Original article

Bifidobacterium reduction is associated with high blood pressure in children with type 1 diabetes mellitus

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ABSTRACT

Children with Type 1 diabetes mellitus (T1DM) have an elevated risk of abnormal blood pressure (BP) measurements and patterns. Both hypertension and T1DM are well-known risk factors for cardiovascular disease and kidney failure. The human microbiome has been linked to both diabetes and hypertension, but the relationship between the gut microbiome and BP in children with T1DM is not well-understood. In this cross-sectional study, we examined the relationship between resting office BP and gut microbiota composition, diversity, and richness in children with T1DM and healthy controls. We recruited 29 pediatric subjects and divided them into three groups: healthy controls (HC, n = 5), T1DM with normal BP (T1DM-Normo, n = 17), and T1DM with elevated BP (T1DM-HBP, n = 7). We measured the BP, dietary and clinical parameters for each subject. We collected fecal samples to perform the 16s rDNA sequencing and to measure the short-chain fatty acids (SCFAs) level. The microbiome downstream analysis included the relative abundance of microbiota, alpha and beta diversity, microbial markers using Linear Discriminant effect size analysis (LEfSe), potential gut microbial metabolic pathways using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) and metabolic pathways validation using Statistical Inference of Associations between Microbial Communities And host phenotype (SIAMCAT) machine learning toolbox. Our study results showed that T1DM-HBP group had distinct gut microbial composition (at multiple taxonomic levels) and reduced diversity (richness and abundance) compared with T1DM-Normo and HC groups. Children with T1DM-HBP showed a significant reduction of *Bifidobacterium* levels (especially *B. adolescentis*, *B. bifidum*, and *B. longum*) compared to the T1DM-Normo group. We also observed unique gut-microbial metabolic pathways, such as elevated lipopolysaccharide synthesis and glutathione metabolism in children with T1DM-HBP compared to T1DM-Normo children. We can conclude that the reduction in the abundance of genus *Bifidobacterium* could play a significant role in elevating the BP in pediatric T1DM subjects. More studies are needed to corroborate our findings and further explore the potential contributing mechanisms we describe.

1. Introduction

The prevalence of T1DM is on the rise worldwide. The SEARCH for Diabetes in Youth study reported a 21.1% rise in the prevalence of T1DM between 2001 and 2009 [1], and World Health Organization (WHO) states that currently about 422 million people are affected with either type 1 or 2 diabetes mellitus [2]. With this increasing prevalence comes a surge in common T1DM comorbidities, including cardiovascular and kidney diseases. Studies have shown that children with T1DM have a higher prevalence of office and ambulatory hypertension (HTN) compared to their healthy counterparts. The prevalence of hypertension in children with T1DM is reported to be between 6% and 16% [3]. Studies have also reported a higher prevalence of abnormalities in 24 h ambulatory BP measurements and patterns in children with T1DM; in fact, BP dipping predicted the risk of future microalbuminuria development [4].

HTN is a known risk factor for diabetes-associated co-morbidities, such as nephropathy, and for each 1 mmHg increase in systolic blood pressure, there is an 8% increase in the odds of developing diabetic nephropathy [5], which emphasizes the importance of correcting the HTN in diabetic subjects. Multiple mechanisms are postulated for the elevation of BP in patients with diabetes [6–8] and, during the last decade, the microbiota gained significant attention due to its unprecedented role in human health [9]. A seminal review paper by Yano and Niiranen summarizes the association connection between the gut microbiome and blood pressure from various studies [10]. Emerging evidence shows that the gut microbiota composition in diabetic subjects differs significantly from their non-diabetic counterparts [11,12]. Such differences can result in changes to the inflammatory milieu and short-chain fatty acid (SCFA) production; both of these factors have been shown to impact pancreatic function in patients with T1DM [13,14]. Investigators found decreased levels of mucin- degrading bacteria (*Prevotella* and *Akkermansia*), butyrate-producing bacteria (*Clostridium clusters IV* and *XIVa*), *Roseburia*, *Faecalibacterium*, and *Bifidobacterium*, and increased levels of *Lactobacillus spp.* in diabetic patients compared to non-diabetic subjects [15–19]. We and others have previously described mechanisms through which dysbiosis may contribute to elevated blood pressure [20], among which, SCFA-induced renin release mediated via Olfr78, GPR41, and other receptors, and the modulation of endothelial-derived nitric oxide pathways [21] could be crucial to elevated BP. However, our current knowledge of the relationship between BP and the richness and composition of the gut microbiome in diabetic patients remains limited. Clarifying this relationship may open new avenues for disease treatment by identifying specific factors that impact diabetic BP dysregulation. In this study, we examine this relationship in children with T1DM and compare it to healthy controls. We also explore mechanisms by which dysbiosis may contribute to elevated BP in this population.

2. Patients and methods

2.1. Study population

Patients with T1DM followed at the Sidra Pediatric Endocrinology clinic were approached to participate in this study. Volunteer healthy controls were recruited from the family members of Sidra employees. The study was approved by the Sidra Medicines Institutional Review Board (IRB, #1708012734) and all participants were recruited after having consented with written parental consent and child assent.

Study participants who met the following criteria were included: age 6–12 years, no known chronic medical condition(s) other than T1DM, diabetes of more than one-year duration, and no history of receiving antibiotic treatment in the last three months at the time of signing the consent. Healthy controls were eligible for recruitment in this study if they had no chronic diseases, body weight and height in the normal range, and no history of receiving antibiotic treatment in the last three

months. A total of 29 children met the inclusion criteria; of whom 24 had T1DM and 5 were healthy controls.

The participants' clinical histories, such as medication, family history of diabetes, insulin treatment, and diabetes duration were provided by the treating clinician. In addition, the dietary intake was determined by a 24 h food recall. Anthropometric measurements were also collected, including body weight, height, and waist circumference. BMI percentile score was calculated and classified as per the Centers for Disease Control and Prevention (underweight < 5th percentile; normal weight 5th-84th percentile; overweight 85th-94th percentile; obese > 95th percentile) [22]. Blood samples for biomedical tests including lipid profile and HbA1c were collected.

2.2. Blood pressure measurements

An average of 3 resting, seated blood pressure measurements, obtained by a pediatric nurse using an appropriately sized cuff, were used to classify participants into two groups; normal BP (< 90%) and elevated/abnormal BP (≥ 90%). BP Indexes (an index of 1 corresponded to the 95th percentile for BP for age, gender and height) were calculated to allow for statistical analysis.

2.3. Dietary data calculation

Nutrient intake was computed from the 24 h food recall data using Nutritionist Pro™ software (Axxya Systems LLC, Texas, USA). Results were compared among groups.

2.4. Sample collection

Two stool samples were collected by the subjects at home, one with an OMNI Gut Stool collection tube, and the other with a 15 ml stool collection Thermo Fisher Scientific tube. Samples received were stored at − 80 ºC.

2.5. Gut microbiome profiling and analysis

Microbial genomic DNA (gDNA) was isolated from fecal samples using QIAamp® Fast DNA Stool Mini Kit (Qiagen, Germany). The library preparation using the gDNA was subsequently performed according to an Illumina protocol targeting the V3-V4 region of 16S rDNA as described previously [23]. This library was then processed for sequencing on the Illumina MiSeq platform using a MiSeq Reagent Kit v3 - 600 cycles (Illumina, California, USA) at Sidra Medicine. Base-calling was carried out directly on the MiSeq.

The raw data were demultiplexed using MiSeq Control Software (MCS). PEAR was used to merge both forward and reverse end sequences for each sample, and the reads with a quality score of ≥ 30 were considered for further analysis [24]. FASTQ files were then converted into FASTA files using the QIIME v1.9.0 (Quantitative Insights Into Microbial Ecology) pipeline [25]. Operational taxonomic units (OTUs) were obtained by aligning the sequence against the Greengenes database (gg_13_08) with a confidence threshold of 97% [26].

2.6. SCFA analysis by liquid chromatography-Tandem mass spectrometry

The SCFA analysis was performed by adaptation of the method published by Han et al. [27] as previously described [23]. Briefly, the collected stool sample was homogenized with a spatula, weighed, and diluted with 50% aqueous acetonitrile (Fluka, Switzerland). A portion of the supernatant was taken for further analysis along with mixed standard calibration solutions representing a range of concentrations for each fatty acid. All SCFA from C2 to C6, along with any iso- and anteiso-methyl branched-chain fatty acids, were tested. Samples and standards were derivatized with 3-nitrophenylhydrazine (Sigma Aldrich, Missouri, USA) and then diluted by a factor of 10 with 10%

aqueous acetonitrile. An internal standard (a mixture of SCFA derivatized as above with $^{13}C_6$-3-nitrophenylhydrazine (IsoSciences, Pennsylvania, USA)) was added. To test if the stool matrix affected recovery, controls were prepared by spiking isotopically labeled straight-chain SCFA derivatized with $^{13}C_6$-3-nitrophenylhydrazine to a mixture of stool sample supernatants from this study. These were analyzed along with the same mixture of isotopically-labeled SCFA prepared in 50% aqueous acetonitrile, and a comparison was performed. All samples were analyzed with a liquid chromatography-triple quadrupole mass spectrometer operated in negative ion scheduled MRM mode. A C18 column allowed the chromatographic separation of all derivatized SCFA. The peak area for all chromatographic peaks was calculated and used for generating calibration curves and for calculating unknown concentrations of SCFA in the stool.

2.7. Gut microbial computational analysis

2.7.1. Microbial diversity indices

Gut microbial richness and abundance were estimated by using Observed (species richness) and Chao1 (rare species richness), and Shannon and Simpson methods (species abundance). Alpha diversity was measured using the R package (Phyloseq and ggplot2). Beta diversity is presented as principal coordinate analysis as proposed in QIIME 1.9.0.

2.7.2. Gut microbial markers

Linear discriminant analysis effect size (LEfSe) was used to find the gut microbial markers for each group [28] as described previously [23]. It was used for a non-parametric factorial Kruskal-Wallis sum-rank test to identify features with significant differential abundance among different groups, followed by Linear Discriminant Analysis (LDA) to calculate the effect size of each differentially abundant microbial feature. Features are considered significant if the LDA value is > 2.0.

2.7.3. Functional profiling of gut microbiota

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis is a bioinformatics software package designed to predict metagenome functional content from marker gene surveys and full genomes, and it was performed according to the literature review from Langille et al. [29].

2.7.4. Statistical Interference of Associations between Microbial Communities And host phenoTypes (SIAMCAT) analysis

SIAMCAT provides a full pipeline supporting data pre-processing, statistical association testing, statistical modeling (logistic regression) including tools for evaluation and interpretation of these models (such as cross-validation, parameter selection, ROC analysis, and diagnostic model plots). Here we used the ridge logistic regression analysis and it was performed according to Wirbel et al. [30]. The predicted functional pathways from the PICRUSt analysis data were validated using the SIAMCAT displaying the cross-validation error as a receiver operating characteristic (ROC) curve, with a 95% confidence interval shaded in grey [30]. The area below the curve gives the receiver operating characteristic curve (AUROC). An AUROC value of more than 0.7 is considered fairly good, in terms of measuring the test's discriminative ability [31].

2.7.5. Statistical analysis

A normality test was used to check the data distribution of the patients' phenotypic data. Unless otherwise specified, data are presented as the median and interquartile range (IQR). Comparisons between the groups were performed by Kruskal-Wallis non-parametric one-way analysis of variance followed by Dunn's multiple comparison test on Prism Software version 8, (GraphPad, California, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Hypertension in children with T1DM

Twenty-nine children, of whom 24 had T1DM and 5 were healthy controls, were included in this study. Seven children (29%) in the T1DM group had elevated/abnormal BP (percentile > 90 for age, gender, and height; T1DM-HBP), compared to none in the healthy group. These three groups - healthy controls (HC), T1DM with normal BP (T1DM-Normo), and T1DM with high BP (T1DM-HBP), had comparable demographic and anthropometric characteristics except for HbA1c levels and diabetes duration between T1DM and HC (Table 1). BP measurements and indexes were significantly higher in T1DM-HBP compared to T1DM-Normo and HC (Table 2).

3.2. Consumption of micro- and macro-nutrients in the study participants

To evaluate the correlation of dietary components with T1DM-associated hypertension, we recorded the dietary habits using a single 24-hour recall and calculated the consumption of micro-and macro-nutrients in HC, T1DM-Normo, and T1DM-HBP subjects. We found that lipid and saturated fat consumption was significantly lower in T1DM-Normo than in HC. In addition, cholesterol consumption was significantly higher in T1DM-HBP compared to T1DM-Normo, while food folate consumption was significantly lower in the T1DM-Normo and T1DM-HBP groups than in HC (Table 3).

3.3. Gut microbial taxonomic composition and BP in children with T1DM

We measured gut microbial composition in the HC, T1DM-Normo and T1DM-HBP groups. Bacteroidetes were more abundant in T1DM-HBP compared with T1DM-Normo (69.24% vs. 56.87%; $p<0.005$). Interestingly, the phylum Firmicutes was more abundant in T1DM-Normo (35.29%) than both HC (27.58%; $p<0.05$) and T1DM-HBP

Table 1

Demographic, anthropometric, and biochemical parameters of HC, T1DM-Normo, and T1DM-HBP subjects.

	HC	T1DM-Normo	T1DM-HBP	One-way ANOVA
Number of subjects (n)	5	17	7	NA
Mean age (yrs)	8.8 ± 1.3	9.1 ± 2.2	9.4 ± 1.5	NS
Gender				
Male	4	10	5	NS
Female	1	7	2	
BMI (percentile)	63 (16.5–80.5)	69.0 (36.0–85.5)	57.0 (41.0–91.0)	NS
OW/OB (%)	20.0	23.5	28.6	NS
Male	1	1	1	
Female	0	3	1	
HbA1C (%)	4.85 (4.65–4.97)	7.6 (6.8–8.25)*	8.1 (7.1–9.4) **	0.0042
Duration of diabetes (yrs)	NA	4.0 (2.0–8.5)	3.0 (2.0–7.0)	NS
CSII therapy	NA	8	0	
Insulin injection	NA	9	7	
TG(mmol/l)	0.8 (0.4–2.75)	0.90 (0.65–1.15)	1.10 (0.9–1.9)	NS
LDL (mmol/l)	2.1 (1.55–2.5)	2.4 (2.1–2.7)	2.7 (1.4–2.7)	NS
HDL (mmol/l)	1.2 (1.15–1.55)	1.7 (1.3–1.95)**	1.9 (1.6–2.1) *	NS

Except for age, number of subjects, and gender, all values are expressed as median and IQR. One-way ANOVA, non-parametric analysis using the Kruskal-Wallis test and multiple comparisons using uncorrected Dunn's test were applied, $p<0.05$ was considered statistically significant. NA = not applicable. NS = not significant. *$p < 0.05$ and **$p < 0.01$ when compared with HC group using Dunn's test.

Table 2

Blood pressure parameters in HC, T1DM-Normo, and T1DM-HBP subjects.

	HC	T1DM-Normo	T1DM-HBP	One-way ANOVA (*p* value)	T1DM-normo vs T1DM-HBP *Dunn's* test (*p* value)
Number of subjects (n)	5	17	7	NA	NA
SBP (mmHg)	98.0 (91.0–105.0)	101.0 (97.5–104.0)	113.0 (110.0–116.0)	0.0004	0.0013
DBP (mmHg)	61.0 (59.5–67.5)	66.0 (61.5–67.5)	69.0 (69.0–77.0)	0.0275	0.0209
SBPI (mmHg)	0.85 (0.80–0.89)	0.87 (0.85–0.89)	0.97 (0.97–0.99)	0.0003	0.0013
DBPI (mmHg)	0.82 (0.80–0.87)	0.86 (0.80–0.89)	0.90 (0.90–0.99)	0.0232	NS

Values are expressed as median and IQR. One-way ANOVA, non-parametric analysis using the Kruskal-Wallis test, and multiple comparisons using uncorrected Dunn's test were applied. $p<0.05$ was considered statistically significant. NA = not applicable. NS = not significant.

(22.69%; $p<0.05$) (Fig. 1a). Furthermore, among the major phyla, Actinobacteria was found to be much more abundant in T1DM-Normo (3.11%) when compared with HC (0.42%; $p<0.05$) and T1DM-HBP (0.139%; $p<0.027$), while the phylum Proteobacteria was significantly less common in T1DM-HBP (1.75%) than in T1DM-Normo (4.00%; $p<0.01$) (Fig. 1a).

At the genus level, we observed many microbial genera (*Bifidobacterium, Collinsella, Odoribacter, Alistipes, Clostridium, Ruminococcus, Blautia, Oscillospira, Holdemania, Sutterella Erwinia,* and unclassified

Table 3

Consumption of macro- and micro-nutrients by the HC, T1DM-Normo, and T1DM-HBP subjects.

Micro- and Macro-nutrients	HC	T1DM-Normo	T1DM-HBP	One-way ANOVA (*p* value)
Number of subjects (n)	5	17	7	NA
Energy intake (kcal/day)	1693 (1480–1784)	1500 (1126–1622)	1310 (1123–1440)	NS
Proteins (g/day)	56.46 (48.32–58.52)	54.94 (46.78–77.25)	57.52 (36.80–72.05)	NS
Carbohydrates (g/day)	198.8 (177.7–209.8)	163.2 (130.2–251.7)	179.3 (129.3–186.6)	NS
Sugar (g/day)	83.69 (52.28–103.2)	59.38 (38.94–79.31)	44.40 (29.28–57.91)	NS
Fructose (g/day)	5.72 (1.30–12.17)	8.55 (1.64–14.68)	8.86 (8.17–11.59)	NS
Lipids (g/day)	75.04 (55.04–82.27)	42.27 (37.53–53.82)*	56.99 (35.69–65.49)	0.0404
Cholesterol (mg/day)	136.2 (75.15–306.6)	131.1 (86.79–257.3)	271.9 (223.9–602.8)**	NS
Saturated fat (g/day)	24.27 (19.23–26.65)	15.60 (11.74–18.55)&	14.01 (11.48–17.89)#	0.0208
MUFA (g/day)	19.97 (12.40–28.20)	14.73 (10.66–21.19)	15.10 (10.32–24.59)	NS
PUFA (g/day)	19.60 (10.50–21.68)	9.42 (6.18–11.56)	9.06 (5.51–16.13)	NS
Fiber (g/day)	15.23 (11.40–21.91)	9.33 (5.33–20.24)	12.09 (8.22–21.61)	NS
Salt (g/day)	5.28 (3.61–6.64)	3.55 (2.61–5.05)	4.59 (3.49–6.66)	NS
Sodium (mg/day)	2113 (1446–2658)	1422 (1045–2022)	1834 (1393–2667)	NS
Potassium (mg/day)	2592 (1344–2755)	2054 (1160–2352)	1624 (1042–1654)	NS
Calcium (mg/day)	452.7 (292.0–678.3)	555.6 (400.2–856.7)	337.3 (260.9–872.2)	NS
Vitamin D (μg/day)	2.33 (1.31–6.41)	4.16 (1.01–6.97)	3.52 (0.65–5.02)	NS
Food folate (μg/day)	176.4 (148.2–309.2)	84.73 (50.77–117.2)$	93.14 (74.28–183.7)	0.0055

Values are expressed as median and IQR, NS = not significant. *p = 0.0344, T1DM-Normo vs HC, Dunn's multiple comparison test. **p = 0.019, T1DM-HBP vs T1DM-Normo, Mann Whitney-test. &p = 0.0272, T1DM-Normo vs HC, Dunn's multiple comparison test. #p = 0.0405, T1DM-HBP vs HC, Dunn's multiple comparison test. $$p$ = 0.0052, T1DM-Normo vs HC, Dunn's multiple comparison test.

genera from the family of Rikenellaceae, Lachnospiraceae and Ruminococcaceae) that were significantly ($p < 0.05$) less abundant in T1DM-HBP compared to T1DM-Normo (Supplementary Fig. 1S). We also found significant differences in the abundance of ten microbial genera when comparing the T1DM-Normo and HC groups. The majority of these genera (*Faecalibacterium, Bifidobacterium, SMB53, Oscillospira, Dialister, Phascolarctobacterium,* and *Bilophila*) were significantly higher in the T1DM-Normo, while *Erwinia, Actinobacillus* and *Lachnobacterium* were lower in T1DM-Normo compared to HC (Supplementary Fig. 1S). Corresponding comparison of the T1DM-HBP and HC groups showed that an unidentified genus from the Lachnospiraceae family was less common in T1DM-HBP (1.01% vs 2.44%; $p < 0.015$) (Supplementary Fig. 1S). Finally, comparison of *Bifidobacterium* species among all three groups showed that *B. bifidum* and *B. longum* were significantly decreased in T1DM-HBP than in T1DM-Normo, while *B. adolescentis* was significantly decreased in T1DM-HBP than in the T1DM-Normo and HC groups (Fig. 1b). Further, we evaluated the ratio of Firmicutes/Bacteroidetes (F/B ratio), which suggests that the F/B ratio was significantly higher in T1DM-Normo than the T1DM-HPB and HC (Fig. 1c).

### 3.4. Gut microbial diversity and HBP in children with T1DM

We estimated alpha diversity by measuring the richness (Observed and Cho1 methods) and abundance (Shannon and Simpson indexes) of the gut microbiota in the T1DM-HBP, T1DM-Normo and HC groups. One-way ANOVA analysis indicated that there was a significant shift in richness (Observed, $p < 2.29^{E-08}$; Chao1, $p < 2.72^{E-08}$) of the T1DM-Normo and T1DM-HBP groups in comparison to HC. In addition, there was a significant shift in bacterial abundance (Shannon index, $p < 2.72^{E-08}$; Simpson index, $p < 3.64^{E-05}$) in T1DM-HBP compared to HC, but no corresponding difference was observed in T1DM-Normo compared with HC (Fig. 2a). We next analyzed beta diversity, a measurement of difference in microbial composition between multiple sample groups, using the Bray-Curtis method. However, this analysis did not uncover any significant similarities or differences among the three groups (Fig. 2b).

3.5. Gut microbial differential abundance and HBP in children with T1DM

We used LEfSe analysis based on the Linear Discriminant Analysis (LDA) score to measure the unique microbial profile that distinguishes each group. Our results indicated that the genus cc_115 and the families Veillonellaceae and Tissierellaceae were significantly enriched in T1DM-Normo in comparison to HC, while the genera *Lachnobacterium, Anaerofustis, Weissella, Enterobacter, Butyricimonas,* and *WAL_1855D* were significantly enriched in HC versus T1DM-Normo (Supplementary Fig. 2S(a)). Next, we compared T1DM-HBP against HC and found that the genus *Dehalobacterium* was significantly enriched in the former. Conversely, the genera *Desulfovibrio, Adlercreutzia, cc_115, Anaerofustis, Christensenella, Aggregatibacter, Victivallies, Acidaminococcus, Clostridium,* and *Bifidobacterium* were enriched in HC compared to T1DM-HBP (Supplementary Fig. 2S(b)). Finally, a comparison of T1DM-Normo versus T1DM-HBP showed that *Bifidobacterium* was significantly decreased and *Lachnobacterium* and *WAL_1855D* (along with other
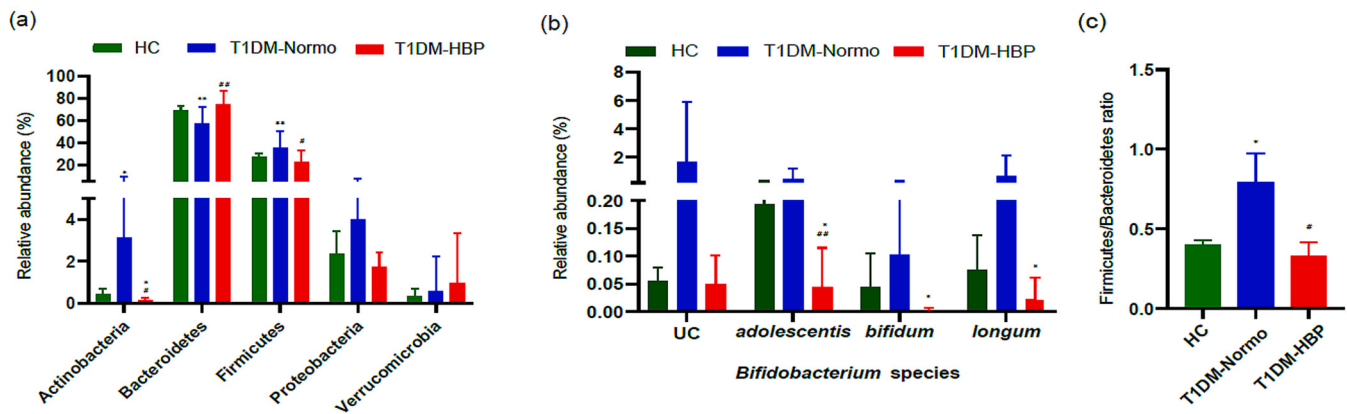
Fig. 1. Gut microbial composition in the HC, T1DM-Normo, and T1DM-HBP groups. (a) Five major bacterial phyla were found in all three groups. Data are shown as relative abundance (percentage of total gut microbiota composed of each phylum). (b) Relative abundance of four *Bifidobacterium* species in all three groups. (c) The ratio of Firmicutes to Bacteroidetes in all three groups. The results are expressed as mean±SD. HC, N = 5; T1DM-Normo, N = 17; and T1DM-HBP, N = 7. $p < 0.05$ considered statistically significant using Student's *t*-test. *$p < 0.05$ and **$p < 0.01$ compared with the HC group; #$p < 0.05$ and ##$p < 0.01$ compared with the T1DM-Normo group. UC, Unclassified bacteria at the species level.
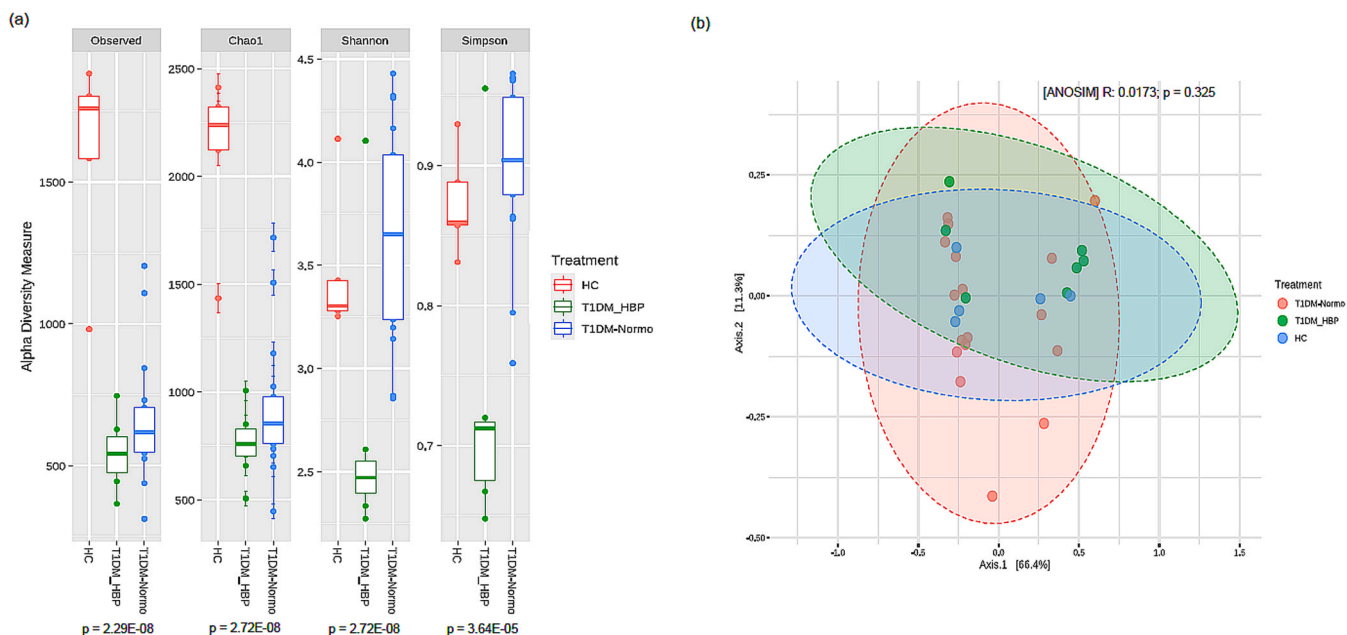

Fig. 2. Gut microbial diversity and microbial markers in the HC, T1DM-Normo, and T1DM-HBP groups. (a) Alpha diversity index measured by Observed, Chao1, Shannon, and Simpson methods. The boxplots are showing interquartile (IQR) ranges with the median and whiskers extending up to the most extreme point within 1.5 folds IQR. (b) Beta diversity index measured by Bray-Curtis method using Principle Co-ordinate analysis (PCoA) based on OTUs relative abundance profile. The two-variance explained by Axis.1 and Axis.2 are 11.3% and 66.4%, respectively.

unclassified genera from various families) were significantly enriched, in T1DM-HBP (Fig. 3).

3.6. Potential pathways and metabolites contributing to the pathophysiology of HBP in children with T1DM

To evaluate the potential effect of differing microbial compositions on gene function, we used the PICRUSt analytical tool to predict potential functional pathways that may be differentially affected by the gut microbial communities in each of the three groups. We identified multiple candidate pathways, including lipopolysaccharide (LPS) biosynthesis, glutathione (GSH) metabolism, amino acid degradation, D-glutamine and D-glutamate metabolism, that were significantly increased in T1DM-HBP compared to HC (Fig. 4b) Comparing the experimental groups, we found that the LPS biosynthesis,

glycosyltransferases, GSH metabolism, RNA degradation, iso-quinoline biosynthesis and D-glutamate and D-glutamine pathways were significantly increased in T1DM-HBP compared to T1DM-Normo, while the transporters pathway, glycerolipid metabolism and nitrotoluene degradation were significantly decreased (Fig. 4c). We performed SIAMCAT and LEfSe analyses to validate the pathways that we observed from the PICRUSt analysis, and both confirmed the significant enrichment of LPS, D-glutamate and D-glutamine, GSH metabolism, glycosyltransferases, and iso-quinoline biosynthesis pathways in the T1DM-HBP group and with the mean AUC of ROC curve is 0.815 (Fig. 5(a) and (b), and Supplementary Fig. 3S). Finally, we measured the concentration of SCFAs, including ethanoic acid, propionic acid, and iso-butanoic acid, in stool samples from all three groups. Contrary to our expectation, we did not find any significant differences among the groups (Supplementary Fig. 4S).
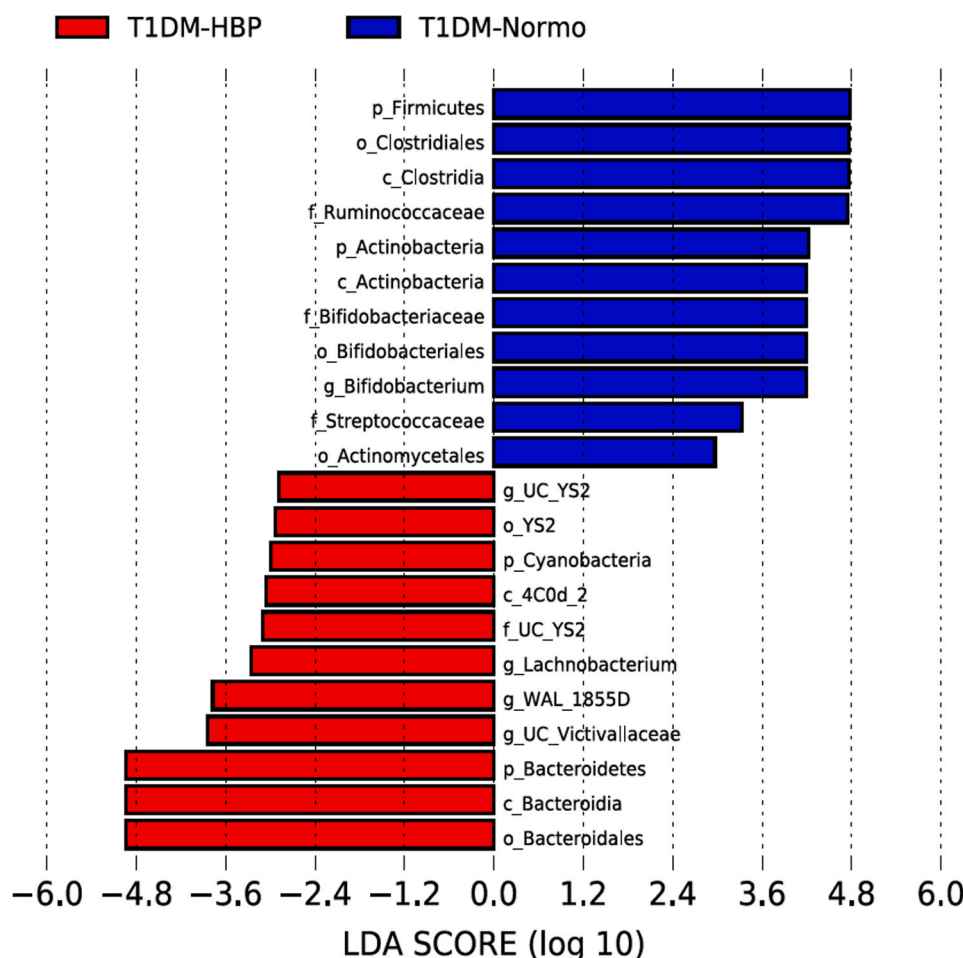
Fig. 3. Gut microbial markers in T1DM-associated HBP. Gut microbial markers measured by LEfSe analysis (cut-off value LDA > 2.0) in the T1DM-Normo and T1DM-HBP groups. HC, N = 5; T1DM-Normo, N = 17; and T1DM-HBP, N = 7. "g_UC" and "f_UC" represent unclassified bacteria at genus level and family level, respectively.

4. Discussion

Many factors are known to contribute to the development of vascular complications in patients with diabetes. Some of these are uncontrollable, including age at disease onset (duration of disease) and genetic susceptibility. However, various other factors, such as diet, lipid abnormalities, and BP, are controllable through changes in patient lifestyle. One particularly important factor is the gut microbiota, which has recently gained significant attention due to our growing understanding of its role in human health and diseases and its relationship to blood pressure. There have been numerous preclinical and translational studies (dietary intervention, probiotics, and fecal transplantation) that demonstrated a strong relationship between gut microbiota dysbiosis and abnormal BP [32–36].

The five primary bacterial phyla in the gut are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia, with Firmicutes and Bacteroidetes composing 90% of gut microbiota [37]. To our knowledge, this is the first study to examine the relationship between the gut microbiome and BP in children with T1DM. We found children with T1DM and elevated BP (T1DM-HBP) to have a distinct microbiota signature compared to normotensive children with T1DM (T1DM-Normo). These observed differences in microbial enrichment were at the phylum, class, order, family and genus levels. During the disease progression T1DM causes gut dysbiosis which would reflect in the compositional change of the dominant phyla, especially Bacteroidetes and Firmicutes. The ratio Firmicutes/Bacteroidetes (F/B) has

been extensively studied by various studies in correlation with T1DM. For example, Murri et al. and Leiva-Gea et al. reported that F/B ratio negatively correlates with the plasma glucose level in the pediatric T1DM group [38,39]. Also, Giongo et al. suggested that F/B ratio could be an early key indicator of autoimmune disorders, such as T1DM [40]. When we examined the F/B ratio in our study population, we found that it was significantly increased in T1DM-Normo compared to HC and decreased in T1DM-HBP ([Fig. 1c). The usefulness of the F/B ratio has recently come under scrutiny, as several studies and analyses have shown no clear trend in its relationship to different disease processes [41–44]. Our results suggest a promising link between these two major phyla and BP control, but more research will be needed to reproduce and interpret our findings on the F/B ratio.

High diversity of the gut microbiota usually indicates a healthy microbiome, which can be estimated by measuring the alpha diversity (Observed and Chao1 indexes for measuring species richness and Shannon, and Simpson indexes for species abundance) and beta diversity (Bray-Curtis dissimilarity index) [45]. In our study the reduced alpha diversity negatively correlating with elevated blood pressure (Fig. 2a) in line with previous report [46].

A fine balance in the gut microbiota is crucial, and any disturbance in its balance may impact the health status due to the complexity and interconnectivity with pathogenic bacteria. The identification of biomarkers by LEfSe analysis showed a significantly lower abundance of the genus *Bifidobacterium* in T1DM-HBP compared to T1DM-Normo (Fig. 3). The genus *Bifidobacterium* is an important probiotic that produces acetic
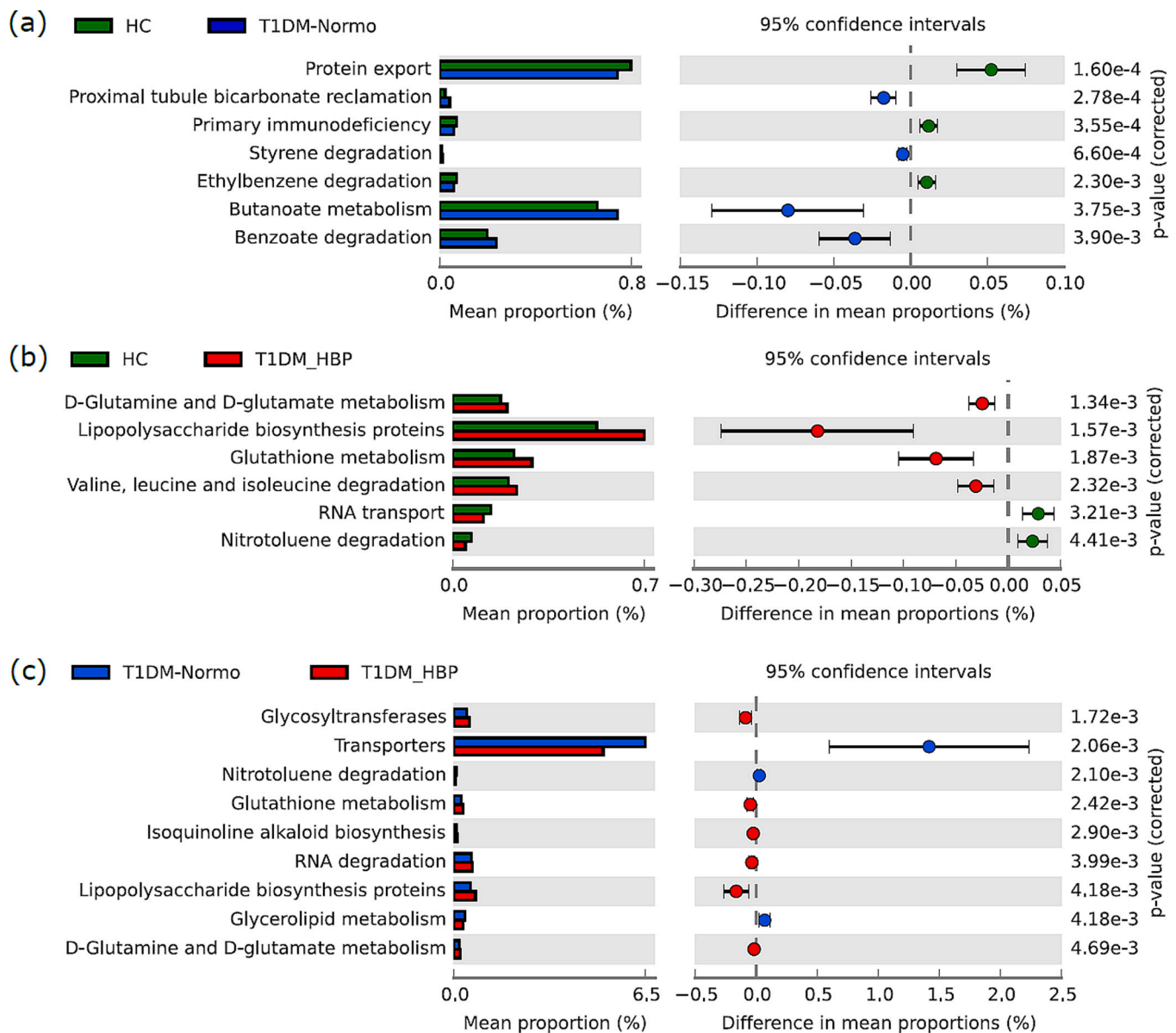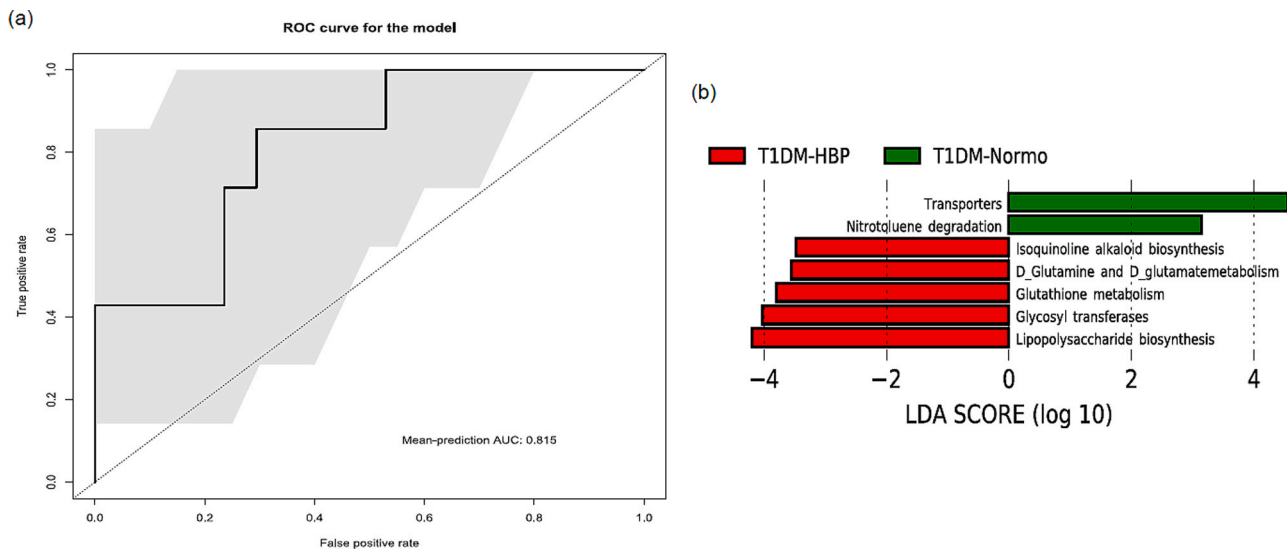
Fig. 4. Predicted functional pathways in the HC, T1DM-Normo, and T1DM-HBP groups. (a-c) Predicted functional pathways were analyzed by the PICRUSt method in all three groups. HC, N = 5; T1DM-Normo, N = 17; and T1DM-HBP, N = 7.

acid and lactic acid, along with butyric acid when cross-feeding [47,48]. It is a commensal bacterium and plays a crucial role in the homeostasis of the immune system [49]. It has been reported to be inversely correlated with systolic and diastolic BP [50] and with chronic diseases, such as obesity and asthma [51]. The genus *Bifidobacterium* contains many species - among these, we found that *B. adolescentis, B. bifidum,* and *B. longum* were significantly decreased in T1DM-HBP compared to T1DM-Normo (Fig. 1b). *B. longum* supplementation increases the level of critical components of the renin-angiotensin system (RAS), such as angiotensin-converting enzyme 2 (ACE2) and mas receptor (MAS-R) in obese mice [52], supporting its potential beneficial effects in reducing blood pressure. Additionally, the genus *Bifidobacterium* has been reported to protect the host intestinal epithelium through various mechanisms, mainly (1) adhesion followed by colonization, (2) lowering of pH that leads to the release of organic acids, (3) immunomodulatory effects, (4) release of antimicrobials called bifidocins, and (5) competitive exclusion of pathogens [53]. Adhesion in particular is a critical process that is necessary for the persistent effects of *Bifidobacterium*. Prebiotics and exopolysaccharides (EPS) are known to promote the

adhesion of *Bifidobacterium* to the intestinal mucosal surface. EPS is released by *Bifidobacterium* strains, particularly *B. breve* and *B. bifidum*, with the former found to bind strongly to an intestinal cell line due to higher EPS production [54].

Bifidobacterium strains, particularly *B. bifidum*, have also been reported to reduce the LDL/HDL ratio via cholesterol assimilation [55,56]. In our study, we found that T1DM-HBP had a significantly higher intake of cholesterol than T1DM-Normo (Table 3). We did not find a significant difference in the LDL level among the three groups (Table 1). However, we did observe a higher, but not significative level of HDL, along with a reduction in the level of *Bifidobacterium* strains (*B. adolescentis, B. bifidum, B. longum*), in T1DM-HBP compared to HC (Fig. 1b). As previously reported, the cholesterol-lowering ability of *Bifidobacterium* can be attributed to the release of SCFA – acetate [57]. SCFAs are the main source of energy for epithelial cells and exert metabolic effects that improve barrier function, reduce mucosal inflammation, and lower blood glucose levels [58]. They have also been reported to promote the development of Th17 cells and cause the release of the pro-inflammatory cytokine IL-17, which has been associated with the pathogenesis of

Fig. 5. Validation of predicted metabolic pathways using SIAMCAT and LEfSe analyses in the T1DM-HBP group. (a) The predicted functional pathways from the PICRUSt analysis data were validated using the SIAMCAT machine learning toolbox displaying the cross-validation error as ROC curve, with a 95% confidence interval shaded in grey. The AUROC = 0.815 is given below the curve and the x-axis and y-axis represent false positive rate and true positive rate, respectively for the pathways that we tested. (b) The LEfSe analysis was used to discriminate the predicted pathways from the PICRUSt analysis with a cut-off value of LDA > 2.0.

hypertension and vascular dysfunction [59,60]. Furthermore, SCFAs can regulate BP through G-protein coupled receptors (GPCRs), mainly the GPR41, GPR43, and GPR109a receptors. It was recently postulated that acetate can also act through the olfactory receptor 78 (in mice) and OR51E2 (in humans) to regulate renin secretion and BP [61]. Ganesh et al. have demonstrated that cecal acetate concentration was decreased by 48% in obstructive sleep-induced hypertension (OSA). Supplementation with probiotics, such as Hylon VII and *Clostridium butyricum*, and acetate has successfully prevented hypertension by restoring proper acetate concentration [62]. While some investigators have found nitric oxide and SCFA to be differentially expressed in hypertensive subjects compared to normotensive subjects [63], in our study, those pathways were not significantly different between T1DM-HBP and T1DM-Normo. To ensure that we did not miss differences in gut microbial SCFA metabolite levels, we examined the concentrations of nine gut SCFAs in all three groups of subjects. We expected higher SCFA levels (particularly butyrate and propionate) in normotensive subjects compared to subjects with HBP, since the phylum Firmicutes – the main producer of butyrate in the gut - was significantly more abundant in T1DM-normo compared to T1DM-HBP (Fig. 1). However, while there was a trend toward higher SCFA concentrations in the normotensive group, these differences were not statistically significant (Supplementary Figure 4S). It is possible that variance in diet (the source of SCFA precursors) between the groups, as well as the small sample size, contributed to these differences not reaching statistical significance.

One of the potential mechanisms by which the gut microbiota may contribute to the pathogenesis of high BP is the activation of the inflammatory response (IR) [64] through gut microbial dysbiosis, which eventually can lead to endothelial dysfunction caused by nitric oxide (NO), as shown in diabetes-associated hypertension [21,64,65]. NO is an unstable free radical that plays a significant role in the regulation of blood flow, smooth muscle relaxation, immunological responses, cell differentiation, and cell death [66]. Under normal conditions, it reduces blood pressure through its vasodilating activity, and the gut microbiota has been reported to regulate its production [67]. During the activation of IR, NO is produced by the inducible NO synthase enzyme (iNOS) and released in abnormally high amounts from immune cells. This NO couples with superoxide radicals (O_2^-) to produce the peroxynitrite ion ($ONOO^-$), causing toxic effects on blood vessels, increased oxidative stress, protein nitration, and increased cellular proliferation. These

effects all cause damage to the endothelium, leading to increased release of vasoconstrictors and eventually causing hypertension [68].

In this study, we found that many predicted functional pathways are perturbed in diabetes-associated hypertension. LPS biosynthesis and GSH metabolism pathways, in particular, were increased in T1DM-HBP compared to T1DM-Normo, while glycerolipid metabolism was reduced (Fig. 4c). LPS is an endotoxin and bacterial cell wall component. It has been extensively studied in the context of IR and has been implicated in many IR-mediated pathological conditions, including hypertension. LPS can increase blood pressure by causing endothelial dysfunction, which leads to vasoconstriction and activating toll-like receptor 4 (TLR4) [69]. This activation causes vasculature inflammation via increased production of NADPH oxidase-dependent free radicals [70]. Interestingly, *Bifidobacterium* has been reported to inhibit LPS-mediated IR in intestinal epithelial cells [71].

Intriguingly, while *Lachnobacterium* – a butyrate-producing bacteria from the Lachnospiraceae family - was significantly enriched in T1DM-HBP compared to T1DM-Normo (Fig. 3), we observed no corresponding change in butyrate levels between these two groups (Supplementary Figure 4S). This suggests that *Lachnobacterium* might exert effects via mechanisms other than butyrate production. A higher abundance of *Lachnobacterium* has been reported in some conditions where the inflammatory pathway takes a central role, such as ulcerative colitis [72] and saturated fat diet consumption during pregnancy [73]. Conversely, other studies on *Lachnobacterium* have described beneficial effects in some diseases, including cancer [74], prediabetes [75], and Crohn's disease [76]. The abundance of *Lachnobacterium* in the gut microbiota varies depending on diet – for instance, diets rich in animal nutrients are correlated with elevated abundance of *Lachnobacterium*, while vegetarian diets are associated with decreased abundance [77]. Interestingly, in our study, we observed no significant difference in the consumption of saturated fat between T1DM-Normo and T1DM-HBP. However, we found that HC did consume a significantly higher amount of saturated fat (Table 3). This suggests that the higher abundance of *Lachnobacterium* found in T1DM-HBP might be due to activation of the inflammatory-mediated response rather than the involvement of SCFAs.

In line with prior studies, we found the T1DM-HBP gut microbiome to be associated with elevated GSH oxidation compared to T1DM-Normo (Fig. 4c). GSH is a major intracellular component of the thiol-disulfide redox system. It functions as a powerful antioxidant against enzymatic

and non-enzymatic oxidative mechanisms via its easily oxidizable sulf-hydryl group. GSH is a dynamic molecule that is oxidized to its disulfide form (GSSH) to protect against oxidant injury, then reduced back to its original form by NADPH and FAD-dependent glutathione reductase enzyme [78]. An optimal concentration of reduced GSH is required for cellular health, and it generally acts through two major enzymes, glutathione transferases [79] and glutathione peroxidases [80], against an oxidant injury. As others have shown, vascular injury due to oxidative stress is the primary cause of hypertension, and prolonged oxidative stress can deplete GSH [81]. Depletion of GSH has been observed in hypertensive subjects compared to normotensive subjects [82]. Anti-oxidant therapy reversed this depletion of GSH and correspondingly reduced arterial hypertension [83]. This phenomenon might link the increased GSH oxidation and elevated BP we observed in T1DM-HBP. The reduced glycerolipid metabolism we found in T1DM-HBP (Fig. 4c) could also cause vascular injury and hypertension through the accumulation of glycerolipids (palmitic, oleic, and linoleic acids are abundant free fatty acids), leading to activation of IR and oxidative stress [84–86]. This pilot study is limited by our small sample size, cross-sectional design, and low power. To our knowledge, however, our findings are the first in this new and rapidly growing field to be reported in the patient population, and we anticipate that they will stimulate more interest and subsequent studies into the link between the gut microbiome and hypertension.

The results that we obtained from the pediatric population are comparable with studies that involved adults. Palmu et al. postulated that the *Lactobacillus spp.* has an inverse association with the BP in Finns population aged between 25 and 74 years [87]. Also, the CARDIA study conducted in the adult population showed that HTN and systolic BP were inversely associated with the measures of alpha diversity along with the several other genera, notably *Anaerovorax* and *Sporobacter*, whereas *Robinsoniella* and *Catabacter* were positively associated with it [88]. Interestingly, a systematic review paper of randomized controlled trials evaluated the potential benefits of probiotics, including *Lactobacillus* and *Bifidobacterium* species, in improving the BP [34].

In conclusion, we found distinct microbial signatures in T1DM-HBP compared to the normotensive group. These differences were found at multiple taxonomic levels and reflected different potential metabolic pathways that may contribute to BP regulation. This study opens the door for further mechanistic studies and calls for future research to confirm our findings and explore possible interventions by manipulating the gut microbiota. Of particular interest was our finding of reduced *Bifidobacterium* genera in T1DM-HBP and its potential mechanistic and therapeutic roles in the management of T1DM-associated hypertension.

Conflict of interest statement

The author(s) report no conflict of interest.

Data availability statement

The data presented in this study can be found here in the NCBI's Bio project repository: [PRJNA693107].

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Authors' contributions

AT and IFS designed the study. SZ, FAK, and GP were involved in the subject's recruitment process. SZ, APL, SAA, DKB were involved in the sample collection process. APL, DKB, and SAA processed samples. APL performed gut microbiome data analysis. SJ performed SCFA analysis. APL and IFS wrote the manuscript. AT and IFS reviewed the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111736.

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