Unveiling the gut connection: Exploring the link between microbiota and type 1 diabetes onset in pediatric patients

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Received May 20, 2025; Accepted August 28, 2025

DOI: 10.3892/br.2025.2074

Abstract. With the increasing occurrence of type 1 diabetes mellitus (T1DM) in younger individuals, it is important to identifying contributing factors. A retrospective case control pilot study involving 31 children within the first 6 months after diagnosis, was conducted in the Elias Hospital in Bucharest, Romania) between January 2019- December 2021. Spearman correlation analysis was performed, evaluating the association between microbiota and early onset T1DM, high-level onset glycaemia, ketoacidosis, thyroid autoimmunity and clinical parameters involving growth. Gut dysbiosis with an over-abundance of bacteria and fungi was observed compared with the healthy control group. *Butyricicoccus* was positively associated with younger age at onset (r=0.6276, P=0.0018) but negatively correlated with markers of disease progression, such as T1DM recent diagnosis (r=-0.517, P=0.0164), homeostasis model assessment of β-cell function) (r=-0.5962, P=0.0034) and C-peptide values (r=-0.5005, P=0.0344). Bacteroides showed a negative association with early-stage diabetes (r=-0.4431, P=0.0442) and *Clostridium leptum* was negatively associated with recent diagnosis (r=-0.6278, P=0.0023). Antibiotic use prior to disease diagnosis was positively correlated with *Candida albicans* (r=0.4431, P=0.0442). Inflammation, determined by the systemic inflammatory index, was positively correlated with *Enterobacteriaceae* (r=0.4331, P=0.0441) and negatively correlated with *Clostridium coccoides* (r=-0.4241, P=0.0492). C-reactive protein levels were negatively associated with Bacteroides (r=-0.4331, P=0.0498). These findings highlighted the role of gut microbiota in pancreatic inflammation and T1DM progression. In conclusion, dysbiosis of children with T1DM was correlated with younger age and a more severe onset. Microbial metabolites levels differed in these patients compared with the healthy control group. To the best of our knowledge, the present study is the first to assess gastrointestinal dysbiosis in T1DM.

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Key words: microbiota, dysbiosis, type 1 diabetes, pediatric patient

Introduction

The total number of patients with type 1 diabetes mellitus (T1DM) is steadily rising. In 2025, an estimated 9.5 million people worldwide are living with T1DM, representing a 13% increase compared with the 8.4 million reported in 2021. Since the genetic transmission of this disease has remained relatively constant, this growing prevalence suggests the involvement of alternative risk factors, including environmental triggers, lifestyle influences, and changes in early-life exposures. These include pollutants, endocrine disruptors, vitamin D deficiency and pathological colonization of the gastrointestinal tract (1,2).

The gastrointestinal microbiota (the colonization of the gastrointestinal tract) comprises all bacteria, viruses and fungi that inhabit this environment. An imbalance of this microbiota (dysbiosis) with more proinflammatory bacteria that affect the immune system is hypothesized to explain the global rise in T1DM, especially the early onset of this disease (1-3).

The balance of the gut microbiota is disrupted by various factors, leading to dysbiosis, which serves a vital role in the development and progression of numerous types of disease, including type 2 diabetes mellitus, autoimmune diseases such as Hashimoto's thyroiditis and Chon's disease (4). The onset of T1DM, especially with onset at younger ages, represents a global health challenge, as it does not affect all individuals with genetic susceptibility (5). The alterations in microbiota and chronic inflammation, due to environmental influences, are hypothesized as explanations for the shifts in the prevalence of this disease (5,6).

Several mouse studies have provided evidence of a potential link between microbiota and T1DM (7-9). For example, exposure to antibiotics in the prenatal period or in the first weeks after birth influences the gut microbiota and the T1DM incidence by reducing the pancreatic inflammation and levels of pro-inflammatory cytokines (10-14).

In the context of T1DM, changes in the gut microbiota have been observed prior to the emergence of systemic signs of islet autoimmunity (15,16). This shift in microbiota could be attributed to the fact that previous studies primarily identified these modifications through gene analysis of 16S rRNA, which may not capture specific structural and functional characteristics potentially involved in disease progression (17,18). Later studies used specialized designs to control all known factors influencing T1DM susceptibility and analyzed microbiome characteristics using longitudinal metagenomic sequencing of stool samples (19,20). Patients with T1DM harbor a proinflammatory environment, irrespective of geographical location, with a higher abundance of *Bacteroidetes* and a lower abundance of *Firmicutes* (21,22).

Decreased levels of Firmicutes (comprising strains of *Clostridium* and *Eubacterium*) may pose a risk to the host as this phylum encompasses numerous producers of the short-chain fatty acid (SCFA) butyrate (23). Butyrate is key for intestinal homeostasis, serving as an energy source for colonocytes and promoting the secretion of mucins. This aids in reducing gut permeability by facilitating the formation of tight junctions (13,17,18,22,23) and protecting against pathogens and harmful substances (21,24,25). The Bacteroidetes phylum contains strains of *Bacteroides* and *Prevotella*. Studies have shown that T1DM is characterized by a dominance of *Bacteroides*, taxa associated with intestinal inflammation, while levels of protective *Prevotella* are decreased (26-28).

Species within the *Bacteroides* genus ferment glucose and lactate to produce propionate, acetate and succinate, but they lack the ability to generate butyrate (29). Additionally, lactate-producing bacteria, including certain probiotic strains such as *Lactobacillus rhamnosus*, *L. reuteri*, *L. johnsonii N6.2*, *L. plantarum* and *Bifidobacterium lactis*, synthesize butyrate, thereby reinforcing the intestinal barrier function (26). These findings underscore the role of the gut microbiota in T1DM and suggest that targeting specific microbial components may have potential for interventions aimed at supporting intestinal health in individuals with T1DM (1,21,27-30).

Similar to autoimmune conditions, T1DM prevalence exhibits a geographical pattern: The EURODIAB Autoimmune Complications of Type 1 Diabetes study (1989-1990) reported incidence rates of ~42.9 per 100,000/year in Finland, compared with 4.6/100,000/year in northern Greece (31). Climate, particularly sunlight exposure, influences microbiota and impacts the immune system. Vitamin D deficiencies and disturbances in the circadian rhythm alter the gut microbiota, contributing to the development of autoimmune disease (2,28,30).

Evidence suggests that vitamin D levels are lower in newly diagnosed patients with T1DM compared with population controls (32,33). Moreover, the supplementation of this vitamin and the risk of T1DM show a dose-response effect (30).

The composition of early-life microbiota undergoes frequent changes influenced by maternal and environmental microbes, as well as exposure to food and animal-borne antigens. The method of birth, whether cesarean or vaginal, has an impact on the neonatal microbiota; findings from the TEDDY study indicate that children delivered vaginally exhibit higher levels of *Bacteroides*, which are associated with increased diversity and accelerated maturation of gut intestinal flora (14,34).

An increasing number of studies underscore the role of environmental factors in the rising incidence of T1DM, particularly among younger children (35,36); to the best of our knowledge, however, there are few studies from Romania or Eastern Europe (37-39). Given the geographical variation in T1DM, the data available may not entirely align with the actual pathogenesis of T1DM.

Investigating the association between T1DM onset and gut microbiota dysbiosis may foster collaborations between researchers, clinicians and policymakers, leading to the development of novel diagnostic tools, therapeutic interventions and tailored public health policies.

Considering the cultural, dietary and environmental changes that have occurred in Eastern Europe over the past three decades, it has been hypothesized that alterations in the gut microbiome may contribute to a decreased age at onset of T1DM, as well as a more severe clinical presentation. Longitudinal microbiome studies have shown that changes in microbial composition and function, including a reduction in short-chain fatty acid producers and enrichment of pro-inflammatory taxa, occur prior to or around the development of islet autoimmunity and overt T1DM (11,14). In parallel, epidemiological studies from Eastern Europe, including Romania and Poland, have reported an increase in T1DM incidence since the 1990s, with diagnosis occurring at progressively younger ages (37,40). At the time of diagnosis, a notable proportion of patients present with diabetic ketoacidosis, reflecting both delayed recognition and a more severe onset of disease, which may be associated with environmental and microbial risk factors (41). Moreover, it has been well documented that T1DM frequently coexists with other autoimmune diseases, such as thyroid autoimmunity and celiac disease, further supporting a role for shared environmental drivers, including gut microbiome dysregulation, in shaping disease course and comorbidity (42,43).

The aim of the present study was to analyze the gut microbiota composition in Romanian children with newly diagnosed T1DM, and to investigate its potential associations with age of onset, severity at presentation, glycemic control, and co-existing autoimmune conditions.

Materials and methods

Study population. The present retrospective case-control pilot study involved 31 pediatric patients (age, 1-18 years, 16 female and 15 male) diagnosed with T1DM within the past 6 months, based on the criteria set by the American Diabetes Association (44).

The inclusion criteria for the patients were as follows: i) Positive clinical T1DM diagnosis and ii) onset <6 months prior to admission. The exclusion criteria were as follows: i) Antibiotic/probiotic administration in the 2 weeks prior to the admission; ii) T1DM diagnosed >6 months previously and iii) other types of diabetes mellitus.

These inclusion and exclusion criteria for patients in the study were established to identify relevant changes in the gut microbiota in patients with type 1 diabetes, in light of observational evidence showing that individuals with type 1 diabetes exhibit altered fecal and oral microbiota composition, reduced butyrate-producing species, and lower plasma levels of acetate and propionate (45).

Patients were referred either by their primary care physician or transferred from other pediatric hospitals in the southern region of Romania to the Pediatric Endocrinology and Diabetes Department at Elias Emergency and University Hospital, Bucharest (Romania). Being one of three referral centers for evaluating and initiating continuous glucose monitor devices and insulin pumps reimbursed by the National Health System in the southern region, most patients with recently diagnosed T1DM sought treatment here. All participants were of Caucasian ethnicity, as defined by Thomson *et al* (46). All eligible pediatric patients, admitted between January 2019 and December 2021, were enrolled in chronological order of admission. The study adhered to the Helsinki Declaration of 2013, with parental approval obtained through written informed consent for the use of medical records in scientific research.

Patients with newly diagnosed T1DM were matched with healthy children of similar age (4-18 years), sex, BMI and urban environment. First-degree relatives with known T1DM were also evaluated to assess the influence of genetic and environmental factors. A comprehensive medical history, including demographics, family history of autoimmune diseases and/or diabetes, personal medical history, birth details, breastfeeding practices, infection history and treatment details, was obtained from all participants. Anthropometric measurements were conducted, including age, weight, height, BMI, blood pressure, Tanner stage (47) and waist-to-hip ratio. Data were collected from medical records and laboratory reports, and compiled in an Excel Spreadsheet (version 16.0 (Microsoft Corporation)

Laboratory procedures. A 10 ml peripheral blood sample was obtained 8 h after the last meal to assess hematological and biochemical parameters. The complete blood count and blood chemistry analyses included measurements of glucose, creatinine, total cholesterol, high- and low-density lipoprotein-cholesterol, triglycerides, glycated hemoglobin (HbA1c), hepatic function and reactive C-protein (CRP). Additionally, levels of anti-thyroglobulin and anti-thyroperoxidase (for autoimmune thyroid disorders) and anti-tissue transglutaminase antibodies (for celiac disease) were measured to assess autoimmune conditions. Thyroid function was evaluated based on thyroid-stimulating hormone (TSH) and free thyroxine (fT4) levels. Furthermore, assessments were made for insulin-like growth factor 1 (IGF1), 25-hydroxy vitamin D [25(OH) vitamin D], and phosphorus-calcium metabolism, including measurements of total serum calcium, phosphorus and parathyroid hormone (PTH).

ELISA. The levels of 25(OH) vitamin D, anti-thyroglobulin, anti-thyroperoxidase and anti-tissue transglutaminase antibodies were determined using ELISA on a Chemwell 2010 ELISA system (Awareness Technology, Inc.) using commercially available kits as follows: 25(OH) Vitamin D ELISA kit (Immunodiagnostic Systems, cat. no. AC-57SF1), anti-thyroglobulin (catalog no. ABIN649023, Antibodies-online), anti-thyroperoxidase ELISA kit (cat. no. MBS3800854), and anti-tissue transglutaminase ELISA kit (both MyBioSource, cat. no. MBS7208318). All assays were performed in strict accordance with the manufacturer's instructions.

Chemiluminometric method. The determination of serum PTH levels were determined using the COBAS E 411 analyzer (Roche Diagnostics), a fully automated, random-access, multichannel instrument designed for immunological analysis.

Spectrophotometric methods. Serum biochemical parameters were assessed using standard enzymatic colorimetric methods. Lipids [total cholesterol (cat. no. 72291UD00), HDL-C- kit number: 67136UQ10, triglycerides- kit number:71068UD00), glucose (cat. no. 67921UQ02, creatinine- kit number: 71560UD00, aspartate transaminase (AST; cat. no. 70068UD00, alanine transaminase (ALT) - kit number: 72494UD00, C-reactive protein (CRP)- kit no. 50519Y600, and calcium - kit number: 74372UD00; all Abbott Diagnostics) were measured on the Architect c8000 Clinical Chemistry Analyzer (Abbott Diagnostics, Abbott Park, IL, USA). Assays were performed according to the manufacturer's instructions and quality control procedures.

Serum phosphorus was determined using the Vitros 5,1 FS Chemistry Analyzer and reagent kit (cat. no. 1203-0398-1506, Ortho Clinical Diagnostics).

Flow cytometry. Complete blood count was determined using a Sysmex XN 1000 hematology analyzer (Sysmex Corporation). Flow cytometry was performed on whole blood samples. Cells were washed twice with PBS and incubated with blocking buffer (2% FBS in PBS) for 15 min at 4˚C. Surface markers were detected with directly conjugated monoclonal antibodies, including CD3-FITC (clone UCHT1; cat. no. 555332; BD Biosciences), CD4-PE (clone RPA-T4; cat. no. 555347; BD Biosciences), and CD8-APC (cat. no. 555369; BD Biosciences). For apoptosis analysis, cells were stained with the Annexin V-FITC Apoptosis Detection kit (cat. no. 556547; BD Biosciences) in combination with propidium iodide (PI; cat. no. P4864; Sigma-Aldrich; Merck KGaA). Where secondary detection was required, biotinylated primary antibodies were visualized using streptavidin-PE (cat. no. 554061; BD Biosciences). Data acquisition was performed on a BD FACSCanto II flow cytometer (BD Biosciences), and results were analyzed using FlowJo software, version 10.8 (Tree Star Inc.).

ECLIA IGF-1, TSH and fT4 concentrations were evaluated by ECLIA on a Roche COBAS® e 411 analyzer (Roche Diagnostics). Commercial reagent kits were provided by Immunodiagnostic Systems, including the IDS-iSYS IGF-1 assay (cat. no. IS-3900), IDS-iSYS TSH assay (cat. no. IS-3100), and IDS-iSYS free T4 assay (cat. no. IS-3300). All assays were performed according to the manufacturer's instructions.

High-performance liquid chromatography (HPLC). HbA1c was measured using the Bio-Rad D-10 Hemoglobin Testing System (Bio-Rad Laboratories, Inc.). The separation was performed on the D-10 cation-exchange cartridge (Bio-Rad Laboratories, Inc.; cat. no. 220-0101) at a controlled temperature of 25˚C. Whole blood samples (5 μl) were injected. The mobile phase consisted of two buffers (Buffer A: phosphate buffer, pH 6.5; Buffer B: phosphate buffer with increased ionic strength). The system was operated at a flow rate of 1.5 ml/min with a programmed gradient increase of Buffer B according to the manufacturer's protocol. An internal calibrator and quality

Table I. Sequences of the primers.

Target	Forward primer (5'→3')	Reverse primer (5'→3')	Target gene
Eubacteria	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC	16S rDNA
Bacteroides spp.	CCTACGATGGATAGGGGT T	CACGCTACTTGGCTGGTTCAG	16S rDNA
Butyricicoccus spp.	ACCTGAAGAATAAGCTCC	GATAACGCTTGCTCCCTACGT	16S rDNA
Gamma proteobacteria	GCTAACGCATTAAGTACCCCG	GCCATGCAGCACCTGTCT	16S rDNA
Akkermansia muciniphila	GCGTAGGCTGTTTCGTAAGTC GTGTGTGAAAG	GAGTGTTCCCGATATCTACGC ATTTCA	16S rDNA
Lactobacillus spp.	ACGAGTAGGGAAATCTTCCA	CACCGCTACACATGGAG	16S rDNA
Clostridium leptum	GCACAAGCAGTGGAGT	CTTCCTCCGTTTTGTCAA	16S rDNA
Clostridium coccoides	GACGCCGCGTGAAGG A	AGCCCCAGCCTTTCACATC	16S rDNA
Faecalibacterium prausnitzii	CCCTTCAGTGCCGCAGT	GTCGCAGGATGTCAAGAC	16S rDNA
rRNA 18S universal primers	ATTGGAGGGCAAGTCTGGTG	CCGATCCCTAGTCGGCATAG	rRNA 18S
Saccharomyces spp.	AGGAGTGCGGTTCTTTG	TACTTACCGAGGCAAGCTACA	rRNA 18S
Candida spp.	TTTATCAACTTGTCACACCAGA	ATCCCGCCTTACCACTACCG	rRNA 18S

control material (Bio-Rad Laboratories, Inc.; cat. no. 220-0102 and 220-0103) were included, and all procedures were carried out strictly according to the manufacturer's instructions. β-cell function and insulin resistance were evaluated using the homeostasis model assessment indices (HOMA-B and HOMA-IR). HOMA-B was calculated as [20 x fasting insulin [μU/ml])/(fasting glucose [mmol/L] - 3.5), and HOMA-IR as (fasting insulin [μU/ml] x fasting glucose [mmol/L])/22.5, according to Matthews *et al* (48)

Microbiota analysis. Stool samples were gathered during hospitalization or at home using a standardized procedure that involved antiseptic handling, collection in sterile tubes (without culture media) and immediate freezing at -20˚C. Subsequently, fecal DNA was extracted utilizing the PureLink Microbiome Purification kit (cat. Number A29790, Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The concentration of DNA was assessed using a Qubit 4 fluorometer (Thermo Fisher Scientific, Inc.). DNA samples were diluted in DNase-free water to a concentration of 3 ng/μl. Quantitative PCR was performed to determine the relative abundance of intestinal microorganisms in stool DNA isolated from both patients with T1DM and healthy controls, using a ViiA7© Fast Real-Time instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). Bacterial or fungal group-specific primers (16S and 18S rRNA, respectively; Table I) were used at their designated annealing temperatures. For all other primers, each set was designed to target the 16S rRNA gene, which is used as a molecular marker for bacterial identification and quantification (49). The 16S rRNA gene contains both highly conserved regions, enabling the design of universal primers, and variable regions, allowing species-level discrimination. The control was18S rRN) gene, which serves as a conserved and widely used molecular marker for fungi. These primers were designed to recognize a broad range of clinically and environmentally relevant yeasts, with specificity toward *Candida* and *Saccharomyces spp*. The primers were previously described in Trandafir *et al* 2024, validated for specificity toward the bacterial taxa of interest, ensuring accurate amplification and minimizing off-target effects (49). Each PCR reaction comprised 2.5 nM forward and reverse primers, 9 ng DNA and 2X SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Negative controls were samples without DNA templates. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec and 72˚C for 1 sec. Universal 16S rRNA and 18S rRNA primers were used for normalization and relative abundance of bacterial abundance was calculated using the $2^{-\Delta\Delta Cq}$ method (50,51).

Metabolite analysis. Fecal content pellets (0.2 g) were suspended in 1 ml sterile saline solution and incubated at room temperature for 2 min. Subsequently, the sample was vigorously shaken for 4 min to create a slurry. After centrifugation at 1,790 x g for 1 h at 4˚C, the supernatant was collected and subjected to further centrifugation at 28,600 x g for 30 min at 4˚C.

Fecal content pellets (0.2 g) were suspended in 1 ml sterile saline solution and incubated at room temperature for 2 min. Subsequently, the sample was vigorously shaken for 4 min to create a slurry. The resulting supernatant was transferred to a new tube and filtered using a minisart-GF filter membrane (Sartorius AG) with a 1 ml sterile plastic syringe. Finally, another filtration step was performed using a Whatman-25 mmGD/X0 filter (Merck Millipore) and a 1 ml sterile plastic syringe. Metabolite levels (including butyrate, acetate, propionate, taurine, succinate and lactate) were determined using commercial kits following the manufacturer's instructions (Abbexa, cat. no. abx258338 for butyrate quantification and Sigma-Aldrich for the other metabolites- Cat. Number MAK355, MAK184, MAK065, and MAK086). Optical densities were measured using a spectrophotometer at 455 nm (Mulsiskan FC, Thermo Fisher Scientific, Inc.) and converted to μg/g feces.

Statistical analysis. All data are presented as the mean ± SEM or SD and were analyzed using the GraphPad Prism 9.0 software (Dotmatics). A total of three independent repeats were

Table II. Biochemical and immunological parameters.

Parameter	Mean	SEM	Std. Deviation	Median	Normal values
Leukocytes, x$10^3/\mu$l	6.68	0.40	2.22	6.15	4.60-15.50
Neutrophils, x$10^3/\mu$l	3.37	0.30	1.69	2.95	1.50-7.00
Lymphocytes, x$10^3/\mu$l	2.62	0.19	1.09	2.31	1.50-10.50
Hemoglobin, g/dl	13.42	0.21	1.15	13.00	11.00-15.30
CRP, mg/dl	3.58	2.11	8.71	0.55	<0.50
ALT, U/l	19.29	1.77	9.84	16.00	13.00-26.00
AST, U/l	23.45	1.28	7.12	22.00	8.00-25.00
Creatinine, mg/dl	0.66	0.03	0.15	0.63	0.57-0.86
Total cholesterol (mg/dl)	172.23	8.16	44.70	164.50	140.00-200.00
Triglycerides (mg/dl)	53.23	3.31	18.15	51.50	>40.00
HDL-cholesterol, mg/dl	63.48	2.45	13.64	61.00	<130.00
LDL-cholesterol, mg/dl	102.90	5.75	32.00	96.40	35.00-150.00
Anti-thyroglobulin antibodies, IU/ml	60.87	29.48	161.44	10.00	1.00-16.00
Anti-thyroid peroxidase antibodies (IU/ml)	51.66	31.90	177.60	9.00	<20.00
Anti-transglutaminase antibodies, IU/ml	0.91	0.40	1.90	0.40	<10.00
TSH, μIU/ml	2.507	0.22	1.18	2.36	0.30-3.60
Free T4, ng/dl	1.185	0.03	0.16	1.20	0.8-1.48
250H vitamin D, ng/ml	28.16	2.10	11.49	24.28	>30.00
Total calcium, mg/dl	9.57	0.07	0.37	9.60	8.40-10.20
Phosphorus, mg/dl	4.83	0.19	0.65	4.85	2.50-4.50
PTH, pg/ml	31.36	2.52	13.31	27.89	15.00-65.00
Insulin, IU/24 h/kg	19.23	4.08	21.58	11.00	0.30-1.00

CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TSH, thyroid-stimulating hormone; T4, thyroxine; 25(OH) vitamin D, 25-hydroxyvitamin D; PTH, parathyroid hormone.

performed. Power analysis was initially performed with a set power (1) of 0.90 and 0.05 for two groups (Control and T1DM) tested using difference in means and SD as parameters. Standardized statistical test methods were used to analyze the results of demography and laboratory tests. The analysis was performed by a normality test ($P<0.05$ was considered to be normal and homogeneous) followed by parametric testing (unpaired t-test) and Welch's post hoc correction. Spearman correlation analysis of the association between proinflammatory taxa and early onset T1DM, high-level onset glycaemia and ketoacidosis, insulin dose, inflammation, thyroid autoimmunity, IGF-1 and other clinical parameters regarding growth was performed using SPSS Windows v. 17.0 (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Among the 31 patients, the ratio male: female was 1.11 and the mean BMI was 17.48 kg/m^2. The mean age of the onset of the disease was 9.0±0.5 years and the mean glycaemia was 350±98 mg/dl (Table II). The vaginal: caesarian birth ratio was 0.36, (13/31) and 10/31 patients with T1DM had a family history of autoimmune disease.

In the T1DM group, dysbiosis was evident, characterized by an overabundance of detrimental bacteria such as *Clostridium coccoides*, *Faecalibacterium*, *Bacteroides* and *Enterobacteriaceae*, as well as fungi including *Candida*, *Saccharomyces* and *Aspergillus spp.*, in contrast to the healthy control group (Figs. 1-3). Furthermore, a notable decrease in *Bifidobacterium spp.* and elevated presence of *A. muciniphila* within the beneficial taxa were observed among patients with T1DM compared with the healthy control group (Figs. 1 and 4).

A decrease in butyrate-producing bacteria was observed in the T1DM group with dysbiosis with increased abundance of *Enterobacteriaceae* in T1DM ($P=0.0193$; Fig. 3). This pattern underscores the alteration in the microbial composition associated with T1DM, suggesting a potential role of gut microbiota dysregulation in the pathogenesis of the disease.

Correlation analysis identified significant correlations between specific gut microbiota taxa and clinical parameters in pediatric T1DM (Fig. 5). The relative abundance of *Butyricicoccus* was positively associated with younger age at onset ($r=0.6276$, $P=0.0018$) but negatively linked to onset within 6 months ($r=-0.517$, $P=0.0164$), pancreatic β cell reserve (HOMA-B, $r=-0.5962$, $P=0.0034$), C-peptide ($r=-0.5005$, $P=0.0344$) and phosphorus levels ($r=-0.5937$, $P=0.0036$). *Bacteroides* relative abundance showed negative correlations with early-stage diabetes ($r=-0.4431$, $P=0.0442$) and inflammation (indicated by CRP levels; $r=-0.4331$; $P=0.0498$). The relative abundance of *Clostridium leptum* was inversely associated with recent diagnosis ($r=-0.6278$, $P=0.0023$) but positively correlated with hemoglobin ($r=0.4765$, $P=0.025$) and PTH levels ($r=0.6047$, $P=0.0029$).

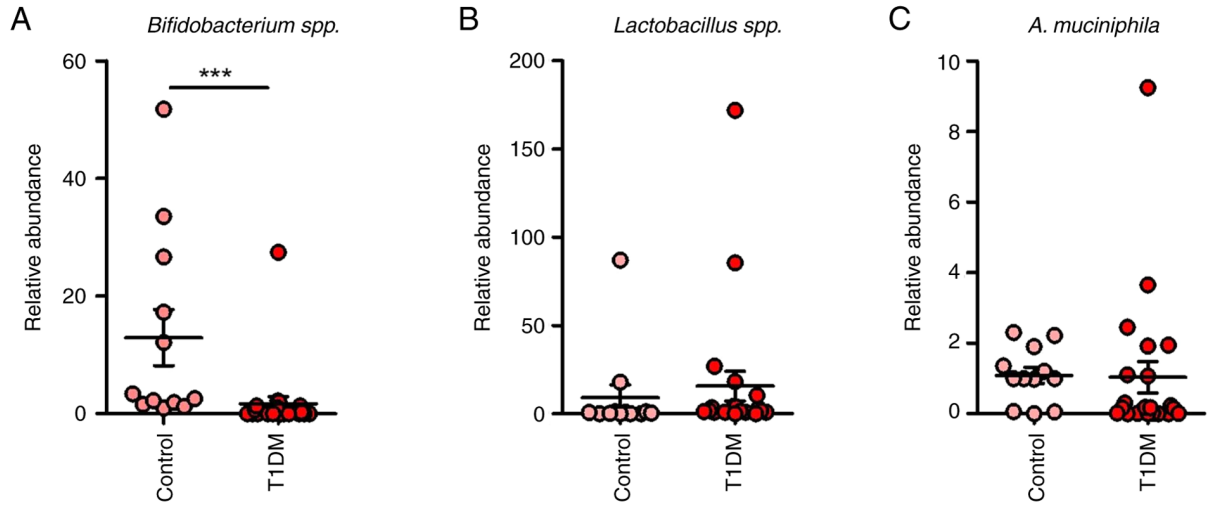

Figure 1. Beneficial bacteria abundance in T1DM. Relative abundance of (A) *Bifidobacterium*, (B) *Lactobacillus* and (C) *A. muciniphila* in patients with T1DM compared with healthy control group. ***P<0.001. T1DM, type 1 diabetes mellitus.

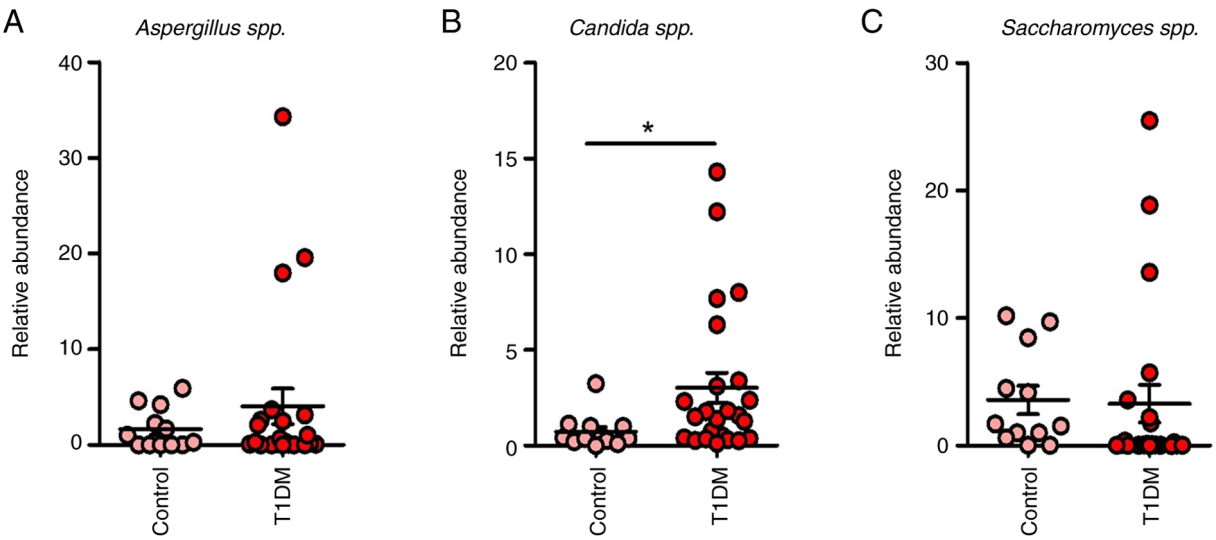

Figure 2. Mycobiome characterization. Dysbiosis with increased abundance of (A) *Aspergillus* (B) *Candida spp*. and (C) *Saccharomyces* in T1DM. The primers were specific to the ribosomal 18S RNA gene and designed tEo recognize various *Candida* and *Saccharomyces spp*. T1DM, type 1 diabetes mellitus. *P<0.05.

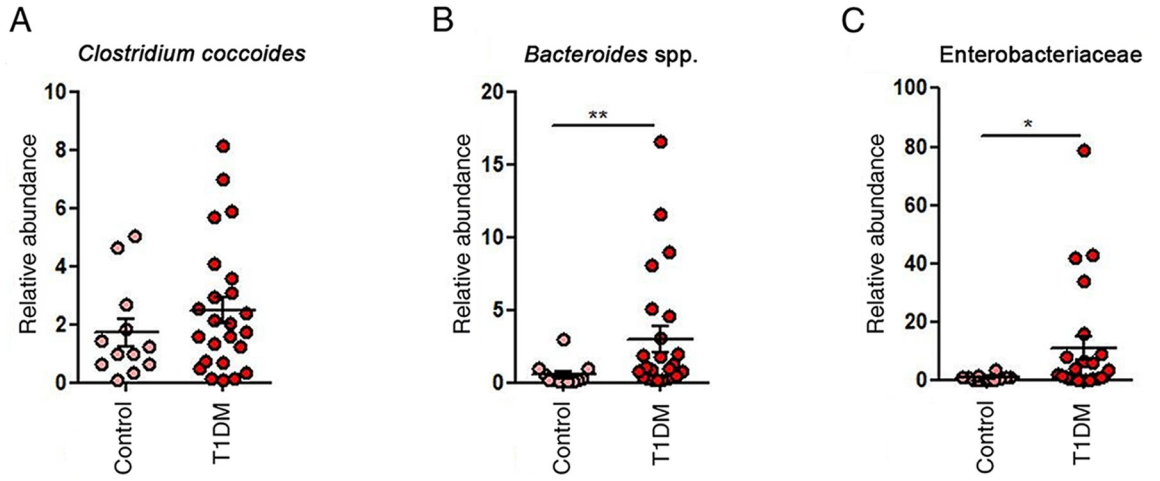

Figure 3. Dysbiosis markers in T1DM. Dysbiosis with increased abundance of (A) *Clostridium coccoides* and (B) *Bacteroides spp*. in T1DM. (C) Dysbiosis with increased abundance of *Enterobacteriaceae* in T1DM (P=0.0193). T1DM, type 1 diabetes mellitus. *P<0.05 and **P<0.01

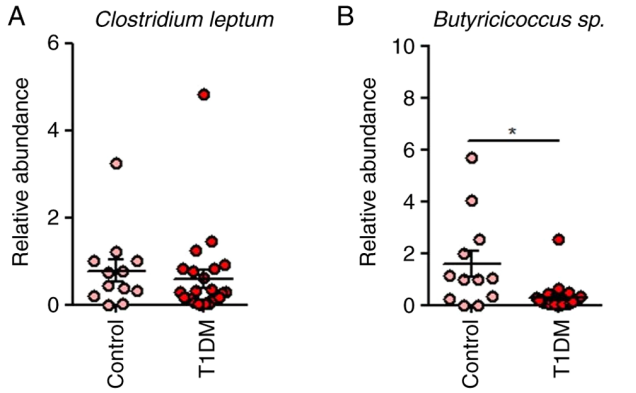

Figure 4. Abundance of butyrate-producing bacteria (A) Relative abundance of *Clostridium leptum*. (B) Dysbiosis with a decrease of *Butyricicoccus spp.* in T1DM group. T1DM, type 1 diabetes mellitus. *P<0.05.

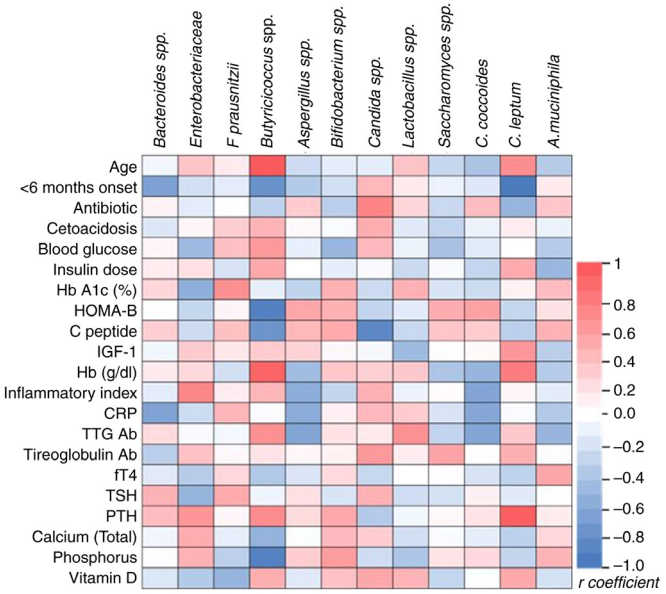

Figure 5. Spearman correlation coefficients between clinical parameters (and relative abundance of bacterial taxa in the microbiome. Red, positive correlation; blue, negative correlations; intensity of color corresponds to the magnitude of the correlation. HbA1C, Hemoglobin A1c (glycated hemoglobin); HOMA-B, Homeostatic Model Assessment of β-cell function; IGF, Insulin-like Growth Factor; CRP, C-reactive Protein; TTG Ab, Tissue Transglutaminase Antibodies; fT4, Free Thyroxine; TSH, Thyroid-Stimulating Hormone; PTH, Parathyroid Hormone.

Antibiotic intake was correlated positively with the relative abundance of *Candida albicans* (r=0.4431, P=0.0442), while the inflammatory index was linked positively to *Enterobacteriaceae* (r=0.4331, P=0.0441) and negatively with *C. coccoides* (r=-0.4241, P=0.0492). Hemoglobin was also positively associated with *Butyricicoccus* (r=0.5857, P =0.0042), highlighting potential microbiota roles in T1DM progression and inflammation.

The microbiota findings were compared with the metabolite levels in stool samples from both patients with T1DM and healthy controls (Fig. 6). Patients with T1DM exhibited notably decreased levels of butyrate and lactate compared with the healthy controls. Conversely, acetate levels were higher in the T1DM cohort.

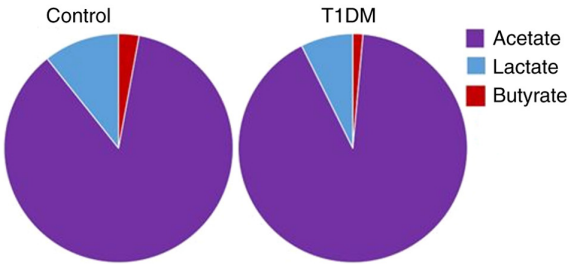

Figure 6. Fecal metabolite analysis in patients with T1DM and healthy controls. In fecal samples, acetate represented the predominant SCFA, accounting for ~85% in controls and ~92% in T1DM patients. Lactate and butyrate contributed ~10 and ~5% in controls, compared with ~6 and ~2% in T1DM patients, respectively. T1DM, type 1 diabetes mellitus.

Discussion

The present retrospective case-control study was conducted in Romania between January 2019 and December 2021, involving 31 children. The timeline of analysis and manuscript preparation was substantially affected by the coronavirus Disease 2019 (COVID-19) pandemic. Institutional priorities during this period were redirected toward pandemic-related clinical and research activities, and access to laboratories and clinical facilities was intermittently restricted. These factors contributed to an extended delay in data processing, analysis, and publication. Nevertheless, all study data were securely stored, curated, and analyzed in accordance with the approved protocol, ensuring the integrity and validity of the findings.

The role of microbiota in T1DM has garnered increasing attention and investigation: Previous studies have linked specific bacterial taxa such as *Bacteroides, Bifidobacterium* and *Ruminococcus* with heightened cytokine levels, pancreatic inflammation, autoimmune reaction and the onset of T1DM (1,2,4). The present study demonstrated dysbiosis characterized by an overabundance of proinflammatory bacteria (including *C. coccoides, Faecalibacterium, Bacteroides* and *Enterobacteriaceae*) and fungi in pediatric patients with T1DM from Romania, in comparison with healthy controls.

Contrary to the healthy control group, patients with T1DM exhibited a microbiota predominantly composed of *Bacteroides* taxa, despite the presence of relatively abundant protective bacteria such as *Bifidobacterium* and *Lactobacillus spp.*, and *A. muciniphila*. Notably, earlier onset and more severe presentation of T1DM were positively associated with specific proinflammatory bacterial species, including *Bacteroides* and *Butyricicoccus spp.*, in alignment with the literature (16,52). *C. leptum* is another taxon correlated with a younger age at onset and although its role may vary depending on the context, host health, and microbiota composition its altered abundance may contribute to disease susceptibility and progression (53). In gut dysbiosis, its abundance may shift, and interactions with other microbial taxa could influence systemic inflammation indirectly. However, it is primarily reported as a contributor to a balanced, healthy gut environment rather than as a proinflammatory agent (21-23).

Studies conducted in a Romanian center on patients with type 2 diabetes mellitus (T2DM) or metabolic syndrome have revealed similar microbial compositions, particularly

the presence of *Enterobacteriaceae*, which is correlated with inflammation and other chronic diseases (53,54).

The Spearman correlation analysis demonstrated associations between specific microbial taxa and clinical markers, including those of inflammation, metabolic status and endocrine function, suggesting a potential role of the gut microbiome in modulating health outcomes in the context of metabolic disorder.

The presence of proinflammatory bacteria such as *Enterobacteriaceae* showed a positive association with the systemic inflammatory index; relative abundance of anti-inflammatory bacteria as *Bacteroides* and *C. coccoides* showed a negative association with CRP levels and with the systemic inflammatory index (23-25).

Antibiotic use during acute infections in the 2 months preceding diagnosis in children with T1DM may have perturbed the microbiota, resulting in an overabundance of *Candida spp* (45,53,54). Additionally, T1DM is associated with an increased susceptibility to recurrent infections and alterations in the immune system within the first year of the disease, as well as lower complement component 4) levels and a higher helper/suppressor T lymphocyte ratio (55,56). These findings are consistent with those reported by other authors (55-57).

Assessing growth and bone density is key in children with T1DM and T2DM, given the link between impaired bone health and dysbiosis characterized by an elevated presence of *Lactobacilli* (58). In the present study, dysbiosis impacted calcium-phosphorous metabolism; phosphorus levels negatively correlated with *Butyricicoccus* and PTH levels were positively correlated with *C. leptum* (56,59,60).

The role of *Bacteroides* in intestinal homeostasis, particularly in T1DM, is complex and context-dependent. *Bacteroides spp.* exhibit both pro-inflammatory and anti-inflammatory properties, depending on species, strain and host factors. On one hand, certain *Bacteroides* strains (such as *B. fragilis,* producing polysaccharide A) have been shown to exert anti-inflammatory effects by promoting regulatory T cell response and maintaining mucosal barrier integrity (61,62) On the other hand, other species or imbalances in *Bacteroides* abundance are associated with pro-inflammatory states, including increased intestinal permeability and heightened immune activation, which are relevant to the pathogenesis of T1DM (11).

Here, the enrichment of *Bacteroides* in patients with T1DM may reflect a shift toward a dysbiotic profile that contributes to inflammation and autoimmune activation. However, not all *Bacteroides* are pathogenic, and their impact may vary with microbial context, age and diet. Future strain-level or functional metagenomic analyses may clarify the specific contributions of *Bacteroides* in the T1DM gut microbiome.

Patients with T1DM exhibited notably decreased levels of butyrate and lactate compared with healthy controls, with higher acetate levels in the T1DM cohort, diverging from other studies that found decreased plasma levels of acetate and propionate and similar levels of plasma butyrate in controls compared with patients with T1DM (11,24). Measurements of SCFA levels (butyrate, lactate and acetate) were obtained from fecal samples, which primarily reflect microbial fermentation activity in the colon and the unabsorbed SCFA fraction. This contrasts with other studies reporting SCFA concentrations in plasma, which are influenced not only by gut microbial production but also by host absorption efficiency, hepatic metabolism and systemic circulation dynamics (62,63). Therefore, direct comparisons between fecal and plasma SCFA levels must be interpreted with caution. The increase in fecal acetate alongside decreased butyrate and lactate in the present T1DM cohort may indicate altered microbial metabolism or impaired SCFA absorption in these patients, rather than a contradiction of plasma findings reported in previous studies (63,64).

The present study demonstrated the role of gut microbiota in pediatric patients with T1DM. It highlighted dysbiosis characterized by an overabundance of proinflammatory bacteria such as *Bacteroides*, *Enterobacteriaceae* and *C. coccoides*, alongside fungi such as *Candida spp*, in patients with T1DM compared with healthy controls. This aligns with existing research, underscoring the association of these taxa with inflammation, autoimmune reaction and metabolic imbalances (11,14). The study also demonstrated correlations between microbial taxa and clinical markers, such as the systemic inflammatory index, CRP levels and calcium-phosphorous metabolism, emphasizing the microbiota influence on systemic inflammation, bone health and disease progression.

Moreover, the perturbation of microbiota following antibiotic use and the increased prevalence of recurrent infections in patients with T1DM highlight the dynamic interactions between external factors, immune function and the gut microbiome. By revealing specific microbiota compositions associated with earlier disease onset, severity and metabolic markers, the present study demonstrated how gut dysbiosis impacts T1DM pathophysiology. These findings may facilitate research into microbiota-targeted interventions, such as dietary modification or probiotic therapy, to improve disease management and patient outcomes in T1DM.

However, the present study had limitations, including the heterogeneous composition of the study group, its retrospective nature with a small patient cohort and the commencement of enrollment a few months before the onset of the COVID-19 pandemic and lockdown, primarily involving an urban population. Additionally, the microbiota study excluded patients who received antibiotics following infections, particularly those with diabetic ketoacidosis.

Future research directions include proteomics and metabolomics profiling to evaluate functional changes in the microbiome of patients with T1DM. Longitudinal studies observing disease onset and progression in patients with dysbiosis are also warranted. Furthermore, the associations between dysbiosis and other autoimmune diseases (such as Hashimoto's thyroiditis and celiac disease) need to be investigated with regard to the correlation between antibody positivity and clinical disease onset.

The present data may be used to estimate the risk of developing T1DM and other autoimmune diseases in the siblings of these patients. Risk calculators that consider specific genetic traits (human leukocyte antigen haplotypes), antibody presence and family history of the disease may predict the likelihood of developing diabetes before symptoms manifest. Early intervention, when a reasonable number of insulin-producing β cells are present in the pancreas, may positively influence immune responses by modifying gut microbiota, potentially slowing disease progression and improving outcomes.

Acknowledgements

Not applicable.

Funding

The present study was supported by Executive Agency for Higher Education, Research, Development and Innovation Funding (project ID no. PN-III-P1-1.1-PD-2019-0499, grant no. 224/2021).

Availability of data and materials

The data generated in the present study are not publicly available due to participant privacy but may be requested from the corresponding author.

Authors' contributions

AAI, SF and GGP conceived the study. AAI and GGP confirm the authenticity of all the raw data. SI designed the methodology. TP analyzed data. AAI and GGP performed experiments. AAI wrote and edited the manuscript, constructed figures and supervised the study. GGP edited the manuscript. SF and GGP acquired funding. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of The Declaration of Helsinki and was approved by the Ethics Committee of University of Bucharest (protocol code CEC reg. no. 235/9.10.2019) and the Institutional Review Board of the Elias Hospital (approval no. 1695, 12.03.2019; both Bucharest, Romania). Informed consent was obtained from the parents of patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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