Original Article

Pilot Study on Gut Microbiota Profile in Indian Children with Type 1 Diabetes

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Abstract

Background: Non-genetic factors like microbial dysbiosis may be contributing to the increasing incidence/progression of type 1 diabetes mellitus (T1DM). **Objectives:** To analyse the gut microbiota profile in Indian children with T1DM and its effect on glycaemic control. **Methodology:** Faecal samples of 29 children with T1DM were collected and faecal microbial DNA was extracted and subjected to 16S rRNA (ribosomal RNA) sequencing and further analysis. **Results:** The dominant phyla in children with T1DM were Firmicutes and Bacteroidetes. Butyrate-producing bacteria *Blautia* and *Ruminococcus* showed a significant negative correlation with the glycosylated haemoglobin (HbA1C) levels ($p < 0.05$). *Coprococcus* and *Propionibacterium* were important negative predictors of glycaemic control ($p < 0.05$). **Conclusion:** Our study suggests that Indian children with T1DM have a distinct gut microbiome taxonomic composition and that short-chain fatty acid-producing bacteria like *Ruminococcus* and *Blautia* (butyrate-producing) may play an important role in the glycaemic control of subjects with T1DM.

Keywords: Children, glycaemic control, India, microbiota, SCFA, short-chain fatty acids, type 1 diabetes

Introduction

Bacteria colonize the human body, including the oral cavity, placenta, vagina, skin and intestinal tract. The microbiome refers to the microbial species along with their genomes within a particular niche. However, the majority of these bacteria (10–100 trillion), commensal as well as pathogenic, reside in the gastrointestinal tract (also called as human gut microbiota).[1] So far, characterization efforts of gut microbiota have deciphered more than 1000 cultured species and more are being discovered as sequencing technologies are advancing.[2] The human gut microbiota is majorly dominated by four bacterial phyla which include Firmicutes, Bacteroides, Proteobacteria and Actinobacteria.[3] Any alteration in the microbial community that results in decreased diversity and reduction in number is termed as dysbiosis. A variety of factors can lead to an alteration in the gut microbiota which includes the type of birthing and infant feeding methods, diet, medications, stages of lifecycle, stress and co-morbid conditions.[4] In the last decade, a lot of research has been undertaken to study the role and relationship of the human gut microbial community in the development and evolution of chronic medical conditions like inflammatory bowel disease, cancer and obesity.[5]

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disorder precipitated by environmental factors in genetically susceptible individuals leading to beta cell destruction resulting in the deficiency of insulin.[6] The various environmental factors that have been postulated (but not proven) in triggering the autoimmune process in T1DM are as follows: viruses (for example, enteroviruses), dietary factors like early introduction of animal milk protein and cereals in infancy and use of antibiotics.[7,8] Numerous studies have

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associated gut microbial dysbiosis with the pathogenesis and progression of T1DM.[9,10]

The role of gut microbiota in T1DM predisposition and progression is complicated and still not very well understood. One of the reasons for altered gut microbiota profile in patients with T1DM may be due to disruption of the intestinal barrier leading to increased intestinal permeability. This could lead to the activation and proliferation of pancreatic-draining lymph node T cells, particularly diabetogenic CD8+ T cells, promoting insulitis.[11] The other important mechanism responsible for gut microbial dysbiosis in T1DM could be due to certain diabetogenic microbes which exist in the gut and may induce or hasten the development of T1DM through molecular mimicry.[12] Finally, the gut microbiome could modulate and affect the innate and adaptive immune system.[12,13]

Despite significant genetic influences, the rise of T1D prevalence and variable incidence rates across different countries suggests that non-genetic factors like microbial dysbiosis may also be contributing to the increasing incidence and progression of T1DM in recent years. However, most of the studies which have implicated the role of the gut microbiota in the development of T1DM have been performed mainly in Europe and America.[9,14-16] Only a handful of these studies have been performed in Asia and no data are available on Indian children with T1DM, despite the fact that India has one of the highest prevalences of children and adolescents with T1DM (0.37 cases per 1000 children) in the world as well as the largest number of new cases in the world (0.43 cases per 1000 children).[17-19] To add to this disease burden, the per annum increase in the incidence of T1DM in India is estimated to be 3% to 5%.[20] Hence, we performed a pilot study to analyse the gut microbiota profile in Indian children with T1DM and the effect of gut microbiota on glycaemic control.

Methodology

Subjects

Thirty children and adolescents with T1DM (aged 10–18 years) and their parents who attended the diabetes clinic at a tertiary care hospital in Pune, India were approached to take part in the study. Due to the fluctuation of weight and metabolic instability, which is usually seen at the onset and during the initial therapy for diabetes, children and young adults with T1DM duration of less than 6 months were not included in the study.[6] Children with other major illnesses or comorbidities (like celiac disease by assessing autoantibodies to tissue transglutaminase, untreated hypothyroidism and/or polyendocrinopathies) were excluded from the study. Of the 30 children with T1DM who agreed to take part in the study, 29 were included for the final analysis (one child was excluded in view of uncontrolled hypothyroidism). None of the children with T1DM included in the study were on any medications except insulin. None of the subjects included were on any treatment with oral medications like antibiotics, prebiotics or probiotics that could potentially influence gut microbiota 3 months before their inclusion in the study.

Ethics declaration

The Institutional Ethics Committee (Jehangir Clinical Development Centre Private Limited) approved the study. Written informed consent was obtained from all parents for children as well as for themselves and children gave assent for the study. This study was conducted between June 2017 and December 2018.

Clinical history and anthropometric measurements

Data on the age of the subjects, age at onset of diabetes, duration of diabetes, current medications, personal medical history, type of insulin regimen and total dose of insulin per day were collected using standardized questionnaires by physicians. The medical history provided by the parents was verified from hospital medical records. Standing height using a portable stadiometer (Leicester Height Meter, Child Growth Foundation, UK) was measured to the nearest millimetre and weight was measured using an electronic scale to the nearest 100 g. Body mass index (BMI) was computed by dividing weight in kilograms by height in metre square. Subsequently, the height, weight and BMI were converted to Z-scores using Indian references.[21]

Biochemical measurements

Glycaemic control was evaluated by measuring glycosylated haemoglobin (HbA1C). A blood sample (5 mL) was collected by a paediatric phlebotomist. HbA1C was measured by high-performance liquid chromatography (HPLC, BIO-RAD, Germany).

DNA extraction, 16S sequencing and bioinformatics

Faecal samples were collected in the morning from all participants in a sterile container at the clinic and preserved at − 80° Celsius until DNA extraction. Faecal microbial DNA was extracted using a QIAGEN Stool mini kit using the manufacturer's protocol. The extracted DNA was eluted in AE buffer (Elution buffer provided in Qiagen Stool mini kit) and subsequently processed for 16S amplicon sequencing using the Ion Torrent PGM platform (Thermo Fischer Scientific, Massachusetts, United States of America). The V3 region of the 16S rRNA gene was amplified using site-specific primers. The sequences generated were processed for quality analysis and primer sequences were trimmed using Mothur.[22] The sequences were then normalized into operational taxonomic units (OTUs) which were used to classify bacteria based on the sequence of the 16S rRNA marker gene at the genus level. The table was generated using the standard QIIME pipeline.[23] Taxonomic relative abundance profiles at the phylum and genus levels were generated based on OTU annotation. Microbial diversity analysis was performed using phyloseq and microbiome package.[24,25] Alpha diversity (variation of microbes in a single sample) analysis was performed using species richness (OTUs), Chao 1 index (abundance-based estimator of species richness), Shannon index (estimator

of species richness and species evenness, more weight on species richness) and Simpson index (estimator of species richness and species evenness, more weight on species evenness).[26]

Statistics

All statistical analyses were carried out using the SPSS for Windows software program, version 26 (SPSS, Chicago, IL). All the outcome variables were tested for normality before performing the statistical analyses. The Spearman correlation coefficient was calculated to estimate linear correlations between variables. Polynomial regression analysis was performed to identify which microbiota taxa were independent predictors of HbA1C levels in the group with T1DM. *P* values < 0.05 were considered as statistically significant.

Ethical Clearance Statement

The study was approved by the institutional ethics committee named as 'Ethics Committee, Jehangir Clinical Development Center Pvt Ltd.' vide letter no NA (our ethics committee does not provide an approval number) on 19th April 2016. Written informed consent was obtained for participation in the study and use of the patient data for research and educational purposes. The procedures follow the guidelines laid down in Declaration of Helsinki 2008.

Results

A total of 29 children with T1DM were enrolled in the study. The median age (± interquartile range) of the children with T1DM was 13.0 ± 3.9 years and the median age of onset was 10.0 ± 7.0 years. The clinical and anthropometric characteristics of the subjects have been illustrated in Table 1. All the children with T1DM were on a basal-bolus regimen of insulin therapy. Among the children with T1DM, 9 children had a disease duration of less than 2 years and 20 children had a disease duration greater than 2 years.

On performing alpha diversity analysis, the diversity was highly varied (irrespective of the type of analysis); the mean observed ASVs (amplicon sequence variants) was 1083.72 with values ranging from 544 to 1712 [Figure 1]. The dominant phyla in children with T1D were Firmicutes and Bacteroidetes followed by Actinobacteria, Proteobacteria and Cyanobacteria [Figure 2]. No significant differences were observed in terms of Bacteroidetes by firmicutes ratio (B/F ratio) on comparing children with disease duration <2 years ($n = 9$) to those over 2 years ($n = 20$) ($p > 0.1$). The relative abundance of different genera in the subjects with T1D has been demonstrated in Figure 3.

On performing correlation analysis between HbA1C and the various phyla and genera, the abundance of *Coprococcus* and *Blautia* genera showed a significant negative correlation with HbA1C concentrations [Table 2]. Similarly, on performing a correlation analysis between the disease duration of diabetes and various phyla and genera, *Ruminococcus* and *Mariprofundus* genera showed a significant negative correlation with disease duration ($p < 0.05$).

Table 1: Clinical and anthropometric parameters of children with type 1 diabetes

Parameter (units)	Children with type 1 diabetes ($n = 29$)
Age (years)	13.0±3.9
Disease Duration (years)	2.9±6.2
Height (cm)	148.5±21.7
Height *Z*-scores	-1.3±1.6
Weight (kg)*	36.0±17.4
Weight *Z*-scores	-0.8±1.5
Body mass index*	16.2±4.3
Body mass index *Z*-scores*	-0.8±1.2
HbA1C*	9.1±2.3

HbA1c: Glycosylated haemoglobin. *$P<0.05$, all values are expressed in median±interquartile range

Table 2: Correlations between various genera with glycosylated haemoglobin (HbA1C)

Genera	Spearman Correlation coefficient	*P*
Ruminococcus	- 0.343	0.068
*Coprococcus**	- 0.404	0.030
*Blautia**	- 0.385	0.039
Vibrio	- 0.334	0.076
Lutispora	- 0.338	0.073
Propinobacterium	- 0.330	0.080

*$P<0.05$

A polynomial regression analysis was then carried out to determine the significant predictors of HbA1C. Increase in *Coprococcus* (adjusted $R^2 = 0.305$, $P = 0.024$) and *Propionibacterium* (adjusted $R^2 = 0.152$, $P = 0.038$) genera led to significant decrease in HbA1C. None of the other genera *Ruminococcus, Blautia, Vibrio, Butyrivibrio* and *Lutispora* were significant predictors of HbA1C.

Discussion

To the best of our knowledge, this is the first study to explore gut microbiome profiles in Indian children with T1DM and its effect on glycaemic control. We have shown that Indian children with T1DM have a distinct gut microbiome taxonomic composition. We also found that short-chain fatty acid-producing bacteria (butyrate-producing) may play an important role in the glycaemic control of subjects with type 1 diabetes.

In our study, the dominant phyla were Firmicutes followed by Bacteroidetes. This is in contrast to findings of other studies, where Bacteroidetes was the dominant phyla followed by Firmicutes.[9,17,18,27] In the studies by Huan *et al*.[18] and Leiva-Gea *et al*.,[9] there was a significant reduction in Firmicutes/Bacteroidetes (F/B) ratio in subjects with T1DM as compared to that of healthy controls. However, contrasting findings have been reported by Qi *et al*.[17] and Meija-Leon

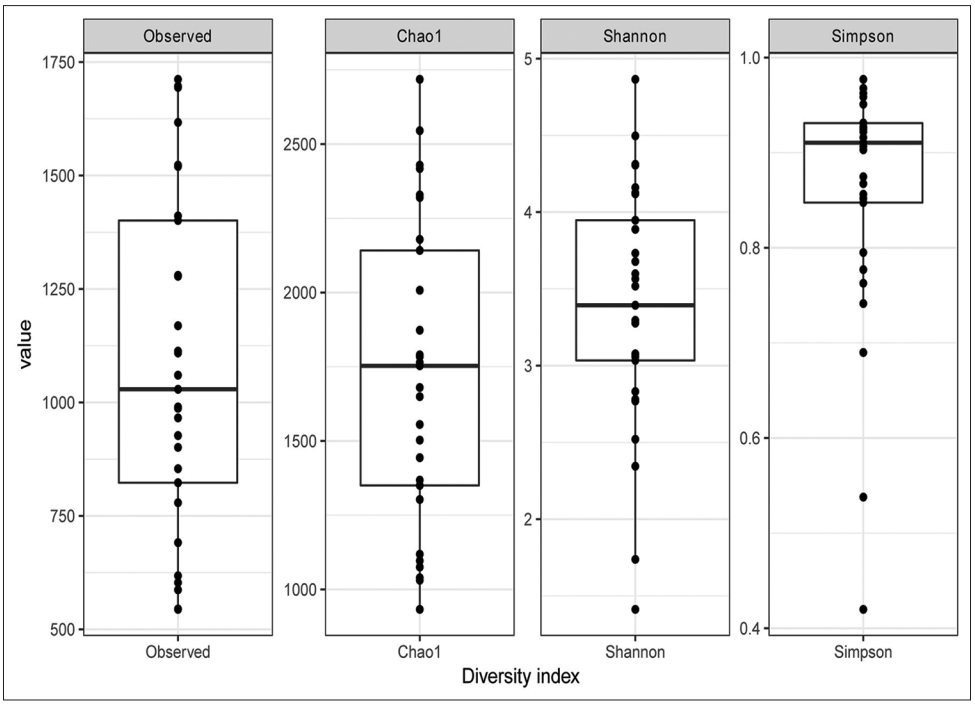

Figure 1: Alpha diversity analysis of Indian children with type 1 diabetes using different indices

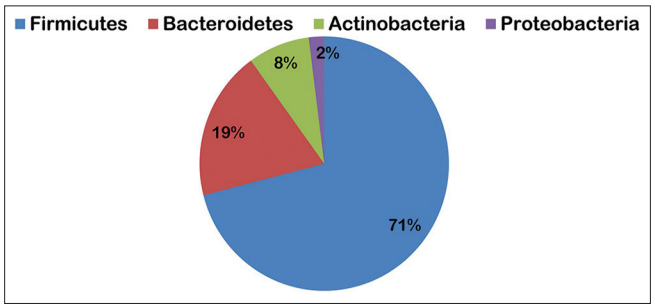

Figure 2: Phyla level distribution of gut microbiome in Indian children with type 1 diabetes

et al.,[27] where the F/B ratio was not statistically different among the two groups. The main reason for the variability in the diversity of the gut microbiota among the different studies could be explained by studying site-specific gut colonization patterns highlighting the importance of country-specific lifestyle-related factors which may play a major role in shaping the composition of the gut microbiome.[28]

Another finding supporting this reason of geographical influence on the gut microbiome is the conflicting reports of alpha diversity reported from various cohorts. No significant differences in alpha diversity have been demonstrated in subjects with T1DM and healthy subjects by Harbison *et al*. (in terms of species richness), Leiva-Gea *et al*. (in terms of Chao index), Pelligrini *et al*. (in terms of Chao index) and also by Huang *et al*. (in terms of the total number of OUT, Chao, Shannon and Simpson indices).[9,18,29,30] However, the studies by Leiva-Gea *et al*.[9] and Qi *et al*.[17] showed contrasting findings of significantly lower alpha diversities in subjects with T1DM using Shannon and Chao indices, respectively. In our study, in subjects with T1DM, the alpha diversity was highly varied.

Short-chain fatty acids (SCFAs) like acetic acid, butyric acid and propionic acid are known to improve glycaemic control in subjects with diabetes by decreasing the inflammatory state and improving the beta cell function.[31] This has been demonstrated by studies in non-obese diabetic (NOD) mice where SCFAs which have been released by gut microbiota have protected these mice against the development of insulitis and hence slowed the progression of diabetes.[32] Another study demonstrated that acetate- and butyrate-yielding diets enhanced gut integrity and decreased serum concentration of diabetogenic cytokines such as IL-21 in NOD mice.[33] This is corroborated by findings in our study where the relative abundance of butyrate-producing bacteria *Blautia* and *Ruminococcus* negatively correlated with HbA1C levels. This was also supported by the fact that propionic acid-producing bacteria *Propionibacterium* was an important negative predictor of glycaemic control in children with T1DM in our study. Surprisingly, two previous studies have shown that the relative abundance of Blautia is positively correlated to HbA1C levels, which is in contrast to our findings, necessitating further long-term large-scale trials to determine whether SCFA-producing bacteria play a role in improved glycaemic control.[9,17] Similarly, *Coprococcus* too is a butyrate-producing bacterium. No studies in subjects with T1DM have explored the role of *Coprococcus* on glycaemic control. However, a study has shown a decreased relative abundance of *Coprococcus* and *Streptococcus* in pregnant mothers with gestational diabetes mellitus (DM) as compared to normal pregnant women.[34]

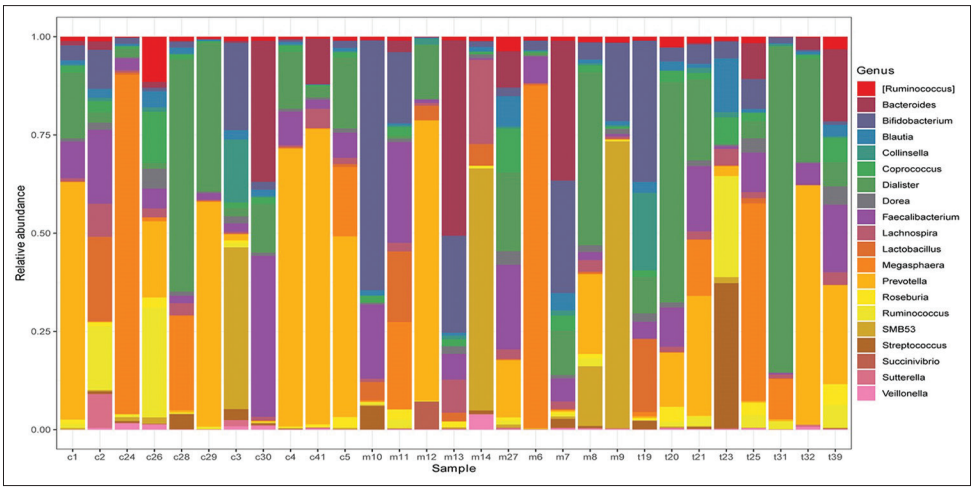

Figure 3: Relative abundance of different genera in Indian children with type 1 diabetes

The limitations of our study include the relatively small sample size of children and lack of healthy controls (10 healthy siblings of children with T1DM were approached, however, only six agreed (mean age-14.6 ± 3.0 years) to be a part of the study). Alpha and beta diversities and B/F ratios were similar in subjects with T1DM and controls. Relative abundances of the genera *Veillonella* and *Streptococcus* were significantly higher and that of *Bilophila* was significantly lower in children with T1DM than in controls (data not shown). In addition, due to the lack of data on type 1 diabetes-related autoantibodies and their titres, the correlation between antibody titres and different microbes could not be studied. Another limiting factor of our study was that there was no specific restriction on the diet of children with T1DM as well as that of healthy controls before collecting the stool samples. Finally, as this was a pilot study where the children had relatively poor control of HbA1c, the findings of this study may not be generalized to other populations of children with T1DM in India.

In conclusion, the gut microbiota profile of Indian children with T1DM and its effect on glycaemic control has been analysed and described for the first time. Larger case-controlled studies are required to determine if any specific 'inflammatory signature' is present in Indian patients with T1DM and whether any intervention may change the microbiota profile and correct dysbiosis to improve glycaemic control.

Authors' contribution

NS and AVK designed the study, contributed to acquisition of data, analysis interpretation of data, manuscript writing and checking. AK, YS DM, KJ, NK, VT, SB, DL, VK and KG contributed to analysis, interpretation of data and writing of manuscript and checking. The manuscript has been read and approved by all the authors, that the requirements for authorship as stated earlier in this document have been met, and that each author believes that the manuscript represents honest work, if that information is not provided in another form.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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