0.99 (± 0.57)</td>
<td>2.29 (± 1.83)</td>
<td>0.98 (± 0.43)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>ALT [U/l]</strong></td>
<td>17.73 (± 8.57)</td>
<td>28.58 (± 16.46)</td>
<td>18.92 (± 6.36)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>HbA1c [%]</strong></td>
<td>8.37 (± 2.72)</td>
<td>8.11 (± 2.72)</td>
<td>5.38 (± 0.24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Bifidobacterium spp.</strong></td>
<td>7.08 x 10^6 (± 2.14 x 10^7)</td>
<td>6.38 x 10^6 (± 9.59 x 10^6)</td>
<td>1.41 x 10^7 (± 1.76 x 10^7)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>6.13 (± 0.96)</td>
<td>6.34 (± 0.77)</td>
<td>6.93 (± 0.43)</td>
<td></td>
</tr>
<tr>
<td><strong>All bacteria</strong></td>
<td>7.27 x 10^11 (± 9.04 x 10^12)</td>
<td>7.29 x 10^11 (± 7.57 x 10^12)</td>
<td>1.48 x 10^12 (± 4.09 x 10^12)</td>
<td>0.397</td>
</tr>
<tr>
<td></td>
<td>11.32 (± 0.96)</td>
<td>11.49 (± 0.73)</td>
<td>11.58 (± 0.59)</td>
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</table>

Table 2. Characteristics of the studied patient groups
species of bifidobacteria varies with age, from childhood to old age. It is a curiosity why in the T1DM group, the correlation of bifidobacteria with age was positive. Perhaps long-term elevated levels of glucose create better conditions for the development of bifidobacteria than, for instance, in T2DM.

Interestingly, in patients with T2DM, a significant negative correlation between the triglyceride levels (TGs) and the number of bifidobacteria was observed. In turn, in patients with T1DM, we documented a negative correlation between the alanine aminotransferase (ALAT) level and *Bifidobacterium* and positive correlation between these bacteria and BMI. There are many studies documenting a lower abundance of bifidobacteria in people with a higher BMI than in lean people [29–31]. However, the exact mechanism that regulates energy metabolism by intestinal microorganisms is not fully explained. It has been suggested that metabolic activity of gut microbiota plays an important role in obtaining calories from digested food, absorbing polysaccharides, and accumulating fat in the host’s fat tissue, so this may somewhat explain the correlations of bacteria with TGs, BMI, or even ALAT [32–34].

### 6. Conclusions

In conclusion, the number of *Bifidobacterium* spp. in the gastrointestinal tract is lower in T1DM and T2DM patients compared to non-diabetic subjects. Interestingly, we observed a negative correlation between the number of bifidobacteria and serum fasting glucose levels. Further studies are needed to clarify the relationship between the amount of these bacteria and some elements of the clinical characteristics of T1DM. This is a pilot study and therefore only includes a general analysis of bacteria of the genus *Bifidobacterium*. Further research is planned on a larger number of patients and with the using of next generation sequencing. A study such as that will make it possible to obtain a complete taxonomic picture of the bacteria colonizing the gastrointestinal tract of patients with diabetes, but in this work, we focused on the genus most often described in the literature.

### Abbreviations

ALT – alanine transaminase; BMI – body mass index; HbA1c – glycated hemoglobin; HDL – high-density lipoprotein; LDL – low-density lipoprotein; T1DM – type 1 diabetes; T2DM – type 2 diabetes; TG – triglycerides; qPCR – quantitative real-time polymerase chain reaction

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### Conflicts of interest

The authors declare no conflict of interest.

### Ethics approval

The study was reviewed and approved by the Jagiellonian University Bioethical Committee: KBET/81/B/2010. All study participants gave written consent to use the data obtained from the biological materials.

### References


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