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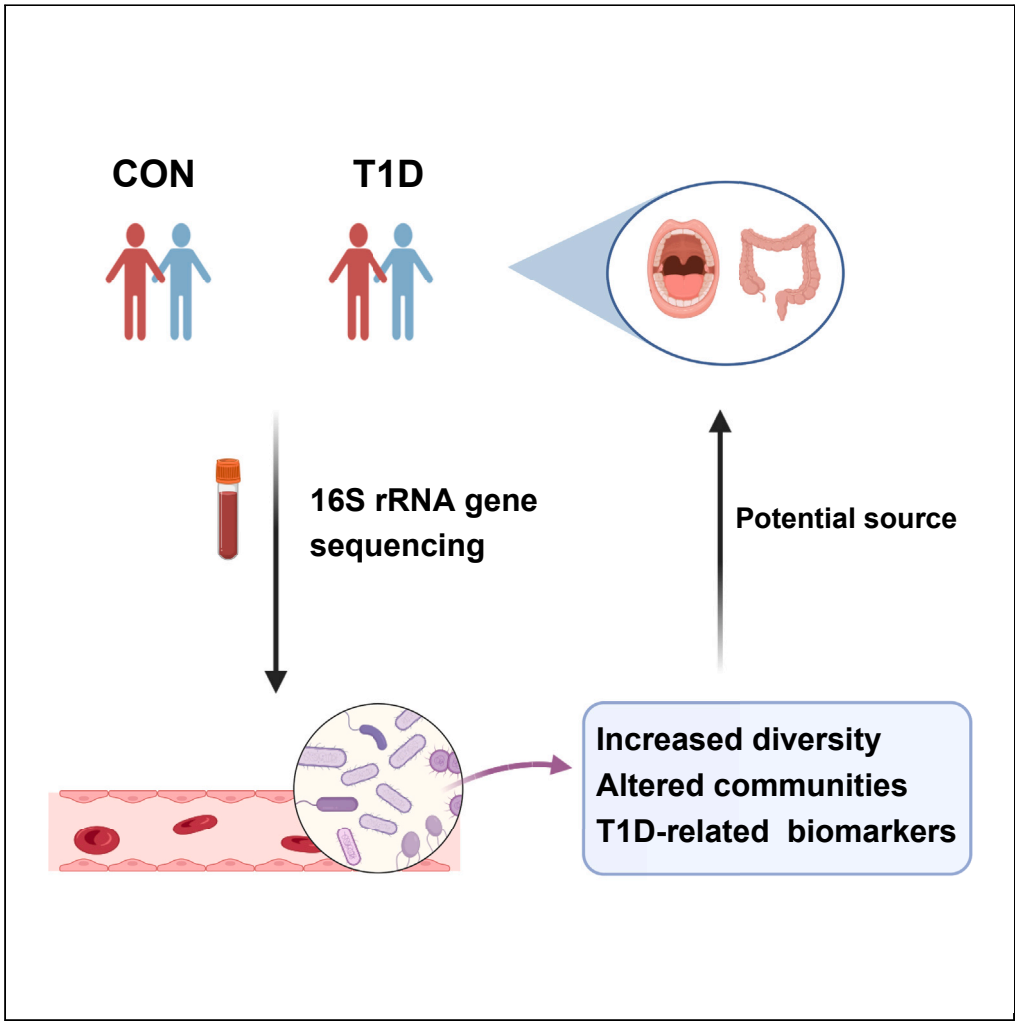

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Highlights

We delineated the blood microbiome signatures in T1D

We assess correlations between blood and gut/oral microbiome signatures in T1D

Identified key blood microbiota exhibited strong discriminatory power for T1D

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The profile of blood microbiome in new-onset type 1 diabetes children

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SUMMARY

Blood microbiome signatures in patients with type 1 diabetes (T1D) remain unclear. We profile blood microbiome using 16S rRNA gene sequencing in 77 controls and 64 children with new-onset T1D, and compared it with the gut and oral microbiomes. The blood microbiome of patients with T1D is characterized by increased diversity and perturbed microbial features, with a significant increase in potentially pathogenic bacteria compared with controls. Thirty-six representative genera of blood microbiome were identified by random forest analysis, providing strong discriminatory power for T1D with an AUC of 0.82. PICRUSt analysis suggested that bacteria capable of inducing inflammation were more likely to enter the bloodstream in T1D. The overlap of the gut and oral microbiome with the blood microbiome implied potential translocation of bacteria from the gut and oral cavity to the bloodstream. Our study raised the necessity of further mechanistic investigations into the roles of blood microbiome in T1D.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by absolute insulin deficiency, with a complex pathogenesis involving multiple genetic and environmental factors, including dietary components, viral infections, and changes in the microbiota.[1,2] Clinically, T1D patients have been reported with gut microbial composition disturbances[3–6] and "leaky gut".[7,8] Increased gut permeability can facilitate the translocation of bacterial products (e.g., gut-derived toxins and lipopolysaccharides) from the gut into the peripheral circulation,[9,10] resulting in an inordinate interaction between the host and microbiome and contributing to a low-grade chronic inflammatory state in metabolic diseases such as diabetes, obesity, and metabolic syndrome.[11]

Blood in healthy organisms is considered a sterile environment because of the lack of proliferating microbes.[12] However, new DNA-sequencing technologies have uncovered the existence of live microbes or bacterial DNA even at low levels in healthy individuals[13] as well as in patients with non-communicable diseases, such as type 2 diabetes (T2D),[14] liver fibrosis,[15–17] rheumatoid arthritis,[18] cancer,[19,20] cardiovascular diseases,[21] Parkinson's disease,[22] immune and inflammatory disorders.[23] A recent study did not support the existence of a consistent core blood microbiome based on a population study of 9,770 healthy humans; however, it supported the translocation of commensal microbes from other body sites into the bloodstream.[24] Blood microorganisms or DNA mostly translocate from other parts of the body (gut, oral cavity, skin

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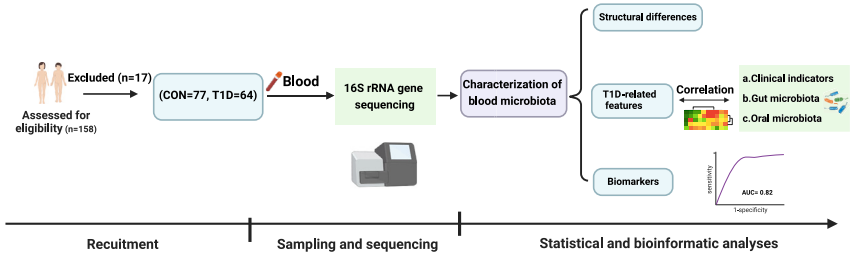

Figure 1. Flow chart illustrating the procedures involved in this study

et al.), and the composition and amount of the blood microbiome are thought to be related to intestinal permeability.[25,26] Previous studies have further suggested that the blood microbiome is involved in the pathogenesis of chronic metabolic diseases, such as T2D, cardiovascular diseases, and inflammatory diseases,[12,27] and antibiotics including antivirals, have been proposed as therapeutic agents.[28]

T1D is an autoimmune disease that is caused as a result of T-lymphocyte-mediated destruction of pancreatic β-cells along with the stimulation of self-antigens and exposure to foreign antigens, such as viruses and bacteria. Bacteria, whether live or dead, may be used as antigenic substances, which raises the question of the existence of the microbiome in the blood and its impact and role in T1D. However, to the best of our knowledge, no study has investigated the presence or composition of the circulating microbiome in T1D.[21] Extensive changes in the blood microbiome and metabolome in T2D indicate that the blood microbiome may play a role in the etiology and development of diabetes. Sato et al. reported gut microbiome dysbiosis and higher blood plasma counts of gram-positive bacteria (specifically, *Clostridium coccoides* and *Atopobium cluster*) in individuals with T2D than in controls.[29] Another study found that participants having members of the genus *Sediminibacterium* in their bloodstream showed a higher risk of developing T2D, whereas individuals having members of the genus *Bacteroides* in their bloodstream had a decreased risk of developing T2D.[14] In a longitudinal study involving 3280 individuals with a 9-year follow-up, a significant association was observed between higher baseline blood bacterial 16S rDNA levels and the onset of T2D,[30] providing evidence that tissue bacteria are involved in the onset of diabetes in humans.

Here, we aimed to delineate the community structure of the blood microbiome in children with new-onset T1D, laying the groundwork for future studies on the association between the blood microbiome and systemic inflammation. We further compared the blood microbiome with the gut and oral microbiomes in patients with T1D to explore the microbial connections.

RESULTS

Study population and clinical parameters

Children with new-onset T1D and non-diabetic controls (CON) were enrolled using a strict pathological diagnostic and exclusion process (Figure 1). Blood samples were collected, and the microbiome was analyzed using 16S rRNA gene sequencing. We recruited 83 healthy subjects and 75 patients with new-onset T1D aged 3–14 years. Finally, 64 newly diagnosed T1D children and 77 age- and sex-matched healthy children were enrolled in this study. As we previously reported[3] (Table S1), the T1D group showed disorders of glucose and lipid metabolism, mainly manifested as low C peptide, high fasting blood glucose (FBG), glycated hemoglobin (HbA1c), and triglycerides (TG). The levels of inflammatory indicators, including white blood cells (WBC) and neutrophils (NEUT), significantly increased in the T1D group.

Significant variation of the blood microbiome profiles in T1D

The taxonomic diversity and profiles of the microbiome DNA were analyzed using high-throughput 16S rRNA gene sequencing. After deleting the unqualified sequences, 9,148,108 valid and trimmed sequences were obtained from all 141 samples, with an average length of 417 bp per sequence. After taxonomic assignment, 1,476 operational taxonomic units (OTUs) were identified. Rarefaction curves were constructed to ensure that sufficient samples were chosen for the alpha diversity analysis (Figure S1). Consistently, we observed an overall increase in microbial diversity in the T1D group (Table S2). The Chao, Shannon, and Invsimpson indices, which measure richness and evenness, were significantly higher in the T1D group than in the CON group (Figures 2A–2C). To evaluate the extent of similarity between the blood microbiome communities, beta diversity analysis based on the Bray Curtis distances showed that the microbiome of the T1D group was distinct from that of the CON group (Figure 2D and 2E). We also observed higher sample-to-sample dissimilarities in the T1D group, indicating a more heterogeneous community among T1D individuals than controls (Figure 2F).

Considering that sex and age may be important confounders of the microbiota, we further investigated blood microbiome community differences by stratifying the CON and T1D groups by sex and age (Figure S2). When the samples were grouped by sex, higher α diversity was observed for the bacterial microbiome in males than in females in the CON group. In the age-stratified analysis, microbial α diversity decreased significantly with increasing age in the T1D group. No significant difference was found in the CON group.

Taxonomic profiles of the blood microbial communities in T1D

To further identify the specific bacterial communities in T1D, we assessed the relative taxonomic abundance of the blood microbiome in the CON and T1D groups (Table S3). Sequences of the overall blood microbiome of T1D belonged mainly to Proteobacteria (82.92%) phyla, to a

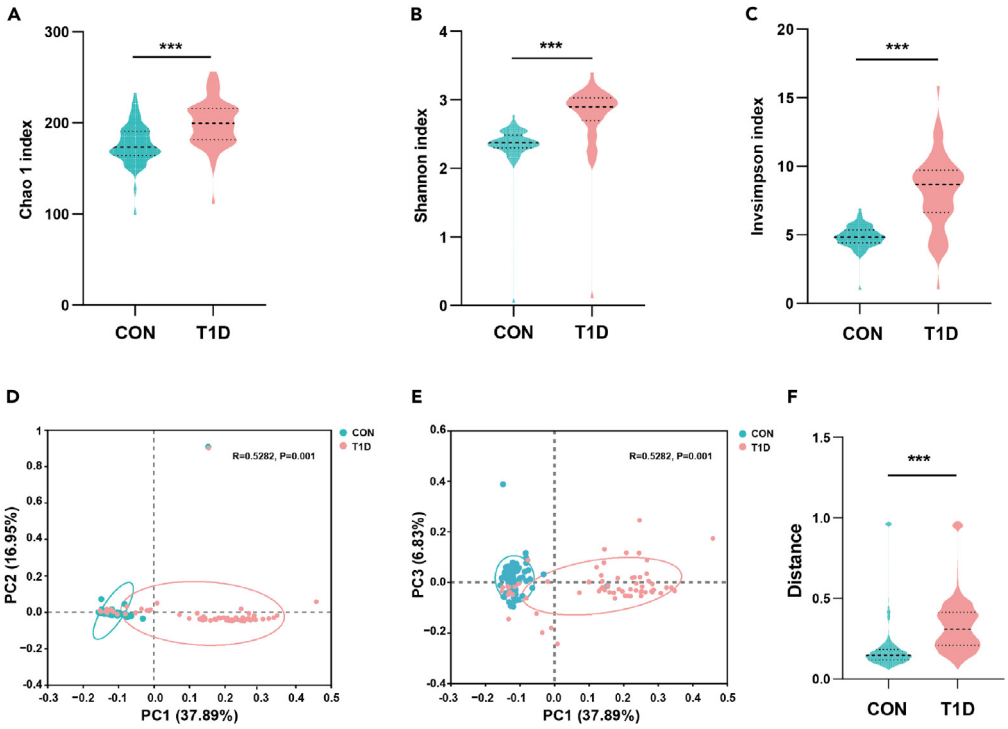

Figure 2. Blood microbiome communities in T1D

(A–C) Microbial community richness (Chao 1, A) and diversity (Shannon, B; Invsimpson, C).

(D and E) Two-dimensional (D, PC1 versus PC2 axes; E, PC1 versus PC3 axes) principal coordinates analysis was performed using ANOSIM with 999 permutations.

(F) Inner-group distance based on analysis of similarities. Violin plots show the median, quartiles, and min/max values. Two-sided Wilcoxon rank-sum test. ***$p < 0.001$.

lesser extent to Firmicutes (4.88%), Bacteroidota (4.31%), unclassified_d__Bacteria (3.43%), and Actinobacteriota (2.75%) phyla. The relative abundance of most phyla, including Bacteroidota, Firmicutes, and Actinobacteriota, significantly increased in the T1D group (Figure 3A; Figure S3). We also compared differences in taxa at the genus level (Table S4; Figure S4). The blood microbiome of the T1D group was significantly enriched in the *Cupriavidus*, *Sphingomonas*, *Brevundimonas*, *Caulobacter*, *Sphingobium*, *Flavobacterium*, *Microbacterium*, etc. In addition, other abundant genera, including *Pelomonas*, *Ralstonia*, *Acinetobacter*, *unclassified_d_Bacteria*, and *Brucella*, etc., were depleted in the T1D group compared to those in the CON group (Figures 3B and 3C; Figure S5). Differential genera were used to construct an interaction network depicting the correlations between CON and T1D-associated blood microbiota. Notably, T1D-enriched genera such as *Stenotrophomonas*, *Sphingomonas*, *Cupriavidus*, *Caulobacter*, and *unclassified_c_Bacteroidia* were positively correlated and more highly interconnected than the T1D-depleted genera (Figure 3D). *Flavobacterium*, *unclassified_c_Bacteroidia*, *unclassified_o_Micavibrionales*, *OM27_clade*, and *Flectobacillus*, which were enriched in the T1D group, negatively correlated with *Ralstonia*, *Pelomonas*, *Lactococcus*, and *Afipia* which were depleted in the T1D group.

Comparative analysis in bacterial profiles between blood and gut/oral microbiota

To further assess the taxonomic association between the blood and oral/gut microbiota, we performed a comparative analysis of the bacterial profiles at the phylum and genus levels, respectively (Figure 4). We found that blood and oral/gut microbiome communities differed significantly from each other (Figures 4A–4C), indicating that the barrier system of the body, such as the intestinal, mechanical, and immune barriers, might affect bacterial translocation. The number and identity of shared genera were evaluated using Venn diagrams. In the T1D group, the proportion of overlapping genera number in blood was 18.01% compared with oral microbiota (Figure 4D), and 21.80% compared with gut microbiome (Figure 4E). The main blood genera that overlapped with the oral microbiome with significant changes between two groups were *Ralstonia*, *Acinetobacter*, *Sphingomonas*, *Brevundimonas*, *Sphingobium*, *Rhodococcus*, *Stenotrophomonas*, and *Enterobacter*, etc. The main overlapping blood genera with gut microbiome were *unclassified_d__Bacteria*, *Escherichia-Shigella*, *Enterobacter*, *Bacteroides*, *Enterococcus*, *Comamonas*, *Staphylococcus*, *Lactobacillus*, *Streptococcus*, and *Faecalibacterium*, etc (Figures 4F and 4G). We further explored the overlap of genera among blood, oral, and gut microbiota. Thirty-one genera were shared among the three groups (Figure S6). The bar plot shows the relative abundance of the differentially overlapping genera among the blood, oral, and gut microbiota, ranked by *Comamonas*, *Enterobacter*, *Bacteroides*, *Staphylococcus*, *Lactobacillus*, *Corynebacterium*, *Romboutsia*, and *Porphyromonas*, etc.

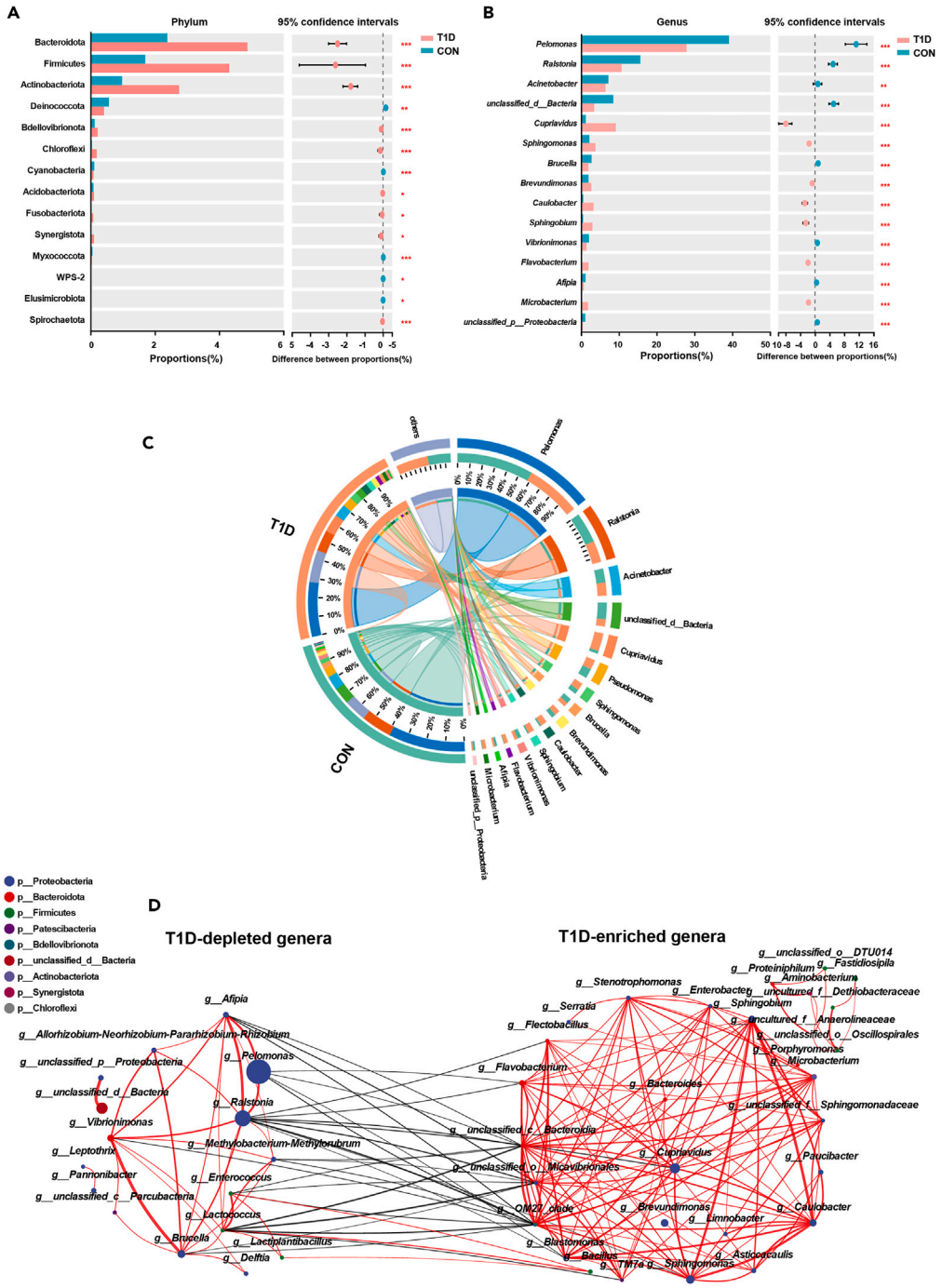

Figure 3. The structural shift of the blood microbiome in T1D

(A and B) Relative abundance of differential microbial taxa at the phylum and genus levels.

(C) Distribution of the microbial community between two groups visualized using the Circos plot at the genus level. The inner circle on the left represents the microbial structural composition at the genus level of each group. The outer circle on the left represents different groups. The outer circle on the right represents the percentage of genera in different groups. The width of the bands represents the proportion or relative abundance of the genus.

(D) Co-abundance network constructed from the differential bacteria identified using the Wilcoxon rank-sum test. The node size indicates the relative abundance of each bacteria genera and color indicates the phylum. The thickness of the line between nodes represents the Spearman coefficient. A total of 46 genera are displayed, with Spearman's correlation values >0.6 between each other. T1D-depleted genera are arranged on the left, while T1D-enriched genera are arranged on the right. Two-sided Wilcoxon rank-sum test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

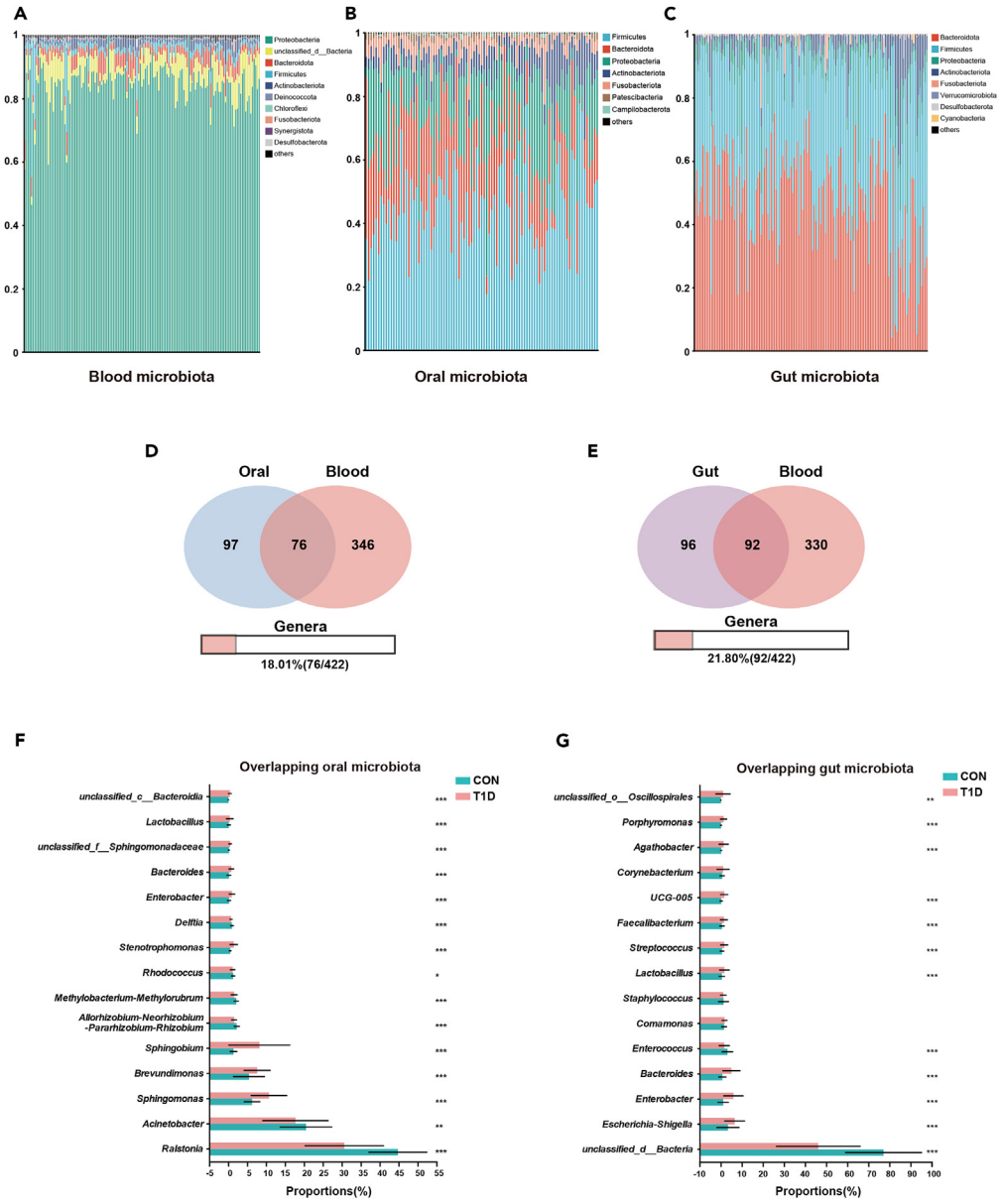

Figure 4. The comparison between the blood microbiome and the gut/oral microbiome

(A) Blood bacterial composition at the phylum level.

(B) Oral bacterial composition at the phylum level.

(C) Gut bacterial composition at the phylum level.

(D) Venn showing the overlap of genera between blood and oral microbiota.

(E) Venn showing the overlap of genera between blood and gut microbiota.

(F and G) The relative abundance of the top 15 overlapping genera between blood and oral/gut microbiota. Two-sided Wilcoxon rank-sum test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(Figure S6C), which might play an important role in the interactions between these key bacterial species and the mechanisms underlying their association with T1D.

Blood microbiota-based biomarkers for T1D

To further reveal the signature blood microbiome profiles and predominant microbiota of patients with T1D, we performed a linear discriminant effect size (LEfSe) analysis (Figure 5A). The cladogram represents the significantly different taxa among the CON and T1D groups according to a hierarchy that reflects the taxonomic rank from phylum to genus (Figure S7). A total of 75 taxa with differential abundance

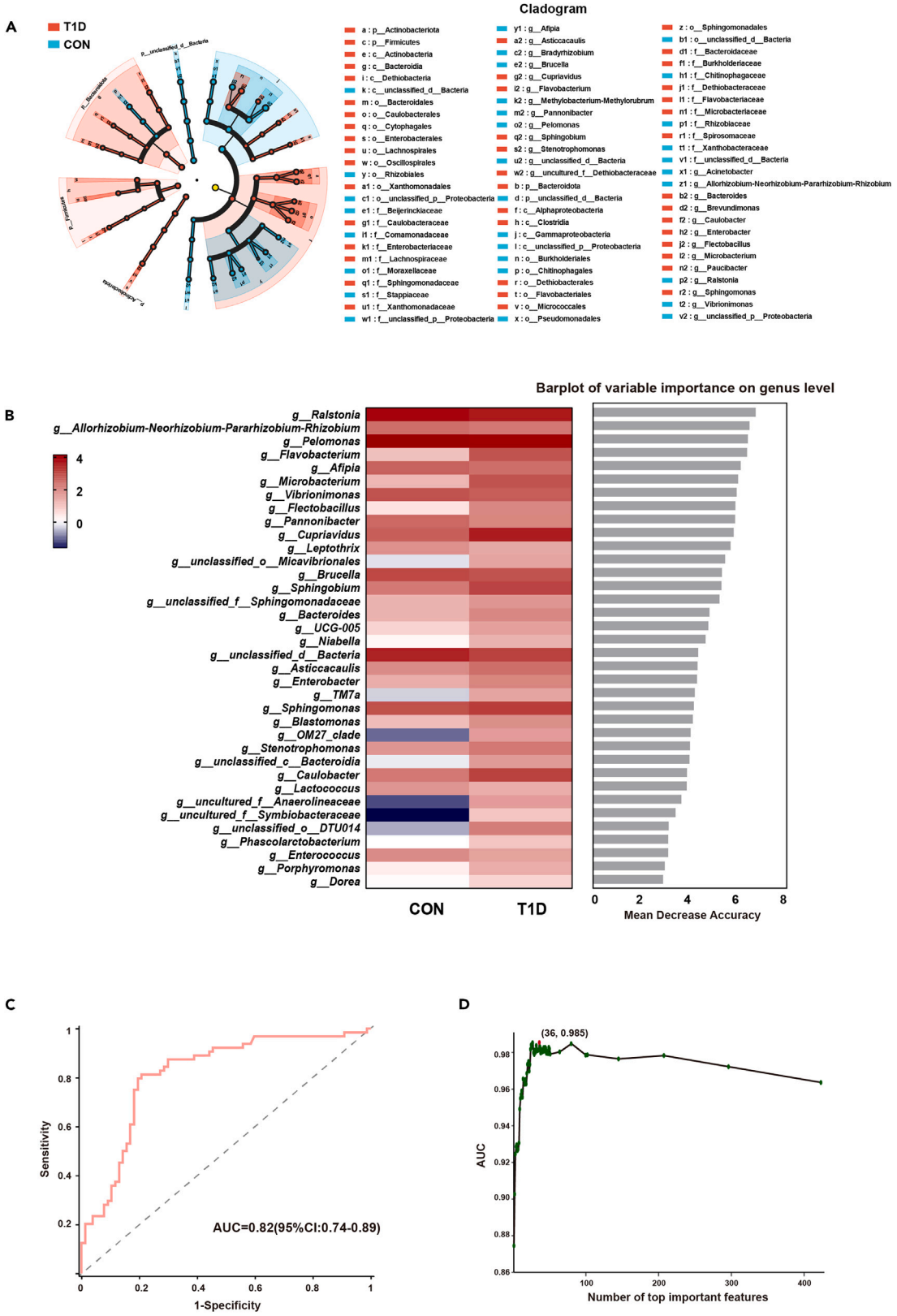
A
T1D
CON
Cladogram
a : p__Actinobacteriota
c : p__Firmicutes
e : c__Actinobacteria
g : c__Bacteroidia
i : c__Dethiobacteria
k : c__unclassified_d__Bacteria
m : o__Bacteroidales
o : o__Caulobacterales
q : o__Cytophagales
s : o__Enterobacterales
u : o__Lachnospirales
w : o__Oscillospirales
y : o__Rhizobiales
a1 : o__Xanthomonadales
c1 : o__unclassified_p__Proteobacteria
e1 : f__Beijerinckiaceae
g1 : f__Caulobacteraceae
i1 : f__Comamonadaceae
k1 : f__Enterobacteriaceae
m1 : f__Lachnospiraceae
o1 : f__Moraxellaceae
q1 : f__Sphingomonadaceae
s1 : f__Stappiaceae
u1 : f__Xanthomonadaceae
w1 : f__unclassified_p__Proteobacteria
y1 : g__Afipia
a2 : g__Asticcacaulis
c2 : g__Bradyrhizobium
e2 : g__Brucella
g2 : g__Cupriavidus
i2 : g__Flavobacterium
k2 : g__Methylobacterium-Methylorubrum
m2 : g__Pannonibacter
o2 : g__Pelomonas
q2 : g__Sphingobium
s2 : g__Stenotrophomonas
u2 : g__unclassified_d__Bacteria
w2 : g__uncultured_f__Dethiobacteraceae
b : p__Bacteroidota
d : p__unclassified_d__Bacteria
f : c__Alphaproteobacteria
h : c__Clostridia
j : c__Gammaproteobacteria
l : c__unclassified_p__Proteobacteria
n : o__Burkholderiales
p : o__Chitinophagales
r : o__Dethiobacterales
t : o__Flavobacteriales
v : o__Micrococcales
x : o__Pseudomonadales
z : o__Sphingomonadales
b1 : o__unclassified_d__Bacteria
d1 : f__Bacteroidaceae
f1 : f__Burkholderiaceae
h1 : f__Chitinophagaceae
j1 : f__Dethiobacteraceae
l1 : f__Flavobacteriaceae
n1 : f__Microbacteriaceae
p1 : f__Rhizobiaceae
r1 : f__Spirosomaceae
t1 : f__Xanthobacteraceae
v1 : f__unclassified_d__Bacteria
x1 : g__Acinetobacter
z1 : g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
b2 : g__Bacteroides
d2 : g__Brevundimonas
f2 : g__Caulobacter
h2 : g__Enterobacter
j2 : g__Flectobacillus
l2 : g__Microbacterium
n2 : g__Paucibacter
p2 : g__Ralstonia
r2 : g__Sphingomonas
t2 : g__Vibrionimonas
v2 : g__unclassified_p__Proteobacteria
B
Barplot of variable importance on genus level
g__Ralstonia
g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
g__Pelomonas
g__Flavobacterium
g__Afipia
g__Microbacterium
g__Vibrionimonas
g__Flectobacillus
g__Pannonibacter
g__Cupriavidus
g__Leptothrix
g__unclassified_o__Micavibrionales
g__Brucella
g__Sphingobium
g__unclassified_f__Sphingomonadaceae
g__Bacteroides
g__UCG-005
g__Niabella
g__unclassified_d__Bacteria
g__Asticcacaulis
g__Enterobacter
g__TM7a
g__Sphingomonas
g__Blastomonas
g__OM27_clade
g__Stenotrophomonas
g__unclassified_c__Bacteroidia
g__Caulobacter
g__Lactococcus
g__uncultured_f__Anaerolineaceae
g__uncultured_f__Symbiobacteraceae
g__unclassified_o__DTU014
g__Phascolarctobacterium
g__Enterococcus
g__Porphyromonas
g__Dorea
4
2
0
CON
T1D
0
2
4
6
8
Mean Decrease Accuracy
C
Sensitivity
1-Specificity
AUC=0.82(95%CI:0.74-0.89)
D
AUC
Number of top important features
(36, 0.985)

Figure 5. Blood microbiome-based biomarkers identified using LefSe and the random forest classification model

(A) LEfSe taxonomic cladogram generated from 16S rRNA gene sequences.

(B) Classification performance of the 36 most discriminant genera of the T1D group by a random forest model and heatmap based on the relative abundance of the genera.

(C and D) The area under the curve (AUC) based on the cross-validation of the random forest model.

between the CON and T1D groups were identified (LDA >3, $p < 0.05$). Higher proportions of *Cupriavidus*, *Caulobacter*, *Sphingobium*, *Flavobacterium*, and *Microbacterium* were observed in the T1D group, whereas *Pelomonas*, *Ralstonia*, *Brucella*, *Acinetobacter*, and *Vibrionimonas* were depleted in the T1D group (Table S5). The differential species identified by the two different analysis methods (Mann-Whitney U test and LEfSe) were consistent, revealing the stability of the blood microbiome profiling data.

Furthermore, to explore the predictive power of the blood microbiome in discriminating T1D status, we used Random Forests to build a predictive model based on genus-level relative abundance and conducted cross-validation tests (Figures 5B and 5C; Table S6). A total of 36 genera were considered predictive of T1D, ranked as *Ralstonia*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Pelomonas*, *Flavobacterium*, *Afipia*, *Microbacterium*, *Vibrionimonas*, *Flectobacillus*, *Pannonibacter*, and *Cupriavidus*, etc. To explore the potential value of the identified key microbiota for clinical discrimination, we constructed receiver operating characteristic (ROC) curves and computed the area under the curve (AUC) values. This model had a robust and statistically significant diagnostic accuracy, with an AUC of 0.82 (Figures 5C and 5D). A heatmap was generated based on T1D predictive biomarker abundance (Figure 5B). These results confirm that the blood microbiota-based classifier can excellently distinguish T1D patients from controls, indicating that the discovered key microbiota may provide the possibility and reliability for clinical transformation and potential value in risk assessment.

Associations between blood bacteria and clinical indicators

Our study demonstrated the compositional changes in the blood microbiomes of patients with T1D. To evaluate whether the T1D clinical parameters were related to the blood microbiota, we performed Spearman's rank correlation analysis (Figure 6A; Figure S8). Glycolipid metabolism indicators such as HbA1c, FBG, and TG were negatively correlated with T1D-depleted genera, including *Pelomonas*, *Ralstonia*, *Brucella*, *Afipia*, *Vibrionimonas*, and *Acinetobacter*, and positively correlated with T1D-enriched genera, including *Cupriavidus*, *Sphingomonas*, *Caulobacter*, *Brevundimonas*, *Sphingobium*, *Flavobacterium*, and *Microbacterium*, whereas HDL showed the opposite trend. WBC, NEUT, and LYMPH, the indices for systemic inflammation, were inversely related to *Ralstonia*, *Afipia*, *Pelomonas*, and *Acinetobacter* but positively related to *unclassified_d_Bacteria*. The redundancy analysis (RDA) plot further showed the correlations between clinic characteristics and the bacterial communities at the genus level (Figure 6B). The broad correlation between metabolic parameters and key bacteria indicates that the blood microbiome may be associated with glucose metabolism, lipid metabolism, and inflammatory response in T1D.

Predicted functions of the blood microbiome in T1D

To characterize the predicted functional profiles of the blood microbiota, a phylogenetic investigation of communities by the reconstruction of unobserved states (PICRUSt) method was used to predict the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways between the microbiomes of the CON and T1D groups (Figure 7). The principal coordinates analysis (PCoA) plot based on the Bray-Curtis distances of the KEGG orthologs (KOs) indicated significant differences between the CON and T1D groups at the functional level, with a higher compositional dissimilarity in the T1D group than in the control group (Figures 7C and 7D). At KEGG level 1 (Figure 7A), the metabolism pathway was the most abundant (74.48% in the T1D group), followed by environmental information processing, cellular processes, genetic information processing, human diseases, and organismal systems pathways (Table S7). At the KEGG level 2, we detected 33 upregulated and 13 downregulated pathways in the T1D group compared to those in the controls, which were mainly involved in carbohydrate, amino acid, nucleotide metabolism, and cellular processes (Figure 7B; Table S8). Interestingly, immune disease-related and infectious disease-related pathways were significantly upregulated in the T1D group compared to those in the CON group (Figure 7E). KEGG pathway analysis showed that metabolic pathways were the most enriched in the T1D group. We further focused on metabolic pathways and found that these functions, including biosynthesis of secondary metabolites, carbon metabolism, butanoate metabolism, valine, leucine and isoleucine degradation, propanoate metabolism, glycolysis/gluconeogenesis, fatty acid degradation, citrate cycle, were primarily upregulated in T1D than controls. However, glyoxylate and dicarboxylate metabolism, pyruvate metabolism, fatty acid metabolism, glycine, serine and threonine metabolism, amino sugar, and nucleotide sugar metabolism, and pyrimidine metabolism were significantly downregulated in T1D (Figure S9; Table S9).

DISCUSSION

In this cross-sectional study, we explored the blood microbial landscape of T1D and, more importantly, assessed the correlations shared microbial signatures between the blood and gut/oral microbiome profiles in T1D. Our study revealed blood microbial structures and signatures in T1D, which were characterized by increased bacterial richness and perturbed microbiome structures, with a significant increase in pathogenic bacteria such as *Sphingomonas*, *Caulobacter*, and *Stenotrophomonas*. Blood microbiome communities were partially similar to those of the corresponding gut and oral microbiota, suggesting that the gut/oral microbiome is a potential source. Functional prediction showed that inflammatory and immune disease-related pathways were upregulated in T1D and that the key blood microbiome was strongly associated with inflammatory and glycolipid metabolism indicators, indicating the involvement of the blood microbiome in inflammatory states in T1D. The identified key microbiota exhibited remarkable discriminatory power in differentiating patients with T1D from controls.

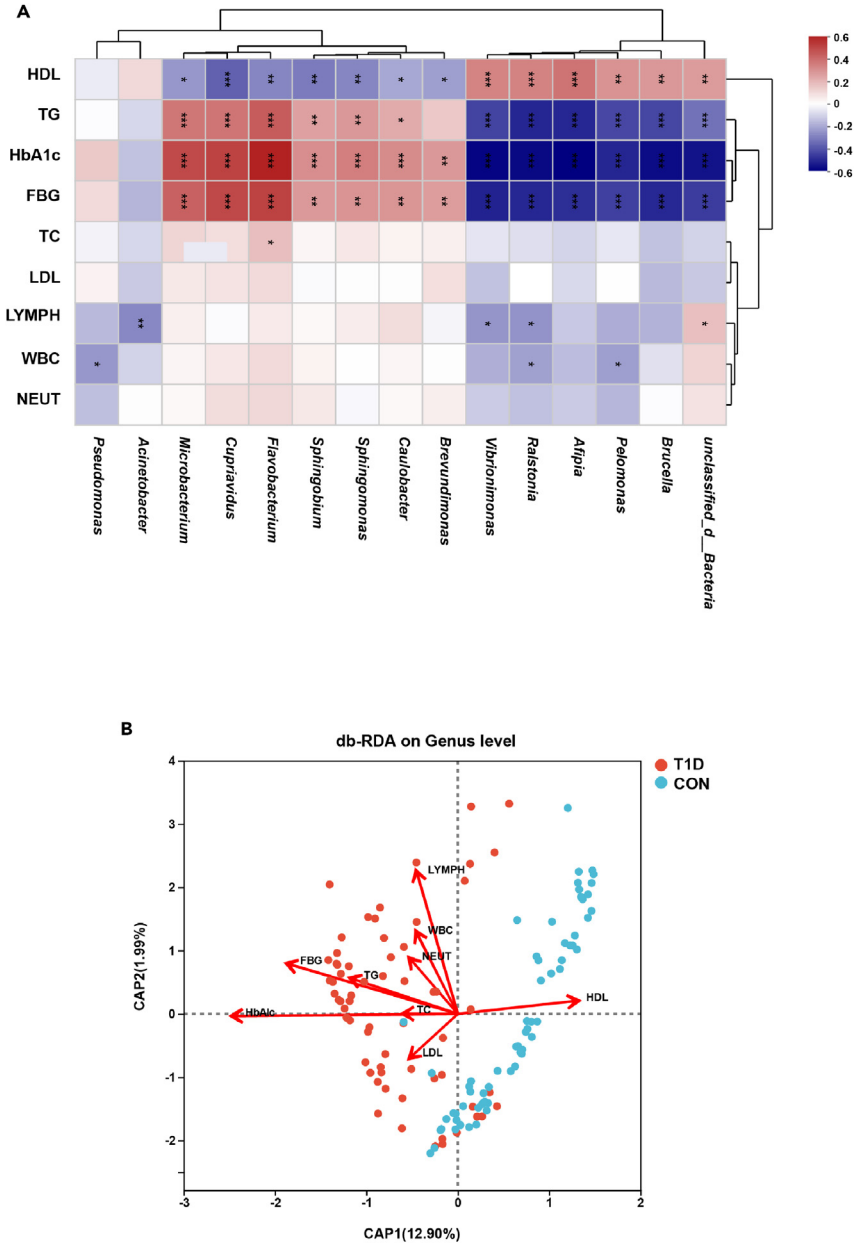

Figure 6. Association of the blood microbial genera with clinical indicators

(A) Heatmap of the Spearman's correlation between clinical indices and genera. Red squares indicate positive correlations, whereas blue squares indicate negative correlations.

(B) Redundancy analysis (RDA) of the relationship between the clinical indices and genus-level composition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The microbiome plays a fundamental role in the regulation of immune response and inflammation.[31] The blood microbiome in healthy individuals is considered dormant because it does not induce inflammation or sepsis; however, it may play a vital role in physiology and immunity.[12] In 2001, Nikkari et al. reported that blood specimens from healthy individuals contained bacterial DNA in healthy individuals.[32] An increasing number of studies have shown that blood contains an authentic microbiome and is one of the major microbial niches in humans.[30,33,34] We observed a remarkable change in the blood microbial community of patients with T1D. We found an overall increase in blood microbial α and β diversity in the T1D group than the CON group, indicating a more heterogeneous microbial community in T1D. Correlation network analysis also indicated that more positive correlations occurred within blood bacteria in the T1D group than in controls. The relative abundances of most differential phyla, including Firmicutes, Bacteroidota, and Acidobacteriota, were significantly increased in the T1D group. The increased abundance of Actinobacteria in diabetic nephropathy is considered an independent risk factor for cardiovascular

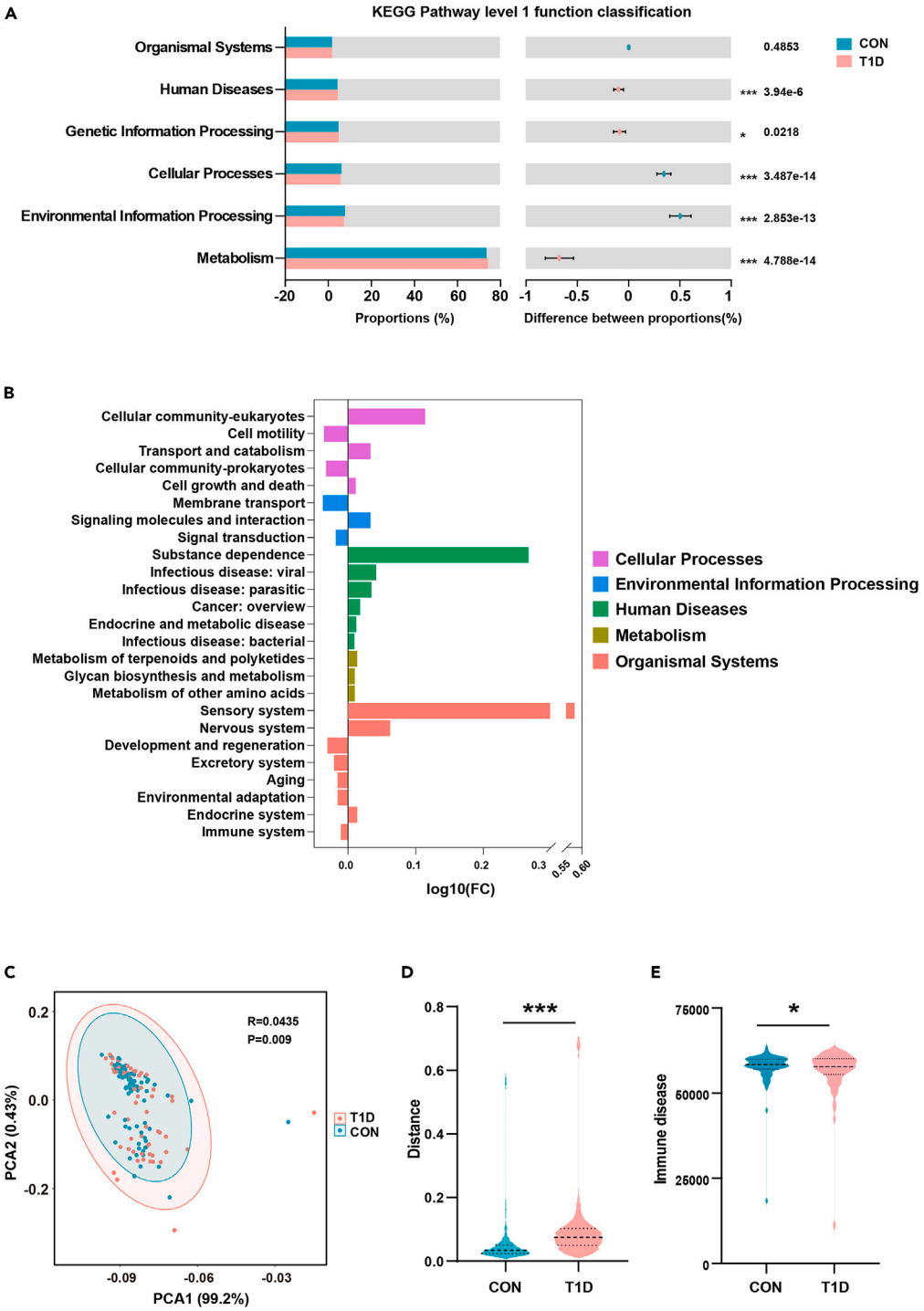

Figure 7. Alteration of predicted blood microbial functions in children with T1D

(A and B) KEGG pathway level 1 (A) and level 2 (B) function classification predicted by PICRUSt between the CON and T1D groups. Foldchange of the top 25 pathways with significant differences in KEGG Level 2 are shown.

(C and D) PCoA plot based on the Bray-Curtis distances of KOs (C, left) and inner-group distance by ANOSIM (D, right).

(E) The abundance of genes involved in the immune disease based on the KEGG database. Violin plots show the median, quartiles, and min/max values. Two-sided Wilcoxon rank-sum test. *$p < 0.05$, ***$p < 0.001$.

mortality.[35] Remarkable differences in blood microbial composition are also found in acute and chronic coronary syndrome, whereby the former has more phyla Proteobacteria and Acidobacteriota, while the latter has more phyla Firmicutes and the genus *Lactobacillus*.[36] Proteobacteria was the most abundant phylum in the blood, and no difference was found between the two groups. This result is similar to the findings reported in T2D studies.[21] Some potentially pathogenic bacteria were also enriched in T1D, such as *Sphingomonas*, *Caulobacter*, and *Stenotrophomonas*. Correlation analysis revealed that the differential blood microbiome was strongly associated with glycolipid metabolism and inflammatory indicators. Previous studies have revealed that decreased oxygenation, reduced immunity, and hyperglycemia in patients with diabetes could increase susceptibility to blood infection induced by pathogenic microbiomes such as *Pneumonia*, *Staphylococcus*, and *Mycobacterium*.[37,38] Our KEGG annotation revealed significant differences in the predicted biological functions of the blood microbiome in T1D, characterized by increased inflammatory, immune-related, and metabolic pathways. Similar functional differences have been observed in some metabolic diseases, such as myocardial infarction and atherosclerosis, where posttranslational modifications, protein turnover, amino acid transport, and metabolic pathways were significantly increased in the blood microbiota of myocardial infarction.[39,40] However, the blood microbiome, such as *Pelomonas*, is an understudied genus[41] with few reports concerning its function and metabolism. Therefore, further studies on the interactions between key blood bacterial species and the mechanisms underlying their association with T1D could help us understand the roles of the blood microbiome in T1D.

Increasing attention has been paid to the association between the gut microbiome and diseases; however, the role of the blood microbiome and microbial connections in disease pathogenesis remains obscure. We assessed the taxonomic association between the blood and gut/oral microbiota. We found that the genera in the blood were partly similar to the microbiome detected in the feces and oral cavity, suggesting that the blood microbiome may originate from other parts, such as the oral cavity and intestinal tract. The gut microbiome has been reported to translocate into the blood in non-infectious diseases, including T2D,[29] obesity,[42] portal hypertension,[43] liver cirrhosis,[44] atherosclerosis, and schizophrenia. Studies have shown that changes in the diversity and composition of the blood microbial species can influence systemic inflammation.[34] Our functional prediction of blood and gut microbiomes both showed that immune and inflammation-related pathways, such as immune disease and infectious disease pathways, were upregulated in the microbiota, suggesting that bacteria capable of inducing inflammation are more prone to entering the bloodstream. In addition, our studies have found that the serum levels of inflammatory parameters, including LPS-binding protein, IL-1β, WBC, NEUT, and LYMPH were all significantly elevated in the T1D group compared with controls, suggesting the increased systemic inflammation in T1D may originate from gut/oral bacterial translocation into the blood. Additionally, as previously reported,[15,45] our study found that blood, oral, and gut microbiome communities differ from each other, indicating that the intestinal barrier, immune cells, and liver might play a role in filtering and affecting bacterial translocation.[46] Our study, together with previous studies, suggests that the microbiome present in the blood may originate from the gut and oral microbiomes as a result of bacterial translocation. A longitudinal study of paired saliva, stool, and blood specimens is required to determine the true prevalence and molecular mechanisms of the gut/oral blood translocation of bacterial microbiota.

Recently, next-generation sequencing techniques and metabolomic technologies have been used to investigate variations in blood microbiome communities and circulating metabolites. The blood microbiome has been reported to be a biomarker and target for diabetes and cardiovascular diseases.[21] A longitudinal study revealed a significant association between higher baseline blood bacterial 16S rDNA levels and the onset of T2D, indicating that high concentrations of blood-derived bacterial DNA could potentially be considered an independent biomarker of the risk of T2D.[30] Another prospective cohort study with primarily Chinese participants revealed the predictive value of the blood genera *Sphingomonas*, *Acinetobacter*, and *Staphylococcus* for hypertension.[47] In our study, among the altered abundances of genera, the combination of 36 genera, including *Ralstonia*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Pelomonas*, *Flavobacterium*, *Afipia*, *Microbacterium*, *Vibrionimonas*, *Flectobacillus*, *Pannonibacter*, and *Cupriavidus* yielded an efficient discriminating performance for T1D, with an AUC of 0.82. These candidate biomarkers may be powerful tools for the clinical diagnostic and prognostic screening of T1D. Common blood microbiota have been identified in certain diseases, for example, *Bacteroides* in cirrhosis, *Legionella* and *Devosia* in kidney diseases, and *Staphylococcus* and *Escherichia/Shigella* in inflammatory diseases.[23] Moreover, the significant correlation between these key circulating bacteria and clinical manifestations offers evidence that the blood microbiome may play an important role in T1D, even in the absence of gut/oral-blood translocation. Disease-specific alterations in the blood microbiome could constitute a relevant, inexpensive, and easy-to-sample approach for screening and characterizing T1D in high-risk populations.

In summary, to our knowledge, for the first time we profiled the blood microbiome in T1D and assessed the taxonomic association between the blood and gut/oral microbiota. T1D is characterized by increased blood bacterial richness and perturbed microbiome structures, with a significant increase in the abundance of pathogenic bacteria, such as *Sphingomonas*, *Caulobacter*, and *Stenotrophomonas*. Blood microbiome communities were partly similar to the corresponding oral and gut microbiomes, suggesting that oral and gut microbiomes are potential sources of the blood microbiome. The identified key blood microbiota exhibited excellent discriminatory power for differentiating patients with T1D from controls. Here, we comprehensively delineated the blood microbiome signatures of T1D, but the physiological and pathophysiological functions of the blood microbiome in T1D require further investigation.

Limitations of the study

Nevertheless, this study has some limitations that deserve discussion. First, our study was based on the sequencing of 16S rRNA genes, which is not sufficient to determine whether a live microbiome is present in the circulation. Further studies are required to validate the absence of a live bacterial microbiome in the blood as well as its functionality and potential role in host metabolism. Moreover, the cross-sectional nature of the study prevented us from elucidating the relevant mechanisms and longitudinal views. Further studies using

metagenomics, metabolomics, and transcriptomics are needed to explore the potential mechanisms underlying the association between blood microbiome and T1D.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110252.

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AUTHOR CONTRIBUTIONS

F.L. and R.W. applied for the grant and designed the study. X.Y. performed the experiments, analyzed the data, and wrote the manuscript. X.Y., Z.X., J.L., and C.S. performed data analyses and interpretations. R.C., H.W., L.C., H.D., G.L., Y.Y., X.C., L.C., J.F., J.W., Z.C., X.F., Z.S., M.Z., J.W., X.C., J.Z., Y.L., and L.Z. recruited subjects and collected samples. All the authors have reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human blood samples	Nine sampling regions including Harbin, Changchun, Taiyuan, Jinan, Zhengzhou, Suzhou, Shanghai, Nanchang, and Fuzhou in China	N/A
Critical commercial assays		
OMEGA Soil DNA Kit	Omega Bio-Tek, Norcross, GA, USA	Cat# M5635-02
Takara Ex Taq	Takara, Japan	Cat# RR820A
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen, Carlsbad, CA, USA	N/A
Deposited data		
Blood microbiome sequencing raw data	This paper	NCBI Sequence Read Archive database: PRJNA1075939.
Gut microbiome sequencing raw data	This paper	NCBI Sequence Read Archive database: PRJNA664632.
Oral microbiome sequencing raw data	This paper	NCBI Sequence Read Archive database: PRJNA876606.
Oligonucleotides		
Forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′)	This paper	N/A
Reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′)	This paper	N/A
Software and algorithms		
FastQC v0.11.7 Babraham Bioinformatics	FastQC v0.11.7 Babraham Bioinformatics	FastQC v0.11.7 Babraham Bioinformatics
QIIME2	https://qiime2.org	Version 2019.4
R version	http://www.r-project.org	Version 3.1.0
SPSS	Chicago, IL, USA	Version 21.0
GraphPad Prism	GraphPad Software Inc., USA	Version 8

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Feihong Luo (luofh@fudan.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact on request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact on request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study participants

This was a cross-sectional, observational, multicenter study. A total of 158 participants were recruited between January 2018 and June 2019, including 75 children with new-onset T1D and 83 age-matched healthy individuals. Finally, 64 T1D patients and 77 healthy subjects were

enrolled in this study. Profiling of the gut microbiome of this cohort has been described in our previous study.[3] Patients in this study were diagnosed with T1D according to the diagnostic criteria of the American Diabetes Association.[48] Patients were recruited and samples were collected within one week of the diagnosis of T1D. Participants were excluded if they suffered from acute infections, chronic diseases, or metabolic diseases, or if they took medications regularly or any other medical treatment within 1 month. After fasting the participants for 10 h, 3 mL peripheral venous blood was collected from each participant in the early morning and frozen at -80°C as per detailed instructions. This study was approved by the Institutional Review Board and Ethics Committee of the Children's Hospital of Fudan University ([2016]210 and [2019]210). All participants were informed of the purpose of the study and provided written informed consent.

METHOD DETAILS

Sample Collection and DNA Extraction

Blood samples from each participant were collected and immediately stored at -80°C at each hospital until they were shipped to the Children's Hospital of Fudan University by a cold-chain shipping company. FBG, HbA1c, C-peptide, WBC, NEUT, LYMPH, HDL-C, LDL-C, TC, and TG levels were measured using standard procedures.

16S rRNA gene amplification and sequencing

Total blood microbial DNA was extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. The quantity and quality of extracted DNA were measured using a NanoDrop 2000 spectrophotometer and agarose gel electrophoresis, respectively. The extracted DNA was used as a template for the PCR amplification of bacterial 16S rRNA genes using barcoded primers and Takara Ex Taq. PCR amplification of the V3-V4 region of bacterial 16S rRNA genes was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR amplicons were purified using Vazyme VAHTSTM DNA Clean Beads and quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×250 bp sequencing was performed using the Illumina NovaSeq platform with the NovaSeq 6000 SP Reagent Kit at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequencing and bioinformatic analysis

Bioinformatics of the microbiome were analyzed using QIIME2 (2019.4), with slight modifications according to official tutorials.[49] Raw sequence data were demultiplexed using the demux plugin, followed by primer cutting using the Cutadapt plugin.[50] The sequences were trimmed, quality-filtered, denoised, merged, chimeric, and dereplicated using the DADA2 plugin.[51] The raw 16s rRNA gene data were processed to form OTUs with 97% identity using UPARSE. Beta diversity analysis was performed to investigate the structural variation in microbial communities across samples using Bray–Curtis metrics[52] and UniFrac distance metrics.[53,54] Beta diversity was visualized via principal coordinates analysis. The differential abundance of bacterial taxa at different levels (phylum, class, order, family, and genus) between the groups was calculated using the Wilcoxon rank-sum test. Linear discriminant analysis effect size (LEfSe) was used to detect differentially abundant taxa between groups using the linear discriminant analysis (LDA) score.[55] The random forest classifier model was generated using the AUC-RF algorithm for feature reduction and maximizing model performance.[56] Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (http://picrust.github.io/picrust) was used to predict the abundance of functional categories in the Kyoto Encyclopedia of Genes and Genomes orthologs using the MetaCyc (https://metacyc.org/) and KEGG (https://www.kegg.jp/) databases. Raw reads were deposited in the NCBI Sequence Read Archive database under the accession number PRJNA1075939. The acquired data were further reprocessed using the following criteria: (1) OTU with sequence numbers greater than or equal to 1 in at least 10% of samples; (2) sequences were aligned against Greengenes to remove contaminated sequences, including mitochondrial and chloroplast sequences that also contain 16S rRNA gene.

Retrieval of sequencing data

16S rRNA sequencing data of the gut microbiome were obtained from the same cohort as in our previous study[3] and sequencing data of the oral microbiome were obtained from our previous study[57] for comparison. Gut and oral microbiome sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra), with the accession numbers PRJNA664632 (gut microbiome data) and PRJNA876606 (oral microbiome data).

Statistics

Statistical analyses were performed using SPSS version 21.0 (IBM SPSS Statistics, Chicago, IL, USA), GraphPad Prism version 8, and R version 3.1.0. The investigators were not blinded to the allocation during the experiments, but the outcome assessments were blinded. The age-stratified analysis focused on three age subgroups, i.e., the young-age subgroup (age $\leq$7), the middle-age subgroup (7< age $\leq$11), and the high-age subgroup (age >11). Outliers were identified as values outside of mean$\pm$3SD and removed from further analysis. Non-parametric Wilcoxon rank-sum tests were used to compare continuous variables between groups. The non-parametric Spearman rank correlation test was used to evaluate the correlations between parameters. A p-value < 0.05 was considered significant.