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Impact of diet and nutritional status on gingival crevicular fluid metabolome and microbiome in people with type 1 diabetes: a cross-sectional study

Michał Kania[1], Zuzanna Drab[2], Michalina Dąbrowska[3], Emilia Samborowska[4], Natalia Żeber-Lubecka[3,5], Maria Kulecka[3,5], Tomasz Klupa[6] & Iwona Gregorczyk-Maga[2]✉

People with type 1 diabetes (PwT1D) are at higher risk of developing periodontal diseases. We investigated the impact of dietary habits on the gingival crevicular fluid (GCF) microbiome and metabolome in PwT1D. The oral and periodontal examination was performed and GCF sampled. Genomic DNA was extracted, bacterial 16S rRNA sequenced and concentrations of short-chain fatty acids and trimethylamine derivatives determined. Pro-healthy (pHDI) and non-healthy diet indices (nHDI) were calculated using the validated Questionnaire of Eating Behaviours. In total, 110 participants were included (mean age 27.1 ± 6.0 years, 60.0% male). In 65 PwT1D, the mean duration of diabetes was 15.5 ± 8.4 years and mean Hba1c% 6.97 ± 0.95% (53 ± 2.2 mmol/mmol). 22 cases of mild gingivitis (G) were identified, all in the T1D group. There were no significant differences in the frequencies of pHDI categories between study groups (T1D with G, low 19 [86.4%] and moderate 3 [13.6%]; T1D without G, low 28 [66.7%] and moderate 14 [45.2%]; control, low 30 [68.2%], moderate 14 [31.8%]; $p = 0.213$). GCF microbiome composition did not differ between pHDI categories. In PwT1D and G caproic acid was higher in low vs. moderate pHDI category (3.5 [0.9–4.9] vs. 0.64 [0.49-NA] umol/l, $p = 0.04$). In people with T1D without G, isocaproic acid and glycerophosphorylcholine were lower in low vs. moderate pHDI category (0.14 [0.13–0.46] vs. 0.45 [0.18–1.24] umol/l, $p = 0.032$, and 71.23 [32.83–120.40] vs. 129.8 [70.5–228.1] ng/ml, $p = 0.013$). This was the first study to report on the impact of diet on GCF in PwT1D. The state of periodontal tissues was worse in people with T1D, overweight and with a worse quality diet. Alterations in the concentrations of selected GCF metabolites suggest an indirect association between the quality of diet and contents of pro-healthy and non-healthy products on the state of periodontium.

Keywords Type 1 diabetes, Continuous subcutaneous insulin infusion, Gingival crevicular fluid, Microbiome, Metabolome, Gingivitis, Diet

The impact of diet and nutritional status on gut metabolome and microbiome is subject to an extensive research. The oral microbiome has been linked to the risk of obesity, with differences in composition between obese and non-obese individuals[1]. Multiple links have been found between dietary components (i.e. macronutrients: protein, carbohydrates, fats, micronutrients, and pre- and probiotics) and types of diet (i.e. mediterranean, low- and very-low-carbohydrate etc.), the state of gut microbiome and diseased states in humans[2,3]. On the contrary, the impact of diet on the oral microbiome remains under researched, even though it is known that diet is an

[1]Chair of Metabolic Diseases, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland. [2]Faculty of Medicine, Institute of Dentistry, Jagiellonian University Medical College, Montelupich 4, 31-155 Krakow, Poland. [3]Department of Genetics, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland. [4]Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. [5]Department of Gastroenterology, Hepatology and Clinical Oncology, Centre of Postgraduate Medical Education, Warsaw, Poland. [6]Chair of Metabolic Diseases, Faculty of Medicine, Center of Advanced Technologies in Diabetes, Jagiellonian University Medical College, Krakow, Poland. ✉email: iwona.gregorczyk-maga@uj.edu.pl

important factor affecting the oral health status and the prevalence of caries and periodontal diseases (PDs)[4]. PDs are a common medial problem, affecting 20–50% of the global population[5]. In susceptible individuals they can lead to life-threatening systemic diseases or cause their exacerbation, including as cardiovascular disease and diabetes[5]. PDs also lead to the reduced quality of life[6] and pose a substantial economic burden on health-care systems[7].

The oral cavity consists of different microbiologically separate niches, including dental surfaces, mucosae, saliva and gingival sulci filled with gingival crevicular fluid[8]. In periodontal health, the gingival sulci are shallow and enclosed. Easily accessible and sampleable saliva and supragingival dental plaque have been studied extensively. Contrasting with is the rarely investigated GCF[9,10], possibly due to more elaborate equipment needed and training required to sample the subgingival space. Moreover, as the GCF in periodontal health does not come in a direct contact with ingested food, its link with diet seem to be less clear that those of saliva or dental plaque. However, in PDs, local inflammation makes the subgingival space and gingival crevicular fluid more susceptible to exogenous compounds from ingested food from the oral cavity.

Although the interplay of protective and harmful factors in oral niches such as the supragingival space is recognized as a driver of oral pathology, the specific influence of diet and nutritional status on these processes remains insufficiently understood[11]. Previous research has primarily focused on the effects of diet on the salivary microbiome and metabolome, while the gingival crevicular fluid (GCF)—a unique and less accessible microenvironment—has been largely overlooked[12,13]. This presents a critical gap in our understanding, particularly given that GCF, distinct from saliva and dental plaque, may reflect localized periodontal changes more sensitively.

Notably, individuals with type 1 diabetes (T1D) are at higher risk for periodontal diseases[14], yet the mechanisms underlying this susceptibility, especially how diet might modulate the GCF microbiome and metabolome in this group, are poorly characterized. Only recently have studies begun to explore GCF profiles in well-controlled T1D populations, revealing early metabolic and microbial alterations, associated either with concomitant T1D and G or T1D on its own[10]. However, the potential mediating role of dietary habits and nutritional status in these changes remains unexplored. Lastly, there are conflicting reports on the role of specific metabolites in the oral cavity and the gut, making this subject even more intriguing.

Our study addresses this evidence gap by investigating the impact of dietary quality and nutritional status on the GCF microbiome and metabolome in people with T1D. By focusing on this under-researched oral niche, we aim to identify novel pathways linking diet, metabolic health, and periodontal diseases. This approach could also inform tailored dietary interventions to improve the oral health and, eventually, mitigate periodontal complications in at-risk populations such as people with T1D.

In this study, we investigated the impact of diet and nutritional status on gingival crevicular fluid metabolome and microbiome in people with type 1 diabetes.

Methods

We consecutively recruited 65 people with T1D treated with continuous subcutaneous insulin infusion (CSII) and matched them with 45 non-diabetic controls. People with T1D were recruited from the Outpatient Clinic of the Department of Metabolic Diseases and Diabetology of the University Hospital in Krakow, an academic referral center for diabetes in southeastern Poland. Patients attending the clinic were offered the opportunity to participate in the study. The inclusion criteria were age 18–35 years, duration of T1D of at least 1 year, duration of CSII treatment of at least 6 months, and informed consent to participate. Patients during pregnancy, breastfeeding and with history of comorbidities including metabolic syndrome, cardiovascular disease, cancer, severe liver failure, or kidney failure were excluded. Diagnosis of T1D was confirmed based on the Diabetes Poland criteria. Data on age, sex, diabetes duration (on the day of sampling) and treatment, comorbidities, smoking, glycated hemoglobin (HbA1c), and T1D treatment were collected. HbA1c levels were measured using high-performance liquid chromatography. Participants height, weight, body mass index (BMI), waist and hip circumferences, and waist-to-hip ratio (WHR) were recorded. The body composition (total body fat [TBF, %] and total muscle mass [TMM, kg]) were assessed using InBody570 system (InBody Poland).

Information on dietary habits was gathered utilizing the Questionnaire Eating Behaviours (QEB), a tool developed by the Science Committee of Human Nutrition of the Polish Academy of Sciences[15]. QEB consists of 21 questions regarding eating habits and 21 questions regarding the frequency of consumption of specific groups of food products. Frequencies encompass six categories from 'never' to 'multiple times daily'. QEB was proved to be an effective and quick instrument in the assessment of the quality of diet[15]. Data gathered through the QEB tool can be analyzed by calculating pro-healthy and non-healthy diet indices. The pro-healthy diet index (PHDI) defines more frequent consumption of whole-meal bread, milk and fermented milk products, cottage cheese, fish, foods from legume seeds, fruit and vegetables. The non-healthy diet index (NHDI) refers to more frequent consumption of fast food, fried food, cheese, sweets, canned food, sweet fizzy drinks, energetic drinks and alcoholic beverages.

The sampling took place in the morning. Participants refrained from using toothpaste, mouthwash, or oral topical agents for 12 h before the visit, and abstained from brushing, eating, drinking, or smoking before sampling. A trained dentist conducted the examination in a private, fully equipped dental room. Periodontal parameters, including Gingival Index[16], Plaque Index[17], and Pocket Probing Depth (PPD), were assessed using standard periodontal probes. Gingival crevicular fluid (GCF) was collected from the buccal aspect, mesial and distal at each gingival sulcus, up to the PPD using PerioPaper Strips placed in the gingival sulcus for 30–45 s and later stored in 1ml of Liquid Amies solution (COPAN ESwab™). Four PerioPaper strips were collected, 2 for metabolome and 2 for microbiome analysis.

Microbiome and metabolome analysis

Genomic DNA was extracted and purified from GCF samples collected on PerioPaper Strips using a modified bacterial protocol with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). DNA purity and concentration were assessed using a NanoDrop™ 2000 Spectrophotometer and quantified via the Qubit dsDNA High Sensitivity Assay (Thermo Fisher Scientific, Carlsbad, CA, USA). Bacterial 16S rRNA libraries were generated with the Ion 16S™ Metagenomics Kit and Ion Plus Fragment Library Kit, following previously described methods[18]. To account for potential variability in microbial DNA content, the amount of DNA used for 16S rRNA library preparation was adjusted according to the Ion 16S™ Metagenomics Kit protocol. Specifically, 1–2 µL of DNA was used for samples with high levels of non-microbial DNA, and pooled short amplicons were normalized to a total input of 10–100 ng. By adjusting the DNA input according to protocol guidelines, we ensured uniformity across samples and improved the reliability of sequencing data. Sequencing was performed on the Ion Torrent Personal Genome Machine (PGM) platform using the Ion PGM™ Hi-Q™ View Kit (Thermo Fisher Scientific, Waltham, MA, USA). MA, USA). To minimize batch effects, all samples, including those from individuals with type 1 diabetes and healthy controls, were sequenced together within the same sequencing runs. This approach ensured that group assignment did not confound technical variation. Additionally, blank extraction controls (NTCs) were included during DNA extraction and sequencing to monitor potential reagent contamination.

The quantification of short-chain fatty acids (SCFAs) and trimethylamine derivatives was conducted using liquid chromatography-mass spectrometry (LC–MS/MS) with Waters Acquity Ultra Performance LC and Waters TQ-S triple-quadrupole MS systems. MassLynx and TargetLynx software (Waters) were utilized for data acquisition and processing. Analyte extraction involved incubating PerioPaper Strips with 50 µL PBS for 30 min. SCFA and lactic acid analysis relied on derivatization using 3-nitrophenylhydrazine and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide–pyridine solution, performed on 20 µL of each sample. The concentrations of short-chain fatty acids (SCFAs) were determined using calibration curves prepared from a series of calibrator samples, which were obtained by spiking standard stock solutions into water. The calibration curves were constructed by plotting the ratio of the peak area of each analyte to that of the internal standard against the known concentrations of the analytes. The limits of quantification (LOQ) were 5 µM for lactic acid, and acetic acid, 1 µM for propionic acid, 0.1 µM for butyric acid, isobutyric acid, 2-methylbutyric acid, isovaleric acid, 3-methylvaleric acid, 4-methylvaleric acid and caproic acid, 0.2 µM for valeric acid. Separation was achieved using a Waters BEH C18 column (1.7 µm, 2.1 mm × 50 mm) and guard column. Aqueous formic acid and formic acid in acetonitrile served as mobile phases at a flow rate of 0.6 mL/min. For trimethylamine (TMA) and related metabolites, a 20 µL sample volume and butyl bromoacetate derivatization method were employed, following established LC–MS/MS protocols[19].

Statistical analysis

Participants responses to QEB questions were analyzed. Frequency of product consumption were transformed into two categories––at least once daily, and less than once daily. PHDI and NHDI calculated values and after categorization were analyzed. PHDI and NHDI values of 0–33 were regarded as low, 34–66 as moderate and 67–100 as high[15]. Participants from three groups were compared: people with T1D and G, people with T1D without G, and healthy controls. Additionally, participants were divided based on BMI category (< 25 kg/m^2 vs. ≥ 25 kg/m^2). Microbiome analyses included comparisons between low and moderate category of PHDI, regardless of T1D or G status with an additional sub analysis of participants with T1D with and without G were performed. Microbiome analyses included also comparisons between BMI categories < 25 kg/m^2 and ≥ 25 kg/m^2. GCF metabolites concentrations were compared between participants from low and moderate category of PHDI and between BMI categories < 25 kg/m^2 and ≥ 25 kg/m^2, regardless of T1D or G status. The analysis of impact of singular QEB components on GCF metabolites concentrations was performed regardless of T1D and G status. The minimal number of participants required to pursue statistical analysis was arbitrarily set to 5.

Univariate ANOVA regression was performed to identify individual factors associated with the concentrations of individual GCF metabolites. Due to non-normal distribution of GCF metabolites concentrations, log(n)-transformed values were used for analyses. Factors included in the models were: age, sex, BMI, WHR, TMM, TBF, current smoking, the frequency of teeth brushing, the frequency of oral rinsing, HbA1c%, T1D, gingivitis, pHDI, and nHDI category. Based on the identification of significant factors of the univariate analysis, the multivariate model was to be built for each individual GCF metabolite. Due to multiple comparisons, the level of significance was set to < 0.001 and only factors reaching this level of significance were to be introduced to the final multivariate model.

The PS Imago Pro ver. 10 was used for all statistical analyses. When data were missing, a complete case selection approach was used. Normality of the continuous variable distribution was assessed using the Shapiro–Wilk test. Differences between groups were analyzed using Student's t-test or nonparametric tests (Mann–Whitney *U* test, Kruskal–Wallis ANOVA), when appropriate. Continuous variables were presented as arithmetic means ($\bar{x}$) ± standard deviations (SD) or as the median with interquartile range (IQR) when the data were not normally distributed. The distribution of categorical variables was described as counts and percentages. Statistical testing was performed to compare categorical variables using an independent sample chi-square test or Fisher's exact test, when appropriate. Statistical significance was set at $p < 0.05$. The Bonferroni method was used to correct multiple comparisons.

Power calculations, performed using the RNASeqPower package, estimated a statistical power of 90% for a sample size of 45 per group, assuming a 10 × coverage depth, a coefficient of variation of 0.5, and a fold-change effect size of 1.5. Power calculations performed using the MEtSizeR[20] package in R for targeted metabolic studies, with projected number of investigated metabolites of 15, and projected 33% significantly different metabolites, revealed a sample size of 34 (17 per each) group would be required to achieve a power of 95%. BAM files containing unmapped reads were converted to FASTQ format using Picard's SamToFastq tool[21]. Further

sequence processing and analysis were conducted in Mothur version 1.47[22]. FASTQ files were converted to FASTA format, and sequences meeting the criteria of 200–300 bp length, a minimum average base quality score of 20 within a sliding 50-base window, and a maximum homopolymer length of 10 were included in the analysis. Chimeric sequences were detected using VSEARCH with default settings[23] and removed before classification of the remaining 16S rRNA sequences. Sequence classification utilized the Wang method and the SILVA database (release 138)[24] with an 80% bootstrap threshold. Alpha diversity was evaluated using the Shannon index to assess bacterial richness and distribution evenness, along with the Chao1 index to quantify richness. Beta diversity analysis was conducted using Bray–Curtis distance-based principal coordinate analysis (PCoA). Differential taxonomic abundance was identified using a mixed-effects model in LinDA[25]. Nonparametric statistical approaches, including the Shannon diversity index and Chao1 richness index, were applied in Mothur, and group comparisons were made using the Mann–Whitney *U* test. Bray–Curtis distances and PCoA were computed with the vegan package[26] (32). Statistical significance was determined with false discovery rate (FDR) corrections for *P*-values ≤ 0.05[27]. Correlations between bacterial taxa and SCFA or amino acid concentrations were evaluated using Spearman's rank correlation coefficient.

Ethics

This study involving humans was approved by the Jagiellonian University Bioethics Committee (Komisja Bioetyczna Uniwersytetu Jagiellońskiego, approval number 1072.6120.10.2021. The study was conducted in accordance with local legislation and institutional requirements. All the participants provided written informed consent to participate in this study.

Results

We included a total of 110 participants, with a mean age of 27.05 ± 5.95 years, of whom 60.0% were male. There were 65 people with T1D treated with continuous subcutaneous insulin infusion and 45 matched non-diabetic controls. The mean duration of diabetes was 15.5 ± 8.4 years. The mean Hba1c% in people with T1D was 6.97 ± 0.95% (53 ± 2.2 mmol/mmol). Dental and periodontal examinations revealed 22 cases of mild gingivitis, all in T1D group, and no cases of periodontitis in the study population. The study population is characterized in Table 1. The mean BMI of participants was 23.91 ± 3.23 kg/m^2, 36 participants (33.0%) had BMI ≥ 25 kg/m^2. BMI was borderline higher in people with T1D and G (Table 1, $p = 0.054$). BMI ≥ 25 kg/m^2 was significantly more prevalent in people with T1D and G than in the remaining groups (54.5% vs. 33.3% in T1D without G and 22.2% in the control group, $p = 0.033$). There were no differences in PBF and TMM between the study groups (Table 1).

The calculated pHDI and nHDI indices for people with T1D with G, people T1D without G and healthy controls were for pHDI 24.2 (20.3–31.4), 25.7 (17.6–37.2) and 25.9 (18.3–36.3) respectively, and for nHDI 11.4 (6.8–17.8), 12.8 (8.8–19.9) and 9.3 (6.6–14.8) respectively, and they did not differ significantly between the study groups ($p = 0.964$ and 0.166, respectively, Table 2). Participants across the three groups had either low or moderate scores for PHDI, with no differences in the frequencies of these categories (T1D with G, low 19 [86.4%] and moderate 3 [13.6%]; T1D without G, low 28 [66.7%] and moderate 14 [45.2%]; control, low 30 [68.2%], moderate 14 [31.8%]; $p = 0.213$, Table 1). All participants scored low on the NHDI scale (Table 2).

Participants from low PHDI score category had higher WHR and TBF as compared to moderate PHDI score category (0.88 [0.83–0.92] vs. 0.85 [0.78–0.89], $p = 0.024$), and 25.8% [19.0–31.4%] vs. 17.9% (13.5–23.7%), $p = 0.014$, respectively; Table 3).

Participants from low versus moderate PHDI score category had higher GI (2.0 [1.0–3.0] vs. 2.0 [1.0–2.0], $p = 0.038$) and the prevalence of PPD > 3 mm was borderline higher in the low PHDI group (13.3% vs. 0%, *p*-0.058). The selected dental indices depending on PHDI category are summarized in Table 4.

To further analyze the impact of singular components of QEB, we performed analyzes for singular questions included in the questionnaire (Table 2, Table S1). Participants with T1D and G as compared to those with T1D without G and healthy controls less frequently reported snacking (59.1% vs. 95.2% and 95.2%, respectively, $p < 0.001$), reported more frequently everyday consumption of potatoes (31.8% vs. 7.1% and 2.3%, respectively,

	T1D with G (N = 22)	T1D without G (N = 43)	Healthy controls (N = 45)	Adjusted *P* value
Age, years	30 (23–34.5)	25 (22–32)	27 (24–30)	NS
Sex (female)	9 (40.9%)	17 (39.5%)	18 (40.9%)	NS
Smoking	5 (23.8%)	6 (14.6%)	2 (4.7%)	0.061
BMI (kg/m^2)	25.1 (22.5–27.3)	23.7 (22.0–25.6)	22.9 (20.2–24.7)	0.054
BMI ≥ 25 kg/m^2	12 (54.5%)	14 (33.3%)	10 (22.2%)	0.033
WHR	0.87 (0.83–0.90)	0.87 (0.83–0.91)	0.87 (0.80–0.92)	NS
TBF [%]	25.9 (19.0–35.0)	23.5 (17.1–29.2)	22.6 (17.5–27.5)	NS
TMM [kg]	33.9 (24.7–37.6)	32.9 (27.9–39.3)	31.8 (25.0–35.8)	NS
HbA1c, %	7.4 (6.9–8.3)	6.8 (6.5–7.7)	5.1 (4.9–5.3)	< 0.001*
T1D duration, years	18 (9–22)	14 (9–19)	–	NS

Table 1. Basic characteristics of the study population. Data are presented as median (interquartile range) or number (%). *significant difference at $p < 0.001$ in post hoc analysis for T1D with G vs. healthy controls and T1D without G versus healthy controls. T1D, type 1 diabetes; BMI, body mass index; WHR, waist-hip ratio; TBF, total body fat; TMM, total muscle mass; HbA1c, glycated hemoglobin; G, gingivitis; NS, not significant.

	T1D with G	T1D without G	Healthy controls	Adjusted *p* value
pHDI	24.2 (20.3–31.4)	25.7 (17.6–37.2)	25.9 (18.3–36.3)	NS
pHDI category				NS
Low	19 (86.4%)	28 (66.7%)	30 (68.2%)	
Moderate	3 (13.6%)	14 (45.2%)	14 (31.8%)	
High	0	0	0	
nHDI	11.4 (6.8–17.8)	12.8 (8.8–19.9)	9.3 (6.6–14.8)	NS
nHDI category				NS
Low	22 (100%)	43 (100%)	44 (100%)	
Moderate	0	0	0	
High	0	0	0	
Vegetarian	0	5 (11.9%)	1 (2.3%)	NS
Selected QEB questions				
Meat consumption				0.047
At least once/day	11 (50.0%)	10 (23.8%)	10 (22.7%)	
Less frequently than once daily	11 (50.0%)	32 (76.2%)	34 (77.3%)	
Potatoes consumption				0.004
At least once/day	7 (31.8%)*#	3 (7.1%)*	1 (2.3%)#	
Less frequently than once daily	15 (68.2%)	39 (92.9%)	43 (97.7%)	
Snacking Yes vs. no	13 (59.1%)*#	40 (95.2%)*	40 (95.2%)#	< 0.001
Fat contents in milk				0.010
Full-fat	14 (63.6%)	18 (42.9%)	15 (34.1%)	
Skimmed	8 (36.4%)	19 (45.2%)	29 (65.9%)	
No-fat	0	5 (11.9%)	0	

Table 2. Results of the dietary assessment. Data are presented as median (interquartile range). T1D, type 1 diabetes; G, gingivitis; NS, not significant; QEB, Questionnaire Eating Behaviours. *#-significant difference in post-hoc analysis at $p>0.05$

	pHDI–low N = 75	pHDI–moderate N = 31	*p* value
BMI (kg/m^2)	23.9 (22.1–25.7)	22.7 (20.7–25.4)	NS
WHR	0.88 (0.83–0.92)	0.85 (0.78–0.89)	0.024
TBF [%]	25.8 (19.0–31.4)	17.9 (13.5–23.7)	< 0.001
TMM [kg]	32.3 (25.0–37.2)	35.9 (29.6–39.4)	0.060

Table 3. Results of nutritional assessment depending on the pHDI category. Data are presented median (interquartile range). BMI, body mass index; WHR, waist-hip ratio; TBF, total body fat; TMM, total muscle mass; pHDI, pro-healthy diet index; NS, not significant.

Examination score	pHDI–low N = 75	pHDI–moderate N = 31	*p* value
PI	3.0 (2.0–4.0)	3.0 (2.0–4.0)	NS
GI	2.0 (1.0–3.0)	2.0 (1.0–2.0)	0.038
CAL min. 1 mm, N	44 (58.7%)	13 (43.3%)	NS
PPD, min. 3 mm, N	10 (13.3%)	0	0.058

Table 4. Results of dental examination depending on the pHDI category. Data are presented median (interquartile range). PI, plaque index; GI –CAL, clinical attachement level; PPD, pocket probing depth; TN, treatment needs; pHDI, pro-healthy diet index; NS, not significant.

$p=0.004$), reported more frequently everyday consumption of meat (50.0% vs. 23.8% and 22.7%, respectively, $p=0.047$), and more frequently drank full-fat milk (63.6% vs. 42.9% and 34.1%, respectively, $p=0.010$). Apart from fruit and nuts and seed as snacks , which were borderline less frequent in people with T1D and G than in people with T1D without G and healthy controls (27.3% vs. 55.8% and 55.6%, and 4.5% vs. 25.6% and 13.3%), there were no differences in the frequency of consumption of other types of snacks (Table S1).

Microbiome

Ten variables explained more than 0% variance in the composition of microbiome, with gingivitis being the only one statistically significant contributor (2.6%, Fig. 1). Moreover, out of measured nutritional status metrics, only TMM was significantly associated with microbial richness (as measured by Chao index, p-value = 0.016, r = -0.25, Fig. 2, panels A–J).

α- and β-diversity were compared between participants from low and moderate categories of pHDI in the whole study population and, additionally, in people with T1D. There were no differences in the GCF microbiome in these two comparisons, with insignificant differences in the Shannon and Chao indices ($p = 0.120$ and $p = 0.490$ for the whole study population and $p = 0.450$ and $p = 0.890$ for people with T1D, Figs. 3A, B and 4A, B), and the β-diversity ($p = 0.263$ for the whole study population and $p = 0.825$ in people with T1D, Figs. 3C and 4C). There were no differential taxa in microbiomes of participants from low and moderate categories of pHDI in the whole study population and people with T1D.

α- and β-diversity were compared between participants with BMI < 25 kg/m^2 vs. ≥ 25 kg/m^2 in the whole study population, showing insignificant differences in the Shannon and Chao indices ($p = 0.0310$ and $p = 0.170$, Fig. 5A, B), and the β-diversity ($p = 0.102$, Fig. 5C), with no differential taxa.

Metabolome

Analyzes of GCF metabolites concentrations between participants from low and moderate categories of PHDI in the whole study population revealed that the only significant differences were present in GPC that was lower in participants from low when compared to moderate pHDI category (72.3 [39.3–114.7] vs.124.0 (61.3–179.4) umol/l, $p = 0.003$, Table 5, Fig. 6).

The analysis of answers to singular items of QEB and their relation with metabolite concentrations, regardless of T1D and G status, showed that GPC was higher in participants consuming more whole meal bread and vegetables, and less full-fat milk, CA was borderline higher in participants eating fried food more frequently, and ICA was lower in participants consuming barley products less frequently. The full analysis is described in the Supplementary Materials (Table S8).

In the entire study population, weak positive correlations were identified between BMI and the concentrations of TMA (0.214, $p = 0.032$), betaine (0.268, $p = 0.005$), and carnitine (0.216, $p = 0.025$, Table S9). Additionally, weak positive correlations were identified between WHR and the concentrations of betaine (0.216, $p = 0.026$) and carnitine (0.194, $p = 0.046$, Table S9).

Analyzes of GCF metabolites concentrations between participants with BMI < 25 kg/m^2 vs. ≥ 25 kg/m^2 in the whole study population revealed the concentrations of AA were lower, whereas those of TMA, betaine and carnitine were higher in participants with BMI < 25 kg/m^2 vs. BMI ≥ 25 kg/m2 (AA: 137.47 [109.94–202.47] vs. 184.31 [130.67–328.01] mmol/l, $p = 0.034$; TMA: 8.92 [2.65–21.37] vs. 15.82 [8.66–28.72] umol/l, $p = 0.017$; betaine: 109.92 [39.72–215.84] vs. 195.98 [82.52–551.68] umol/l, $p = 0.015$; carnitine: 41.56 [29.66–73.25] vs. 66.45 [50.18–88.52] umol/l, $p = 0.030$; Table 6, Figure S1).

In the univariate regression analysis, the concentrations of AA, VA and CA were significantly associated with T1D ($p < 0.001$), and IBA and IVA with gingivitis ($p < 0.001$, Table S10). Among the remaining factors, only the concentration of CA was significantly associated with HbA1c% ($p < 0.001$). As no new significant factors influencing GCF metabolite concentrations were identified, multivariate analyses were not performed.

Discussion

To the best of our knowledge, it was the first study to investigate the associations between the nutritional status and diet with GCF metabolome and microbiome in people with T1D. Overall, the state of periodontal tissues was worse in people with T1D, with cases of G recorded only in this group. The prevalence of overweight (BMI ≥ 25 kg/m^2) was higher in people with T1D and G. Finally, people with T1D and G tended to have worse quality diet that participants without G. However, the GCF microbiome composition was not affected by the diet

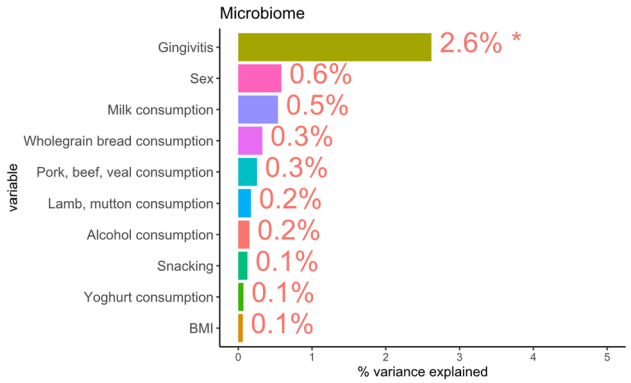

Fig. 1. Percentage of microbiome variance explained by each tested variable. 10 variables explained more than 0% variance in the composition of microbiome, with gingivitis (marked *) being the only one statistically significant contributor. BMI, body mass index.

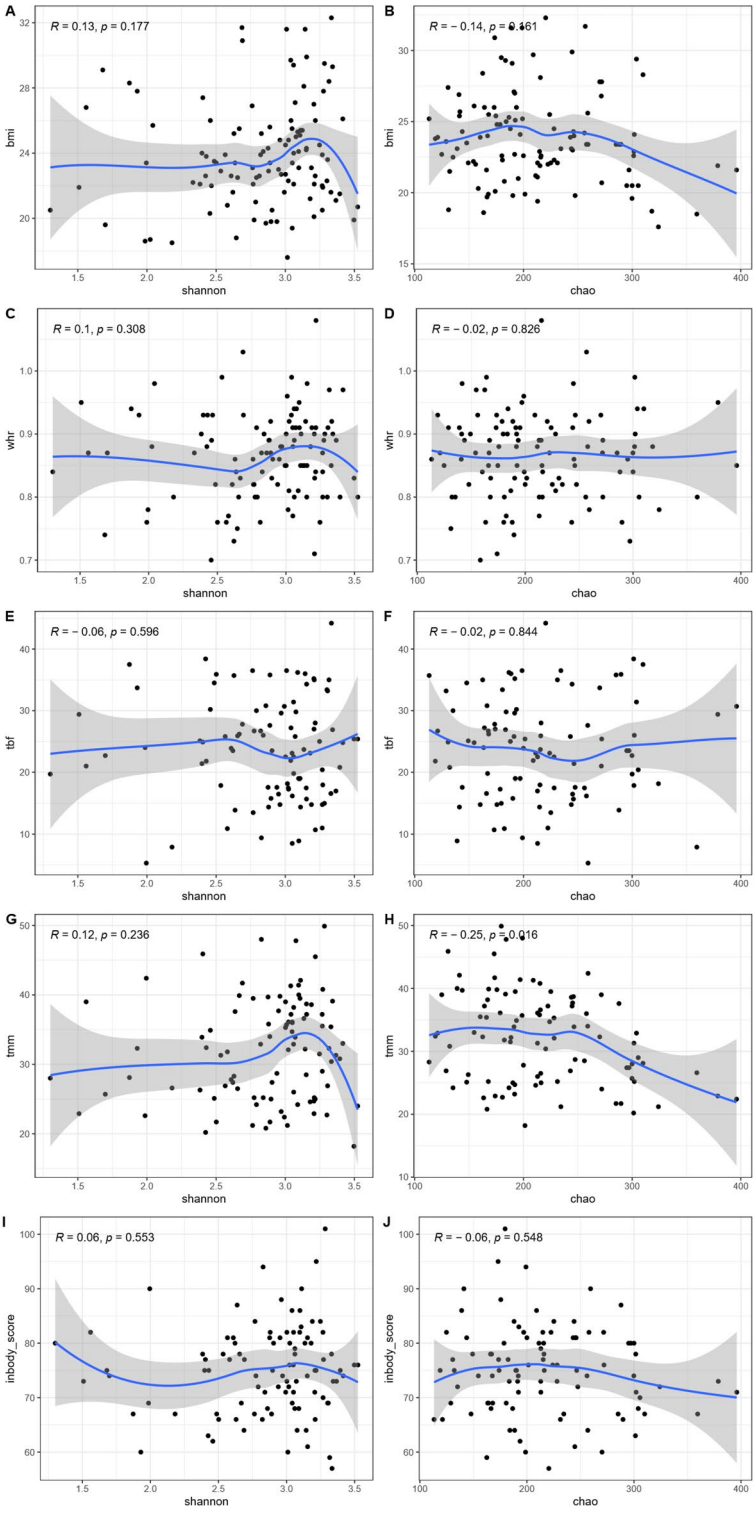

Fig. 2. (**A**–**J**) Correlations between nutritional status measurements, Shannon and Chao indices, as measured by Spearman's coefficient. TMM was significantly associated with microbial richness (as measured by Chao index, p-value = 0.016 , r = -0.25). There were no significant correlation between the remaining analyzed indices. BMI, body mass index; TMM, total muscle mass; WHR, waist hip ratio.

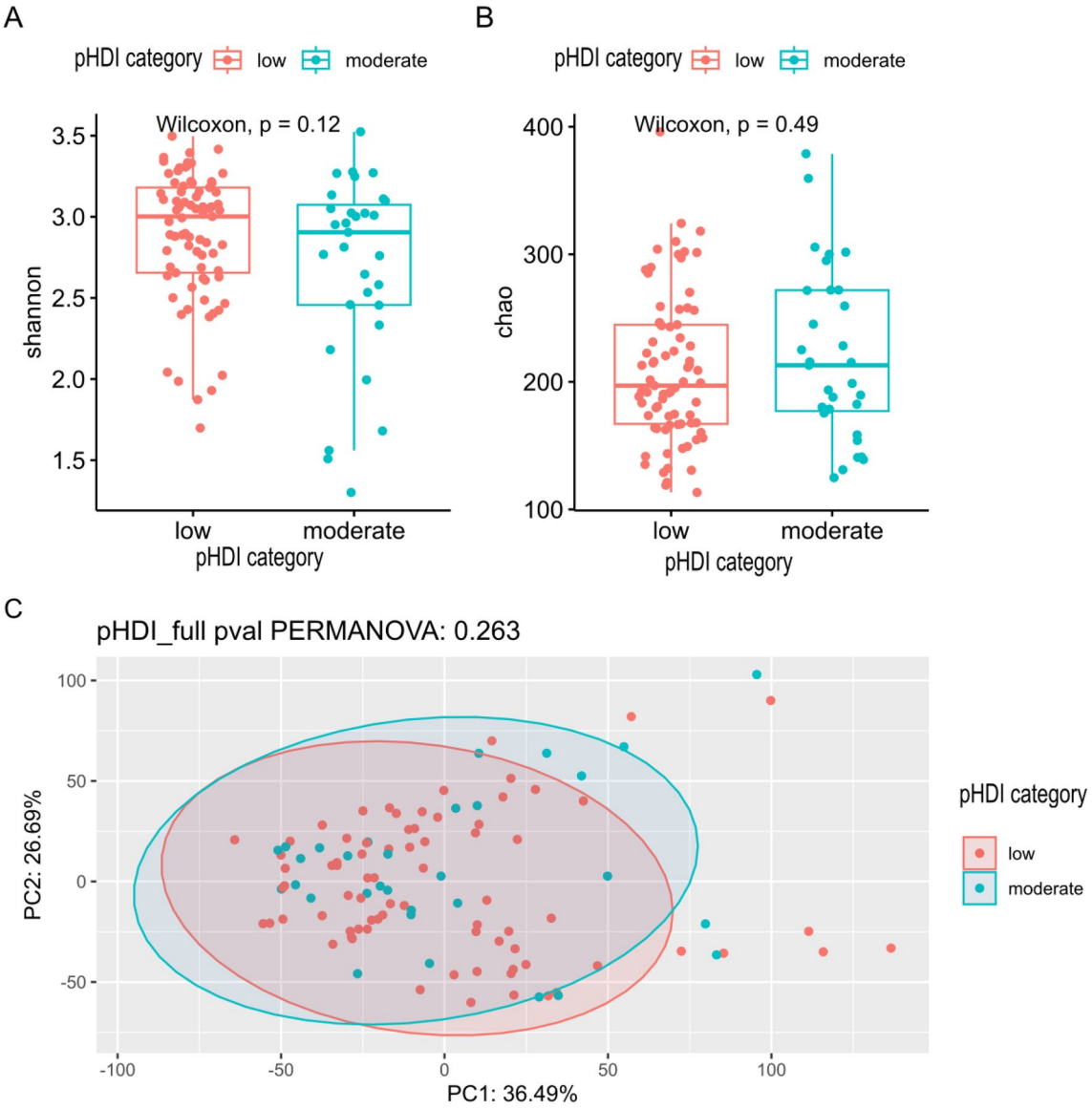

Fig. 3. Differences in bacterial diversity (**A**) and richness (**B**) between low and moderate pHDI groups. Differences in beta diversity between low and moderate pHDI groups (**C**). pHDI—pro-healthy diet index.

quality, BMI, body fat and muscle contents. Concentrations of CA, ICA and GPC, showed differences depending on the quality of diet and nutritional status.

The role of diet, its macro- and micronutrients contents, were reported to play an important role in the development of oral pathology, including PD[11,28]. A healthy and balanced diet, with anti-inflammatory qualities, may have a protective effect on periodontal tissues[28]. Some authors suggest it might have a supportive role on the treatment of PD[28]. Still, the data in people with diabetes, including T1D, is sparse. In one study of patients with the metabolic syndrome, a diet intervention reduced inflammatory markers in chronic periodontitis[29]. Such associations were observed for Mediterranean and whole-food diets as well[30,31]. Interestingly, diabetes may attenuate the positive effects of such diets[32]. In our study, participants tended to have rather healthy diet, rich in unprocessed, complex carbohydrates with low glycemic index like fruits, whole grain, vegetables and legumes, and with low consumption of high glycemic index products, containing processed, fermentable carbohydrates and simple sugars (like refined sugar, white wheat flour, sugary drinks). Our results fit well with these reports[30,33], as in participants with G in our study there was a tendency toward a worse quality diet, with numerically higher percentage of participants with low vs. high pHDI (although this results was not statistically significant than those with no signs of periodontal pathology. The consumption of fermentable carbohydrates was reported to be associated with an increased risk of dental caries and periodontitis[28]. Moreover, diet rich in high-caloric carbohydrates was linked to proinflammatory states[34]. Conversely, raw vegetables consumption, an in general vegetable-based or vegetarian diets, were suggested to reduce the risk of PDs[28].

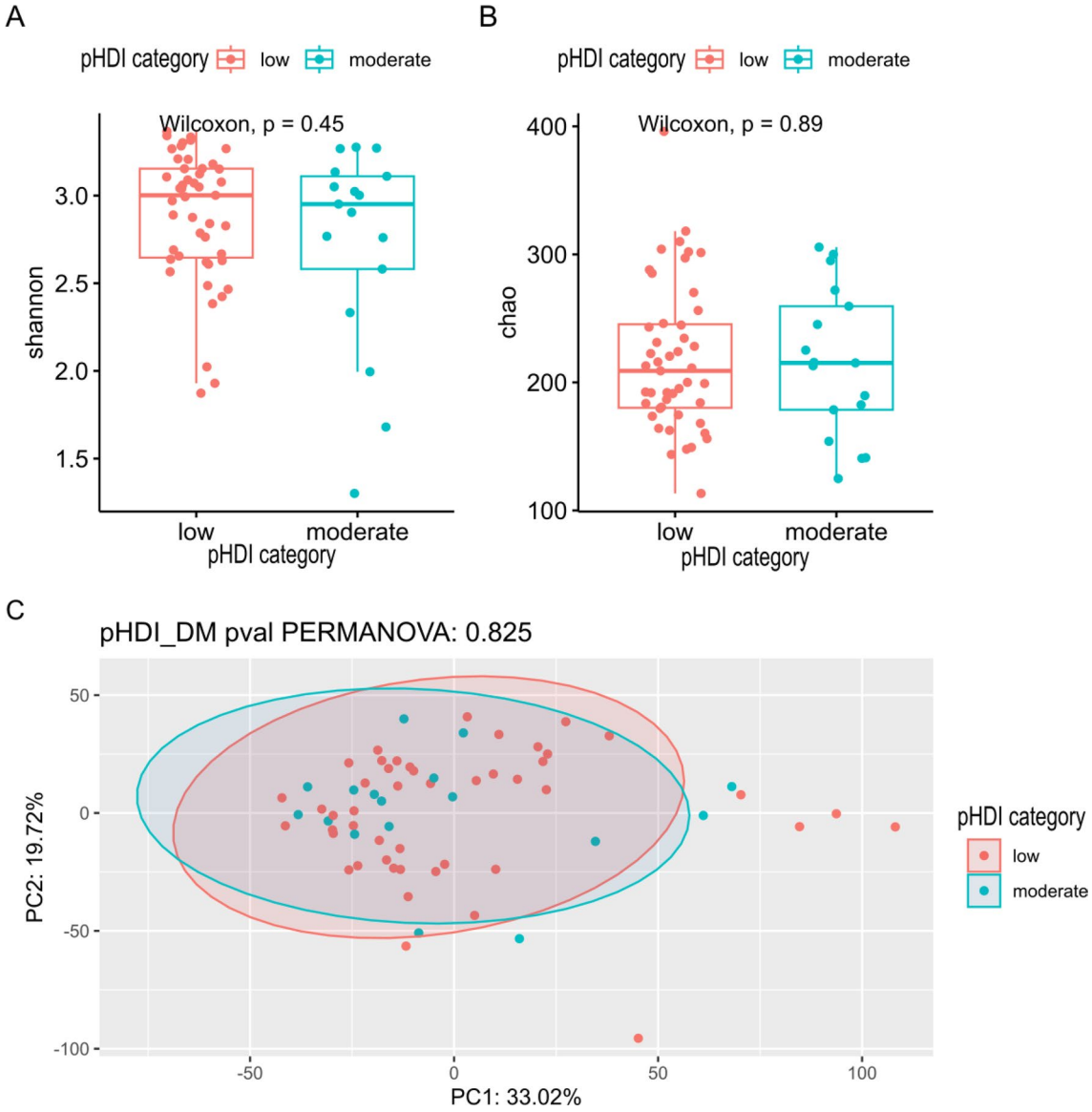

Fig. 4. Differences in bacterial diversity (**A**) and richness (**B**) between low and moderate pHDI groups. Differences in beta diversity between low and moderate pHDI groups (**C**). The figure concerns T1D group only. pHDI—pro-healthy diet index.

A healthy diet can be also described as rich in anti-inflammatory components, such as nuts, fruits, vegetables, seeds, poultry and seafood and low-calorie drinks, and with low contents of pro-inflammatory products—carbonated soft drinks, refined grains and red meat[30]. In a large cohort study, a dietary pattern rich in salad, fruit, and vegetables, and with plain water was associated with lower periodontal indices[4]. Another study assessed the impact of proinflammatory diet on the GCF microbiome and inflammasome, showing that such a diet contributed to periodontal inflammation, modulating inflammatory biomarkers and moderately affecting the subgingival microbial community[35]. Moreover, specific product were also reported to lower the risk of PDs, such as food rich in nitrates[36]. On the other hand, a pro-inflammatory diet has been reported to increase the risk of periodontitis, higher probing-pocket depth and clinical attachment level[31,33].

The impact of protein content in meals on periodontal conditions has not yet been well defined. Possibly the source of protein––plant or animal––may play a major role in its negative or positive impact on health status, both local and systemic one[28]. Some studies suggested that the enrichment of gingival pocket environment in protein, by degradation, causes its alkalization, creating a more favorable environment for the growth of pathogens typical for PD such as *Prevotella*[12,37]. In our study, we observed a higher consumption of meat in participants with T1D and G. Interestingly, vegetarian or vegan diets seem to have a positive role in keeping the systemic and periodontal health but there are other socio-economic and health-awareness-related factors that may be confounding this populations[38].

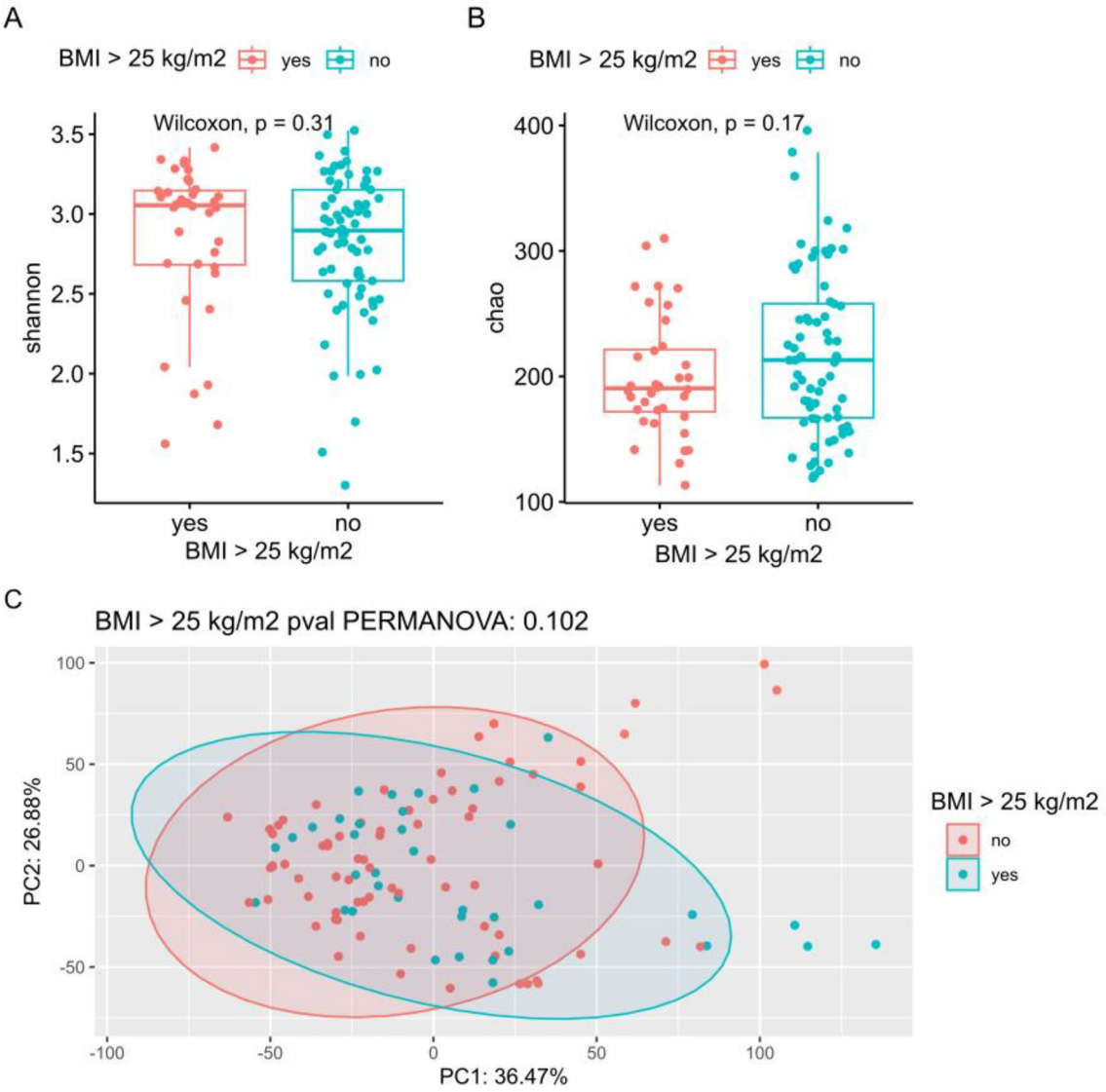

Fig. 5. Differences in bacterial diversity (**A**) and richness (**B**) between normal and elevated (≥ 25 kg/2) BMI groups. Differences in beta diversity between normal and elevated (25 +) BMI groups (**C**). pHDI—pro-healthy diet index.

Considering lipid contents in the diet, we only observed higher fat contents in milk consumed by participants with T1D and G. Overall, participants in our study used fats that are considered as healthy, such as olive oil. Several studies have shown that saturated and trans fats, and omega-6 fatty acids , found in processed meat, dairy products, eggs, vegetable oils and margarine promote inflammation[28].

Although we believe that the diet may have played some role in the development of G in our study population, it seems that other factors were more impactful. First of all, the prevalence and severity of PD are recognized to be higher in people with T1D when compared to non-diabetics[10,14]. These conditions have somewhat shared pathophysiology. T1D is associated with increased inflammation, which in turn lead to microvascular and macrovascular complications, with hyperglycemia further enhancing advanced glycated-end products formation, oxidative stress and inflammation[2,39]. The lack of a more pronounced impact of diet on the state of periodontium in our study population may also be explained by overall good quality of diet in our study participants, as evidenced by scored in the low nHDI category. Furthermore, we included people with T1D with a good metabolic control of diabetes, treated with insulin pumps. As pump therapy is not fully reimbursed in Poland, our participants could have a better socio-economic status and more motivation for treatment. Possibly, in populations with worse diet quality, poorer metabolic control and other types of diabetes, such as type 2 diabetes (T2D), could have given different results. In fact, poor quality diet in T2D was linked to caries and higher bleeding scores on probing[40].

Obesity and overweight have been widely recognized as risk factors for the development and progressions of PD[41]. This has been addressed to chronic inflammation with increased circulation adipokines and oxidative stress observed in obesity. Some studies reported that along with a higher BMI, waist circumference, a marker of

Metabolite	pHDI – low N = 75	pHDI – moderate N = 31	*p* value
Lactic acid [mmol/l]	92.4 (47.4–159.9)	123.5 (59.0–190.7)	NS
Acetic acid [mmol/l]	150.4 (103.6–311.2)	150.1 (109.3–207.1)	NS
Propionic acid [mmol/l]	8.3 (5.5–16.0)	10.7 (4.2–14.1)	NS
Isobutyric acid [mmol/l]	0.6 (0.4–1.5)	0.7 (0.41–1.5)	NS
Butyric acid [mmol/l]	1.8 (1.0–3.2)	1.6 (0.8–4.8)	NS
2-metylobutyric acid [mmol/l]	0.3 (0.2–0.8)	0.3 (0.2–0.7)	NS
Isovaleric acid [mmol/l]	0.3 (0.1–0.9)	0.3 (0.1–0.6)	NS
Valeric acid [mmol/l]	16.4 (13.2–22.5)	19.3 (13.3–20.5)	NS
Isocaproic acid [mmol/l]	0.2 (0.1–0.6)	0.2 (0.1–0.5)	NS
Caproic acid [mmol/l]	1.0 (0.8–3.5)	0.9 (0.6–3.4)	NS
Trimethylamine [umol/l]	11.7 (5.2–22.1)	10.1 (3.4–24.7)	NS
Betaine [umol/l]	109.5 (50.5–306.7)	146.6 (61.4–240.9)	NS
Glycerophosphorylcholine [umol/l]	72.3 (39.3–114.7)	124.0 (61.3–179.4)	0.003
Choline [umol/l]	383.3 (190.9–571.3)	361.7 (182.9–564.2)	NS
Carnitine [umol/l]	50.9 (31.9–83.5)	49.7 (37.8–74.6)	NS

Table 5. Results of metabolomic analyses depending on the pHDI category. Data are presented median (interquartile range). pHDI, pro-healthy diet index; NS, not significant.

highly metabolically active abdominal fat tissue, is also associated with PD[41]. This was again confirmed by our results with the highest prevalence of overweight and obesity in participants with T1D and G. Unsurprisingly, participants with lower quality diet had higher WHR, TBF and lower TMM. Significant positive correlation between BMI and WHR and potentially harmful TMA and its precursors, such as betaine, and L-carnitine in GCF[42], without changes in microbiome composition, add to the evidence for an early anomaly in gingival pockets. An inverse but weak correlation between TMM and microbial richness was observed. To date, reports on the association between the gut microbial diversity and muscle mass were largely inconclusive, focusing mainly on individuals with sarcopenia. Some studies reported differences in either alpha or beta diversity, with the remaining reporting specific shifts in selected bacterial taxa[43]. We did not find any reports on the oral, and specifically the GCF microbiome, and muscle mass. We did not investigate the inflammasome, and, additionally, this analysis was performed in the whole study population, without the stratification for the presence of G and T1D, which are associated with increased inflammation, potentially confounding the results, and warranting further research.

The reported negative correlation between total muscle mass (TMM) and microbial richness is statistically significant yet weak ($r = -0.25$). No mechanistic explanation is provided to support this inverse association, and the result seems biologically implausible without additional context such as inflammatory or metabolic profiling.

Nevertheless, to date most studies showed no difference in the growth rates of oral bacteria depending on the diet, suggesting no or merely a weak relationship between diet and the oral microbiome composition[12]. To date, saliva has been investigated in the majority of studies of oral microbiome and metabolome. These studies did not elicit clear links between the diet, nutritional status, saliva metabolite concentrations and its microbial communities[13]. Small differences in salivary metabolomics were however reported[12]. When compared to saliva, GCF can provide site-specific information about the state of periodontal tissues[44,45]. GCF sampling is a non-invasive and relatively easy technique. Despite these qualities, its analysis is not widely used in studying the periodontal tissues and periodontal examination. Even though GCF contains multiple biomarkers for PDs, there is no consensus on their application in the diagnosis and monitoring of PDs[44]. As a small study showed, food rich in SCFAs can lead to their accumulation in gingival pockets, stimulating its inflammation and paving a way towards the development of PDs[46]. In our study, we strived to fill this knowledge gap, however we only identified singular associations between the quality of diet and its contents with GCF metabolome, with no impact on GCF microbiome. We believe that the observed differences in the GCF metabolome in our study, with limited dependence on the diet quality, further confirm our hypothesis that it forms the first stage in the continuum of the development of an abnormal oral microbiome and, further, the advent of PD.

Regarding the impact of diet on GCF metabolite concentrations observed in our study, GPC was lower in participants with lower quality diet. GPC is a phospholipid containing choline, presents in various foods including fish, meat, milk, eggs, cereals, and peanuts[47]. Some of these products were lacking in participants with lower quality diet in our study. GPC can be converted to a metabolically active phosphorylcholine, increasing metabolically transmission in the brain. GPC is also used to synthesize phosphatidylcholine which is main component of cellular membranes. Multiple trials investigated the potential role of GPC and its derivatives as a dietary supplement[47]. Caproic acid is a fatty acid found naturally in various animal fats and oils, and its synthetic forms are used as artificial flavors. This is in line with our findings, that people from lower quality diet group also consumed more meat and full-fat milk. Previous studies showed that CA is higher in people with T1D and GDM, suggesting connections with glucose metabolism[10,48].

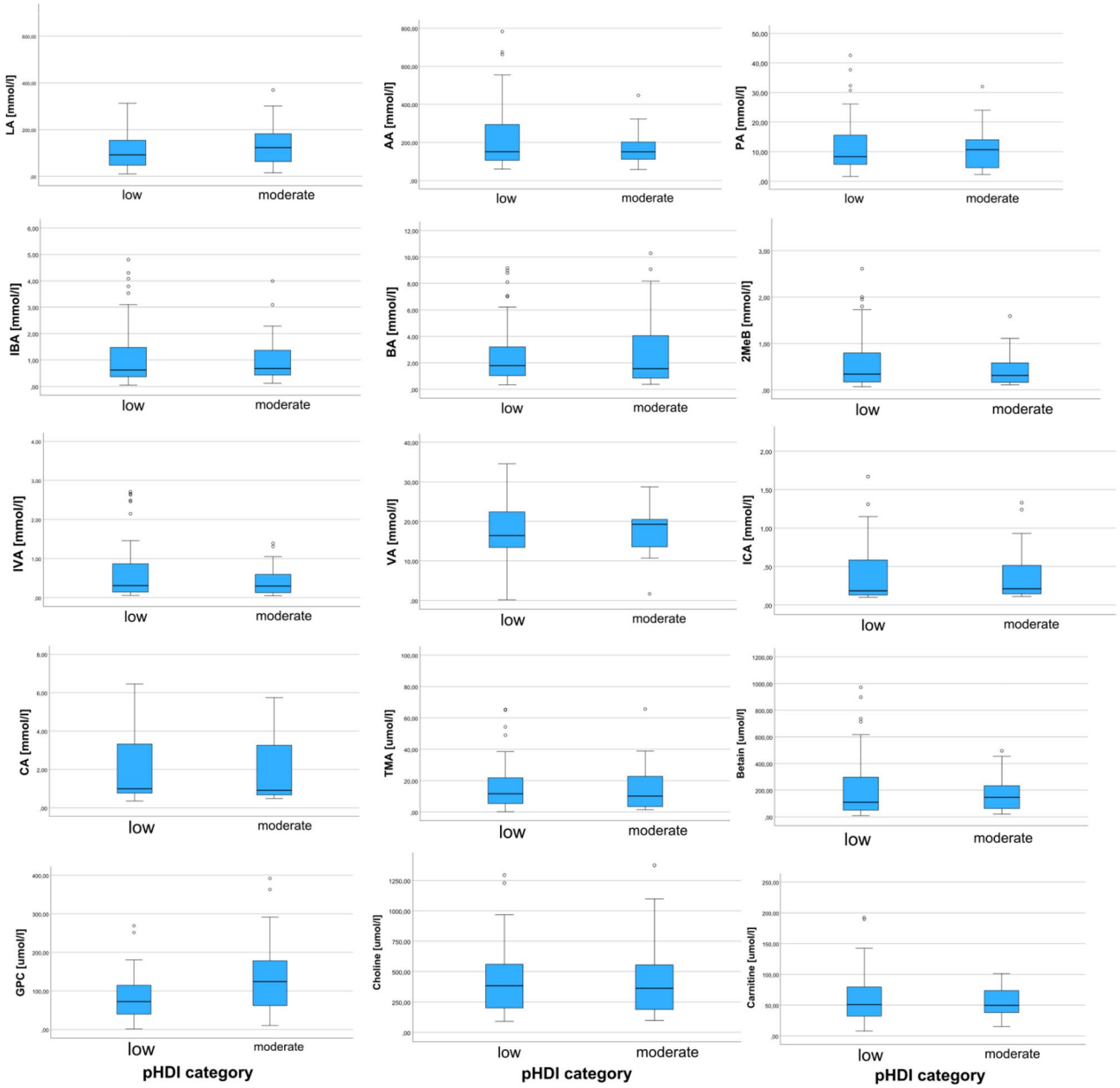

Fig. 6. Differences of GCF metabolites concentrations between participants from low and moderate categories of PHDI in the whole study population. GPC was lower in participants from low when compared to moderate pHDI category (72.3 [39.3–114.7] vs.124.0 (61.3–179.4) umol/l, $p = 0.003$).

Our study has some limitations. We included only relatively young people with T1D, treated with insulin pumps and with good metabolic control. Secondly, our participants has overall good diet quality. Furthermore, due to reimbursement schemes and lack of full coverage of insulin pumps in Poland, our participants might have presented with a better economic status, potentially confounding our results. Moreover, despite a relatively large study sample size, the numbers of participants in selected stratified subgroup analyzes could have been underpowered. Finally, we also did not record information on physical activity.

Conclusions

In this study, we showed that the state of periodontal tissues was worse in people with T1D, overweight and with a worse quality diet. Although the impact of diet itself on the GCF microbiome was not significant, there were alterations in the concentrations of selected GCF metabolites. This suggests an indirect association between the quality of diet and contents of pro-healthy and non-healthy products on the state of periodontium. These

Metabolite	BMI < 25 kg/m² N = 35	BMI ≥ 25 kg/m² N = 72	*p* value
Lactic acid [mmol/l]	113.89 (54.99–160.26)	80.33 (44.17–174.11)	NS
Acetic acid [mmol/l]	137.47 (109.94–202.47)	184.31 (130.67–328.01)	0.034
Propionic acid [mmol/l]	8.30 (5.23–13.84)	11.25 (6.12–18.31)	NS
Isobutyric acid [mmol/l]	0.64 (0.35–1.45)	0.68 (0.44–2.04)	NS
Butyric acid [mmol/l]	1.62 (0.97–3.28)	1.86 (0.97–3.28)	NS
2-metylobutyric acid [mmol/l]	0.32 (0.16–0.63)	0.36 (0.18–1.06)	NS
Isovaleric acid [mmol/l]	0.30 (0.13–0.63)	0.36 (0.18–1.34)	NS
Valeric acid [mmol/l]	17.62 (12.83–22.53)	16.07 (13.83–20.98)	NS
Isocaproic acid [mmol/l]	0.20 (0.130–0.58)	0.18 (0.14–0.60)	NS
Caproic acid [mmol/l]	0.92 (0.75–2.90)	1.07 (0.72–4.27)	NS
Trimethylamine [umol/l]	8.92 (2.65–21.37)	15.82 (8.66–28.72)	0.017
Betaine [umol/l]	109.92 (39.72–215.84)	195.98 (82.52–551.68)	0.015
Glycerophosphorylcholine [umol/l]	79.67 (54.30–133.48)	78.24 (39.78–138.17)	NS
Choline [umol/l]	375.54 (174.56–615.56)	365.75 (229.92–547.47)	NS
Carnitine [umol/l]	41.56 (29.66–73.25)	66.45 (50.18–88.52)	0.030

Table 6. Results of metabolomic analyses depending on the BMI category. Data are presented median (interquartile range). BMI, body mass index; NS, not significant.

metabolome variations may constitute the first stage in the continuum of the development of an abnormal oral microbiome and, further, the advent of PD.

Data availability

The data presented in the study are deposited in the NCBI BioProject repository, accession number ID: 1064953.

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Author contributions

MiK recruited control group participants, performed study procedures during participants' visits, analyzed data, prepared initial draft of the manuscript with selected figures. ZD recruited control group participants, performed study procedures during participants' visits and helped prepare the final version of the manuscript. MD, ES, NŻM and MaK were responsible for the metagenomic and metabolomic analyses, biostatistics, prepared selected figures helped prepare the final version of the manuscript. TK took part in the design of the study, recruited participants, helped prepare the final version of the manuscript. IGM conceived and designed the study, performed dental examination and the remaining study procedures during participants' visits, prepared initial draft of the manuscript with subsequent changes. All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to I.G.-M.