Gut Microbiome Analysis Reveals Characteristics Decrease in *Bifidobacterium* Count in the Adult Type 2 Diabetes Mellitus Patients

Muzammal Sattar

Government College University Faisalabad

Haseeb Anwar (✉ drhaseebanwar@gcuf.edu.pk)

Government College University Faisalabad <https://orcid.org/0000-0001-8438-9700>

Muhammad Umar Sohail

QBRI: Qatar Biomedical Research Institute

Irshad Ahmad

MoPH: Ministry of Public Health Qatar

Muhamm Naeem Faisal

University of Agriculture Faisalabad

Zahid Kamran

Islamia University of Bahawalpur: The Islamia University of Bahawalpur Pakistan

Maha Al Asmakh

Qatar University

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Abstract

Background: Type 2 diabetes mellitus is an idiopathic metabolic disorder in epidemic phase. The current study examines the gut microbiome of diabetic (Diab), diabetic with secondary complications (DSC), and healthy control (HC) subjects enrolled at the district hospital, Faisalabad, Pakistan. The study groups were characterized based on serum glucose and insulin concentrations and the subject's clinical history. Serum IgM, leptin, and amylin concentrations were determined using ELISA. The stool microbiome was analyzed using 16S rRNA amplicon sequencing on the Illumina MiSeq.

Results: Serum IgM, leptin, and amylin concentrations were higher ($p \leq 0.05$) in the Diab and DSC groups compared with the HC group. **Results:** No statistical differences were observed in microbiome composition and diversity at community levels as evaluated by alpha (Kruskal–Wallis $p = 0.16$) and beta (PERMANOVA $p = 0.41$) diversity indexes. Firmicutes (51.2%), Proteobacteria (14.9%), Actinobacteria (12.5%), and Bacteroidetes (7.8%) were the predominant bacterial phyla present in the stool microbiome of the study population. However, no differences ($p \geq 0.05$) in phylum-level taxonomic composition were observed between the study groups. At genus level, *Coprococcus* and *Ruminococcus* from phylum Firmicutes and *Bifidobacterium* from phylum Actinobacteria were significantly different ($p \leq 0.05$) between the study groups. *Bifidobacterium* and *Coprococcus* were higher in the HC group, whereas *Ruminococcus* was higher in the DSC group.

Conclusions: Taken together, in the present study, we report the gut microbiome of diabetic patients, which has specific taxonomic characteristics associated with the incidence and progression of diabetes.

Background

Diabetes mellitus is an idiopathic metabolic disease with severe secondary complications and a life-time disability. In recent years, the global prevalence of the disease has reached an epidemic phase at an alarming pace [1]. The progression of hyperglycemia into type 2 diabetes mellitus (T2DM) in susceptible adults is contributed by genetic and environmental factors [2]. In general, gut microbes exist in a state of symbiosis with their host. However, environmental factors, such as diet and lifestyle habits, may perturb this symbiotic association and lead to unfavorable health consequences [3, 4]. The significance of the gut microbiome in diabetes pathogenesis remains elusive. Although, recently emerged notion of shared genetic material and functional dogma between host and its microbiome highlights the dominant role of the microbiome in the development of T2DM and other obesity-related metabolic diseases [5].

Recently, the gut microbiome has become a key area of research as its manipulation could potentially help to manage several diseases, including obesity and diabetes [6]. Microbial dysbiosis, characterized by a decline in bacterial richness and diversity, may enhance intestinal permeability, glucose homeostasis, and elicit systemic inflammation [7]. Previous diabetes-microbiome studies demonstrate several vital changes in gut microbiome composition, including a decline in microbial diversity and relative richness of specific microbial taxa at different hierarchical levels [5, 6, 8–10]. Literature review also reveals a rise in

Firmicutes to Bacteroidetes ratio (F/B) in the subjects with T2DM compared to the healthy controls. Furthermore, the F/B has been positively associated with serum glucose, whereas, *Bifidobacterium* and *Roseburia* are positively and negatively associated with serum glucose, respectively [8, 10]. Specifically, *Bifidobacterium* [10], *Coproccoccus* [11], and *Ruminococcus* [11, 12] have been involved in energy homeostasis and may be associated in the transition from normoglycemia to pre-diabetes and T2DM [13].

Supporting a causal role of gut microbiome in diabetes, human fecal transplantation studies show improvement in insulin sensitivity in recipients with metabolic syndrome [14]. However, the precise mechanism through which the microbiome contributes to the T2DM is not fully understood. Furthermore, many diabetes-microbiome studies have produced inconsistent observations about the explicit nature of microbiome associations with T2DM. The data from three large cohort studies [15–17]; conducted in America, China, and Europe, suggest a fundamental role of gender and ethnicity in the gut microbial composition, indicating an ambiguity in generalizing diabetes-microbiome association, particularly to ethnic groups underrepresented in research studies and clinical trials. Therefore, to address the critical knowledge gap, we examine the gut microbiome of T2DM patients attending District Headquarter Hospital Faisalabad, Pakistan. We analyze the association of the gut microbiome with serum leptin, amylin, and IgM levels. To the best of our knowledge, this is the first diabetes-microbiome study from Pakistan, a country with an estimated 14.62% prevalence of T2DM [18].

Methods

1.1. Study cohort

This cross-sectional study enrolled twenty-two male adults (age group 40 to 60 years) volunteers from district headquarter hospital, Faisalabad, Pakistan. All participants were divided into three groups; healthy control (HC; N = 6), type 2 diabetes (Diab; N = 9), and Diab subjects with secondary complications (DSC; N = 7). The inclusion criteria for the participants were: age ≥ 30 years, in good general health (HC), or diagnosed with diabetes type 2 with or without secondary complications. Individuals taking antibiotics in the preceding three months or unwilling to provide stool samples were excluded from the study. All the participants were informed about the study objects and protocols. Before the start of sampling, written formal consent was obtained from each participant. The institutional review board of the university approved this study in compliance with participant anonymity, research ethical, moral, and biosafety standards (Reference no. GCUF/ECR/13).

A diabetes study participation questionnaire was designed according to WHO standards to identify the participant's eligibility for the current study. Diab and DSC status was established by the patient's self-reported history. Furthermore, the diabetic status of the patients was confirmed by measuring random blood glucose and insulin concentrations. Information on disease history, secondary complications, and medication were collected from the participants. All study participants from the Diab and DSC groups were on diabetes medication. The selection of patients for the DSC group was based on their proven

medical history in the hospital and their clinical profile. Mild to moderate nephropathy and cardiovascular diseases were the reported secondary complications in the DSC patients.

1.2. Samples collection

Blood samples were collected in a serum collection tube without clot activator. The serum was harvested by centrifugation at $1500 \times g$ for 30 minutes.

A stool collection kit comprised of a sterile tube, spatula, gloves, and collection paper was provided to the participants. Stool samples were self-collected at the house by the participants and transported on ice to the hospital. All samples were preserved at -20°C before DNA extraction.

1.3. Serology biochemical and hormones analysis

Serum insulin, leptin, amylase, and IgM antibodies concentrations were determined using commercially available kits. In brief, serum IgM (E-EL-H1814), insulin (E-EL-H2665), leptin (E-EL-HO113), and amylin (E-EL-HO322) concentrations were measured using human ELISA kits (Elabscience Biotechnology Inc., Wuhan, China). The optical density of these reactions was measured spectrophotometrically at a wavelength of 450 nm. The glucose (monoreagent- K082) concentration was measured by an enzymatic reaction following commercial kit protocol (Bioclin Systems II®, Quisaba, Bioclin, Belo Horizonte, MG, Brazil). The optical density was measured spectrophotometrically at a wavelength of 505 nm.

1.4. Stool microbiome analysis

All stool samples were thawed on ice and weighed for further processing. Approximately 200 to 300 mg of samples were used for DNA extraction using a commercially available kit [19]. In brief, the stool samples were re-suspended in 300 μL of lysis solution containing lysozyme (20 mg/mL) and 100X Triton (1.2%) in 1X TE buffer. The samples were vigorously vortexed and incubated at 37°C for one hour. The quality and concentration of the genomic DNA was examined using NanoDrop-2000 (Thermo Fisher, US).

Purified DNA samples were subjected to amplicon sequencing library preparation protocol, as elaborated previously [20]. In brief, the DNA samples were subjected to PCR amplification using 337F/805R primer-pair and index-primers. All libraries were washed and pooled before loading them in the Illumina MiSeq cartridge (MS-102-3003; Illumina, San Diego, CA, USA) [21]. Sequencing was performed on the Illumina MiSeq instrument at Applied Biological Materials Inc. Richmond, Canada.

1.5. Bioinformatics analysis

QIIME2 Bioinformatics pipeline was used for quality filtration, annotation, assembly, and FeatureTable construction, as previously described.⁸ In brief, FASTX toolkit was used for removing low-quality reads [22]. Paired-end reads were reassembled using DADA2 plugin to attain maximum overlap length [23]. Taxonomy classification was performed using VSEARCH, and BLAST + tools wrapped in q2-feature-classifier plugin.[24] Greengenes (gg_13_8) was used as a reference database for taxonomy classification.

1.6. Statistics analysis

Only those taxa that had a relative abundance of at least 0.5% in all the three groups were included in statistical analysis, and remaining data were discarded. All data were presented as mean and statistical significance ($P \leq 0.05$) between the groups was determined by one-way ANOVA, followed by Tukey's posthoc test. The Benjamini-Hochberg false discovery rate (FDR) was used to perform multiple comparisons of the P-values for the microbiome data, and an adjusted $P \leq 0.05$ was considered statistically significant. Alpha- and beta-diversity analysis was performed at 15,653 sequencing depth and differences between the groups were observed using the Kruskal–Wallis and PERMANOVA test, respectively.

Results

The mean serum glucose concentration was higher ($p \leq 0.05$) in the Diab and DSC groups as compared with the HC group (data not presented). Concordantly, the serum insulin concentration was lower ($p \leq 0.05$) in the Diab and DSC groups as compared to the HC group. Serum IgM concentration was higher ($p \leq 0.05$) in the Diab group compared with the HC and DSC groups. Serum leptin and amylin concentrations were also higher ($p \leq 0.05$) in the Diab group compared with the HC group. However, the differences between the DSC group and the Diab and HC groups were statistically non-significant (Fig. 1).

Illumina MiSeq sequencing produced 2,243,433 sequences for all the analyzed samples ($n = 22$, median $\pm$ SD = $91,821 \pm 54,999$). After quality filtration and chimera removal, 438,285 sequences of good quality (Phred quality score $\geq$ ASCII 30), belonging to 3,585 features (OTUs), were used for further analysis. These sequences correspond to 50 phyla, 149 classes, 253 orders, 362 families, 522 genera, and 583 species. In descending order, the four most dominant phyla present in the stool microbiome of the study population were Firmicutes (51.2%), Proteobacteria (14.9%), Actinobacteria (12.5%), and Bacteroidetes (7.8%) (Fig. 2). Among these abundantly present phyla, Actinobacteria, Proteobacteria, and Firmicutes to Bacteroidetes were numerically different among the groups. Similarly, at family and genus levels, *Bifidobacteriaceae* ($p = 0.03$) and *Bifidobacterium* ($p = 0.02$) were more abundantly present in the HC group compared with the Diab and DSC groups. Discordantly, family *Ruminococcaceae* ($p \leq 0.05$) and genus *Ruminococcus* ($p \leq 0.05$) were more abundant in the DSC group compared with the Diab and HC groups (Fig. 3). However, when Benjamini-Hochberg FDR was applied to these statistically different clades, their significance was lost, and only family *Bifidobacteriaceae* and genus *Bifidobacterium* were left statistically different ($p \leq 0.05$).

Figure 4a depicts the alpha-diversity index of bacterial communities in the three study groups. The observed_OTUs index was utilized to determine taxonomic diversity (species richness) within the samples. However, no significant differences were observed between the HC, Diab, and DSC groups (Kruskal–Wallis $p \geq 0.05$). Similarly, beta-diversity was also non-significantly different between the study groups. The weighted_unifrac distance matrix-based PCoA plot showed that no distinct clustering pattern was present between the microbiome of the study samples (Fig. 4b). The Permutational multivariate

analysis of variance (PERMANOVA) tests applied in the beta-diversity index did not show significant differences (PERMANOVA $p = 0.41$) among the studied groups.

Discussion

In the present study, we analyzed serum leptin, amylin, and IgM concentrations and stool microbiome of the healthy, diabetic, and diabetic with secondary complications subjects. The microbiome data were presented at phylum, family, and genus levels. Furthermore, the microbiome was also analyzed at the community level using alpha- and beta-diversity methods to classify specific community features that differed between the three studied groups.

In this study, we found that higher serum leptin and amylin levels were associated with diabetes mellitus. Amylin, a peptide hormone, plays an important role in pancreatic beta-cell damage in T2DM patients through islet amyloid formation and progression of secondary cardiovascular and nephropathies [25, 26]. In diabetes patients, the co-secretion of amylin and insulin from beta-cells in response to the glucose decreases [27]. Similarly, leptin secretion is closely related to body fat contents, and it is suggested that serum leptin may be a good biomarker of obesity-associated T2DM. [28] Higher leptin levels in obese subjects may promote the development of T2DM and secondary metabolic complications [28, 29]. Therefore, both amylin and leptin are associated with either loss of beta-cell mass or impaired insulin sensitivity on peripheral tissues in T2DM patients [30]. However, previous studies examine the association between serum leptin concentration and T2DM were not consistent in their findings. Few studies described a significantly positive association between serum leptin and the development of T2DM [31, 32], while others reported positive associations in males only [33, 34] or even negative association [35, 36]. It is possible that these inconsistencies in previous findings might be due to differences in sampling techniques or the subject's inclusion and exclusion criteria.

The gastrointestinal tract microbiome was investigated using 16S rRNA amplicon sequencing, and the data were presented at different taxonomic levels. Comparisons between community composition and taxonomic diversity were made between the study groups. Here we observed that microbial community diversity, as revealed by alpha- and beta-diversity indexes were not significantly different between the study groups. These observations resemble with our previous findings [9], where we report no statistical differences between the salivary microbiome diversity of obese pre-diabetic and healthy lean controls. Contrary to our observations, many researchers hypothesize that the diabetic phenotype is associated with a decline in gut microbial diversity, characterized as species richness and abundance [8, 17, 37]. These studies concluded that diverse gut microbiome could have a protective effect against systemic inflammation and metabolic disorders.

Similarly, no difference was observed between Firmicutes to Bacteroidetes ratio in the study groups, another critical parameter, most often emphasized as a prodrome for metabolic disorders development [9]. However, no significant differences in quantifications of taxonomic composition at phylum were observed in this study among the study groups. In the current study, only a few interesting observations

were reported at the family and genus level; particularly, a decline in population densities of the *Bifidobacteriaceae* and *Bifidobacterium* in the diabetic subjects. Previous diabetes-microbiome studies also correlate genus *Bifidobacterium* from the phylum Actinobacteria with a decreased risk of T2DM [10, 38, 39]. In general, the members of family *Bifidobacteriaceae* are known for lowering metabolic endotoxemia and glucose intolerance [10]. Previous studies show that increase in *Bifidobacterium* level improved glucose-tolerance, insulin secretion, and sensitivity and reduced systemic endotoxemia through enhanced gut barrier function.[40, 41] Particularly, probiotic and prebiotic supplementations in T2DM adults that reported increase in *Bifidobacterium* and *Coprococcus* counts also showed marked improvement in glucose and lipid homeostasis [42]. Besides, restoration of *Bifidobacterium* and *Coprococcus* spp., these supplements were also associated with alleviations of hyperglycemia, hyperlipidemia, and hepatic steatosis in mice fed with HFD [43, 44].

Conclusion

In conclusion, we observed that subjects with diabetic and diabetic secondary complication have different serum biochemical and the gut microbiome profiles. Results of this study reveal a bacterial dysbiosis in stool samples of disease subjects with a significant drop in the concentrations of family *Bifidobacteriaceae*, which may be considered as T2DM confounding factors. These findings suggest that gut microbiome's effects on T2DM would not be mediated through microbiome composition but rather through its function contents as closely related taxa may have widely different functions while distantly related taxa can have similar features.

Declarations

Ethics approval and consent to participate

The study was proved from institutional review board of the Government College University, Faisalabad (Reference no. GCUF/ECR/13).

Consent for publication

All the authors have read the draft before submission and have agreed with the contents.

Availability of data and material

The raw sequencing data in the form of FASTQ is being submitted to NCBI-SRA. Once obtained, the project ID will be mentioned in the draft.

Competing interests

All the authors declare that they do not have any competing interests with the submitted results.

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Authors' Contributions

MS collected samples and perform laboratory procedures. MUS conceived the concept and performed bioinformatics analysis. HA supervised the laboratory work and contributed to writing the manuscript. MNF, IA, and ZK contributed to Statistical analysis, paper draft and technical review. MA performs statistical analysis and contributed to writing the manuscript.

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Figures

Figure 1

Serum concentrations of IgM, Leptin, and Amylin. Groups with different superscripts (a, b, and c) show that the serum levels of the markers differ significantly between the groups.

Figure 2

Taxonomic distribution of the microbiome at the phylum, family and species level. Height of each bar represents the relative proportion of that phylotype. Only microbes with a relative proportion of at least 0.5% are presented in the bar plots. Taxon with asterisks differ significantly ($P \geq 0.05$) between the study groups.

Figure 3

A box and whisker plot of the estimated means of the statistically different bacteria at phylum, family, and genus levels. Groups with different superscripts (a and b) show that the bacterial population differ significantly between the groups.

Figure 4

Alpha- and beta-diversity analysis at 15,653 sequencing depth. No statistical differences are observed between the study groups.