Original Article

Disorders of gut microbiota in children with Tetralogy of Fallot

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Background: Gut microbiota plays an important role in cardiovascular health and disease, including congenital heart disease (CHD). Tetralogy of Fallot (TOF) is the most common form of cyanotic CHD characterized by systemic chronic hypoxia and sustained pressure overload of the right ventricle. It is well-known that hypoxia and pressure overload can affect gut microbiota. However, the effects of TOF on the gut microbiota remain little understood. This study explored the profile of the gut microbiota in children with unrepaired TOF.

Methods: A total of 12 pediatric patients diagnosed with TOF and 9 healthy age- and gender-matched children were enrolled in this study. Fecal samples were collected from every participant and subjected to 16S rDNA gene sequencing. The raw sequencing data were processed using the Quantitative Insights Into Microbial Ecology pipeline.

Results: A comparison of the gut microbiota revealed that pediatric patients with TOF had developed dysbiosis as reflected by the altered taxonomic composition and impaired functional profile. A total of 14 indicative bacterial genera were identified as differential biomarkers capable of distinguishing between healthy children and TOF patients. Furthermore, functional annotations revealed that the gut microbiota in TOF patients was characterized by increased levels of inflammatory, oxidative, and immune responses, and decreased activities of adaptation, synthesis, and metabolism.

Conclusions: Pediatric patients with unrepaired TOF have intestinal dysbacteriosis that is characterized by altered taxonomic composition and impaired functional profile. These findings suggested that the interplay between gut microbiota and the host may be dysregulated in patients with TOF.

Keywords: Gut microbiota; Tetralogy of Fallot (TOF); children; metabolism; inflammation

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Introduction

In recent years, the gut microbiota has attracted much attention due to its important role in human health and disease via interacting with the host (1,2). Gut microbiota is related to cardiovascular health and diseases, such as atherosclerosis, heart failure, and arterial stiffness (3,4). Furthermore, research suggests that there exists a close relationship between the gut microbiota and congenital heart disease (CHD). A recent case-control study suggested that disorders of maternal gut microbiota and plasma metabolites were associated with a higher risk of CHD in the offspring (5). Additionally, intestinal microbiota may be a contributing factor in necrotizing enterocolitis among

term infants with cyanotic CHD (6).

Tetralogy of Fallot (TOF) is the most common form of cyanotic CHD (around 8–9.7%) and is characterized by systemic chronic hypoxia and sustained pressure overload of the right ventricle (RV) (7,8). Studies have shown that hypoxia and pressure overload can affect the gut microbiota (9-11). Thus, it is reasonable to speculate that the gut microbiota in TOF patients may be dysregulated. However, to the best of our knowledge, to date, there have been no studies exploring the changes of the gut microbiota in children with unrepaired TOF compared to healthy subjects.

Herein, a cross-sectional study was conducted to explore the changes in the gut microbiota in pediatric patients diagnosed with TOF who have not undergoing any intervention. It was hypothesized that the combined heart defects can influence the composition and function of the gut microbiota, which may be involved in the disease progression and outcome.

We present the following article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-33/rc).

Methods

Experimental design and study cohort

This cross-sectional study was designed to investigate the changes in gut microbiota in children with TOF. Twelve pediatric patients diagnosed with TOF by echocardiography in the clinic of Guangdong Provincial People's Hospital were enrolled in this study. Since age and gender can impact the gut microbiota (12,13), nine healthy age- and gender-matched children were enrolled as the control group. Comprehensive medical histories were obtained and physical examinations were performed to exclude conditions that were unsuitable for the trial, such as having a cold, diarrhea, constipation, jaundice, or use of any antibiotics within the past month. This study was approved by the Ethics Committee of the Guangdong Provincial People's Hospital (No. KY-Q-2021-091-01) and all procedures were conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consents were obtained from the parents or guardians of the participants.

Fecal sample collection and 16S rDNA gene sequencing

Fecal samples were collected from all participants and frozen at −80 ℃ for subsequent analysis. DNA was extracted using a DNA isolation kit (Findrop Biosafety Technology, Guangzhou, China) according to the manufacturer's protocol, and quantified and qualified by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) amplification of the bacterial 16S rDNA genes V4 region was performed using Premix Taq Version 2.0 (TaKaRa, Dalian, China) with forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR amplicons were then purified with AMPure XP Beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Finally, sequencing was performed using the Illumina NovoSeq 6000 platform.

Bioinformatics analysis

The sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME2, v2020.6) pipeline as previously described (14). Venn diagrams were generated to visualize the shared and unique genera between groups using VennDiagram. Indicator species analysis was performed using the indval function in labdsv (v2.0.1), and genera with a P value <0.05 were considered significant indicators. Alpha diversity (Chao1 and Shannon) was calculated in QIIME2. Beta diversity was visualized via nonmetric multidimensional scaling (NMDS) (15) and further examined using analysis of similarities (ANOSIM). Microbial functions were predicted by PICRUSt2 and Tax4Fun (16,17).

Statistical analyses

Statistical analysis was conducted using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA), and the graphs were plotted with GraphPad Prism (Version 7.0, San Diego, USA). Categorical variables were described as whole numbers and percentages, and comparisons between groups were performed with Fisher's exact test. Continuous variables with normal distribution were presented as mean ± standard error of the mean (SEM) or mean (95% confidence interval), and comparisons between groups were performed with *t*-tests. Continuous variables with non-normal distribution were presented as median and interquartile range (IQR), and comparisons between groups were performed with Wilcoxon rank-sum test. A P value

Table 1 The demographics and clinical characteristics of the study participants

Variables	Healthy group (n=9)	TOF group (n=12)	P
Male	6 (66.7)	9 (75.0)	1.000
Age (months)	8 [5, 10]	6 [4, 11]	0.452
Weight (kg)	8.3 (5.9, 9.3)	6.6 (5.8, 8.9)	0.522
Diameter of the VSD (mm)	–	10.4±0.6	–
Pressure gradient across the RVOT (mmHg)	–	71±4	–
Diameter of the LPA (mm)	–	5.5±0.4	–
Diameter of the RPA (mm)	–	6.1±0.4	–
Over-riding of the aorta (%)	–	49±2	–

Data are shown as numbers and percentages or median (interquartile range) or mean ± standard error of the mean. TOF, Tetralogy of Fallot; RVOT, right ventricular outflow tract; VSD, ventricular septal defect; LPA, left pulmonary artery; RPA, right pulmonary artery.

<0.05 was considered statistically significant.

Results

Subjects and clinical characteristics

Twelve pediatric patients with TOF were enrolled in the study and nine healthy children matched for age and gender were enlisted as the control group. Baseline characteristics are shown in *Table 1*. The gender, age, and weight were not significantly different between the two groups. Specifically, there were six and nine males in the healthy group and the TOF group, respectively. The median ages were 8 and 6 months, and the median weights were 8.3 and 6.6 kg in the healthy and TOF groups, respectively. The echocardiographic indices, such as diameter of the ventricular septal defect (VSD) and pressure gradient across the right ventricular outflow tract (RVOT), were recorded.

Alterations of bacterial taxa and indicative bacterial genera

Venn diagrams were used to illustrate the similarities and differences in the genera observed in the TOF patients and the healthy controls. There were 59 overlapping genera, and 36 and 53 genera were found exclusively in the healthy group and the TOF group, respectively (*Figure 1A*). The taxonomic compositions and abundances of the gut microbiota between the two groups are presented at the phylum level (*Figure 1B,1C*). In the healthy group, the most abundant phyla were *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, with an average abundance of 36.05%, 34.67%, and 14.30%, respectively. In the TOF group, the most abundant phyla were *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, with an average abundance of 47.33%, 24.44%, and 17.90%, respectively. At the genus level, 14 indicative bacterial genera (median relative abundance >0.1% and P<0.05) were identified as biomarkers that may distinguish between healthy children and TOF patients (*Figure 1D*). In the healthy group, *Terrisporobacter*, *Chryseobacterium*, and *Prevotella* were the top three genera with indicator values of 0.66, 0.51, and 0.43, respectively. In the TOF group, *Faecalibacterium*, *Megamonas*, and *Subdoligranulum* were the top three genera with indicator values of 1.00, 1.00, and 0.91, respectively.

Altered beta diversity in the gut bacterial community

The alpha diversity of the gut microbiota was compared using Chao1 and Shannon, and the results showed that TOF patients and healthy children had similar gut microbial alpha diversity (*Figure 2A,2B*). The similarities and differences in the community composition between the two groups were also examined. Notably, the NMDS plot of beta diversity based on Bray-Curtis distance matrix revealed that the differences in the community composition between the two groups was greater than that within the groups (*Figure 2C,2D*), indicating that TOF has a significant impact on gut bacterial community composition.

Altered abundance in Enzyme Commission (EC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KO)

The functional properties of the intestinal microbiota

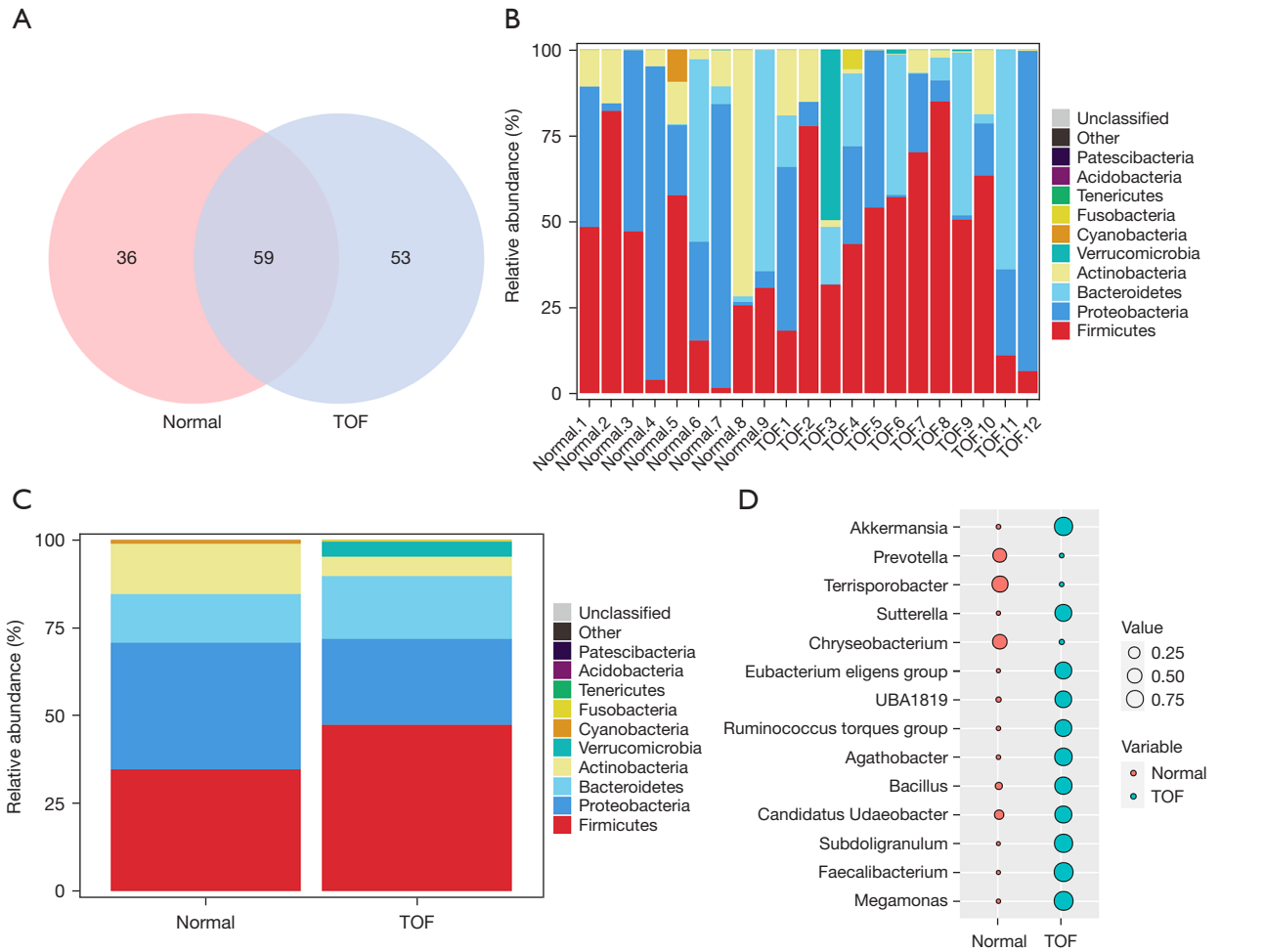

Figure 1 The alterations in bacterial taxa and the indicative bacterial genera in the TOF population and the healthy population. (A) There were 59 overlapping genera between the TOF and healthy population. A total of 36 and 53 genera were found exclusively in the healthy group and the TOF group, respectively; (B,C) the taxonomic compositions and abundances of the gut microbiota between the two groups are presented at the phylum level; (D) fourteen indicative bacterial genera were identified as potential biomarkers for the differentiation between healthy and TOF children. TOF, Tetralogy of Fallot.

were predicted using PICRUSt2. Interestingly, the gut microbiota of the TOF group was characterized by a significant reduction in the abundance of enzyme EC (Enzyme Commission), including transketolase, enoyl-CoA hydratase, and lysophospholipase. The top 20 enzyme ECs in relative abundance (P<0.05) are displayed in *Figure 3A*. Furthermore, the TOF group showed a lower abundance of KOs (KEGG Orthologs), such as LacI family transcriptional regulator, ribose transport system permease protein, and DNA-damage-inducible protein J. The top 20 KOs in relative abundance (P<0.05) are displayed in *Figure 3B*.

Altered abundance in KEGG pathway annotations

KEGG pathway annotations at level 2, as determined by PICRUSt2, showed significant differences between the TOF group and the healthy controls. In contrast to the healthy children, the functional profile in TOF patients exhibited a greater abundance of pathways related to immune diseases (*Figure 4A*) and a lower abundance of pathways related to environmental adaptation (*Figure 4B*), membrane transport (*Figure 4C*), and xenobiotics biodegradation and metabolism (*Figure 4D*). Furthermore, KEGG pathway annotations at level 3, as determined by

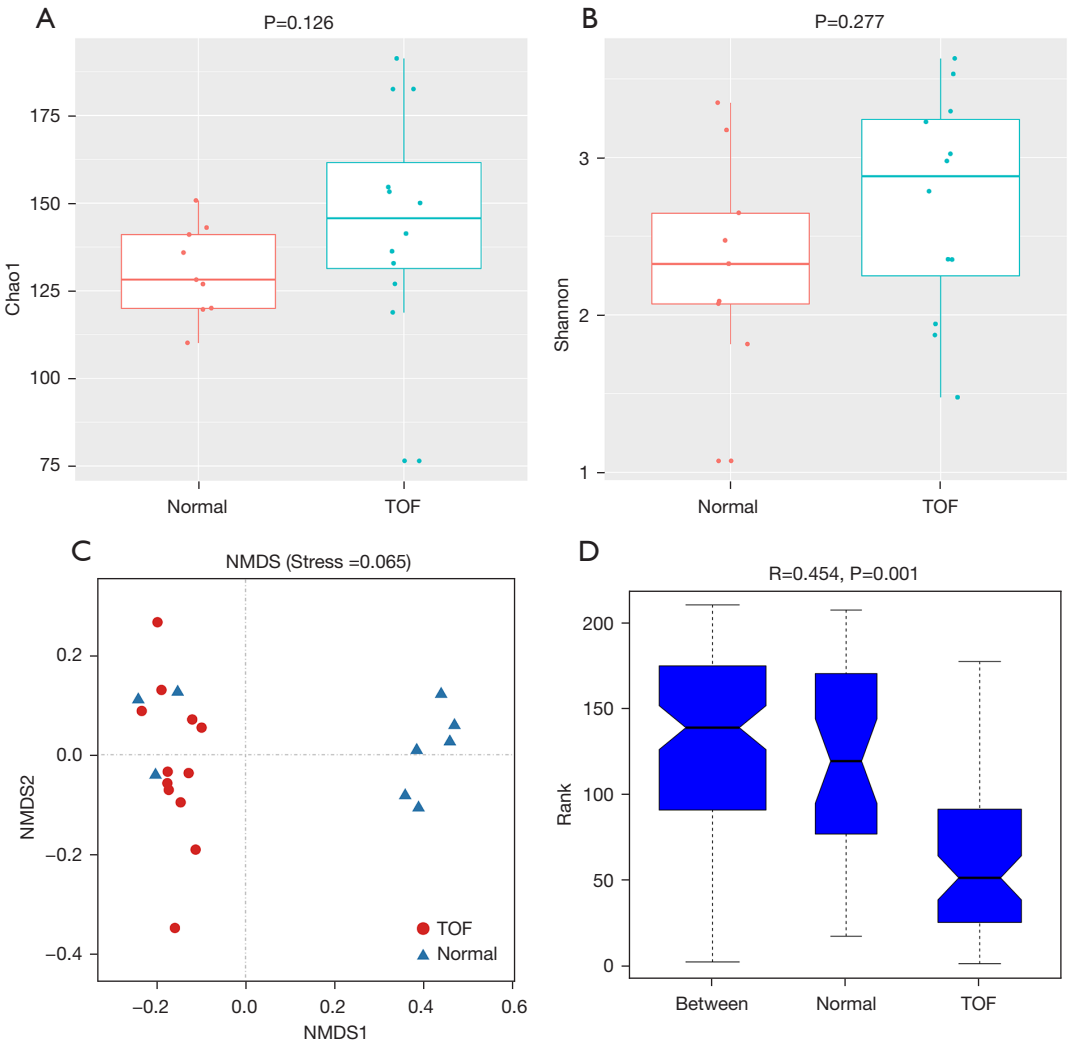

Figure 2 The alpha and beta diversity in the gut bacterial community. The TOF and healthy populations exhibited a similar microbial alpha diversity as estimated by (A) Chao1 and (B) Shannon. (C) The NMDS plot of beta diversity based on Bray-Curtis distance matrix is shown. (D) ANOSIM revealed a significant difference in the community composition between the two groups. TOF, Tetralogy of Fallot; NMDS, nonmetric multidimensional scaling; ANOSIM, analysis of similarities.

Tax4Fun, also presented significant differences between the two groups. In particular, the functional profile in TOF patients showed significant association with the nuclear factor (NF)-kappa(κ)B signaling pathway (*Figure 5A*), oxidative phosphorylation (*Figure 5B*), cytokine-cytokine receptor interaction (*Figure 5C*), and the vascular endothelial growth factor (VEGF) signaling pathway (*Figure 5D*), while there was a lower association with pathways involved in ribosome biogenesis in eukaryotes (*Figure 5E*) and glutathione metabolism (*Figure 5F*).

Discussion

The current study provided evidence that the gut microbiota in pediatric patients with unrepaired TOF differs from that in healthy children in terms of taxonomic composition, beta diversity, and functional profile. These findings suggested that the interplay between the host and the gut microbiota may be dysregulated in children with TOF, and indeed, TOF may influence the composition and function of the gut microbiota.

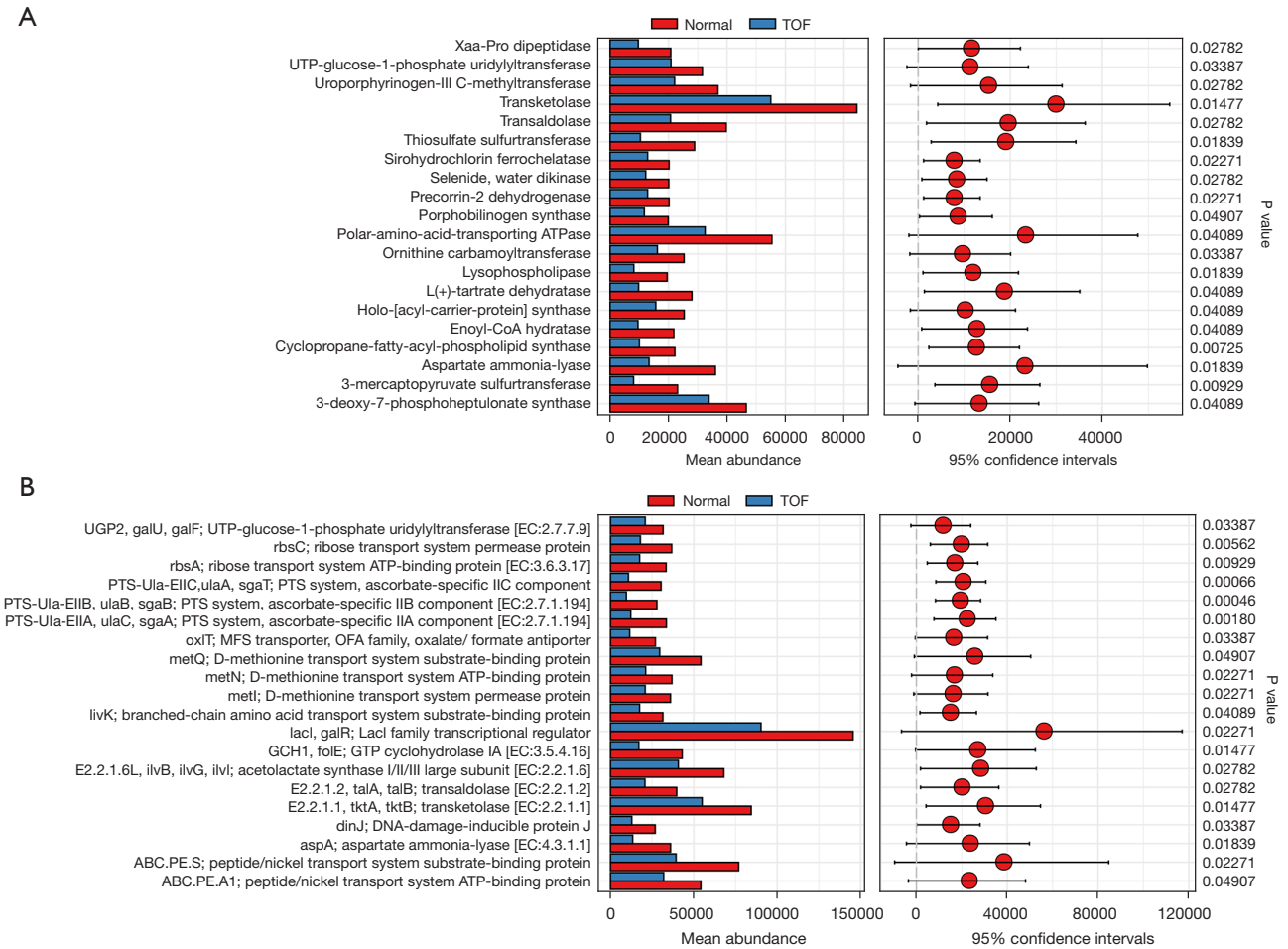

Figure 3 The altered abundance in enzyme EC and KOs. The gut microbiota of the TOF group was characterized by a significant reduction in the abundance of (A) enzyme EC and (B) KOs. EC, Enzyme Commission; KOs, KEGG Orthologs.

TOF is not only a combination of four heart defects, but it is also a systemic disease to some extent since patients are born with systemic chronic hypoxia, sustained RV pressure overload (8), and arterial stiffness (18). It has been reported that, despite having a correction in infancy, children with TOF are at a higher risk of disorders in speech and language compared to patients with VSD and cardiac insufficiency (19). Study has shown that in patients with repaired TOF, serum bile acid levels are positively correlated with the indexed RV end-diastolic volume, suggesting the presence of hepatic congestion (20). In addition, transcriptomic study on children with TOF revealed that hypoxia can lead to enhanced expression of genes related to apoptosis and remodeling, and reduced expression of genes related to myocardium contractility and function (21). However, the alterations in the gut microbiota of TOF patients remain unclear. This current study lends support the concept that TOF is a systematic condition by clearly demonstrating the imbalance and dysfunction in the gut microbiota of TOF patients.

Systemic chronic hypoxia and sustained RV pressure overload are prominent pathological features in TOF, and may be important factors involved in the gut microbiota changes observed in this current study. There is plenty of evidence to suggest that hypoxia can affect intestinal microbiota. Indeed, intrauterine hypoxia can cause changes of the initial microbiota colonization in neonatal rats (22). A recent study revealed that chronic hypoxia caused gut dysbiosis and D-galactose accumulation, thereby inducing premature senescence of bone marrow mesenchymal stem cells (23). Another research suggested that intermittent

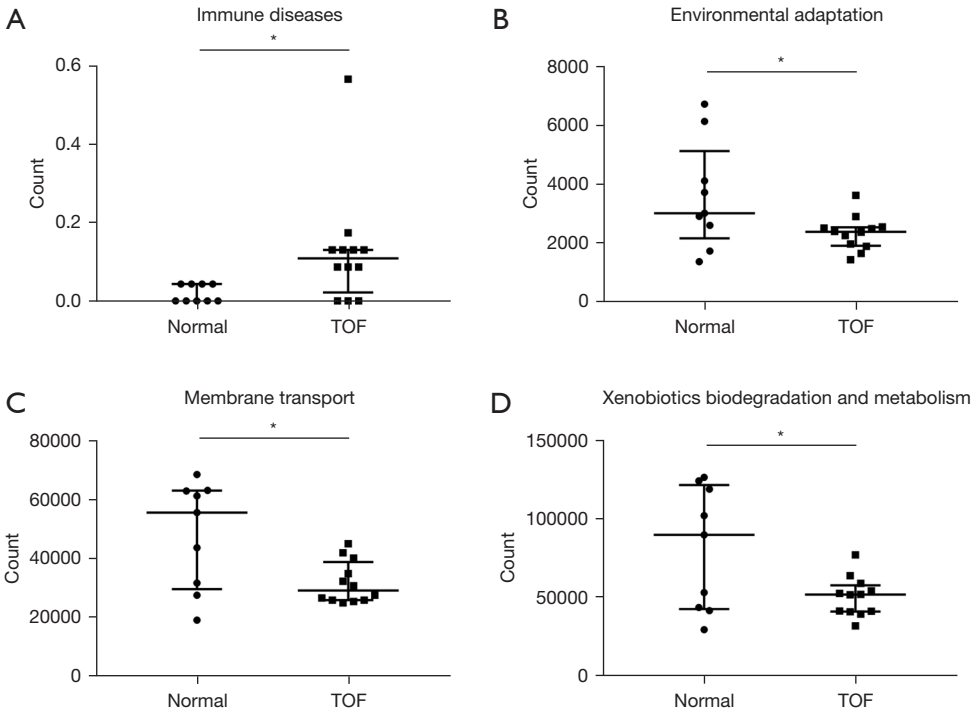

Figure 4 The impaired KEGG pathway annotations at level 2, as determined by PICRUSt2. The functional profile in TOF patients showed (A) a greater abundance of pathways involved in immune diseases and a fewer abundance of pathways involved in (B) environmental adaptation, (C) membrane transport, and (D) xenobiotics biodegradation and metabolism. *, P<0.05. KEGG, Kyoto Encyclopedia of Genes and Genomes; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; TOF, Tetralogy of Fallot.

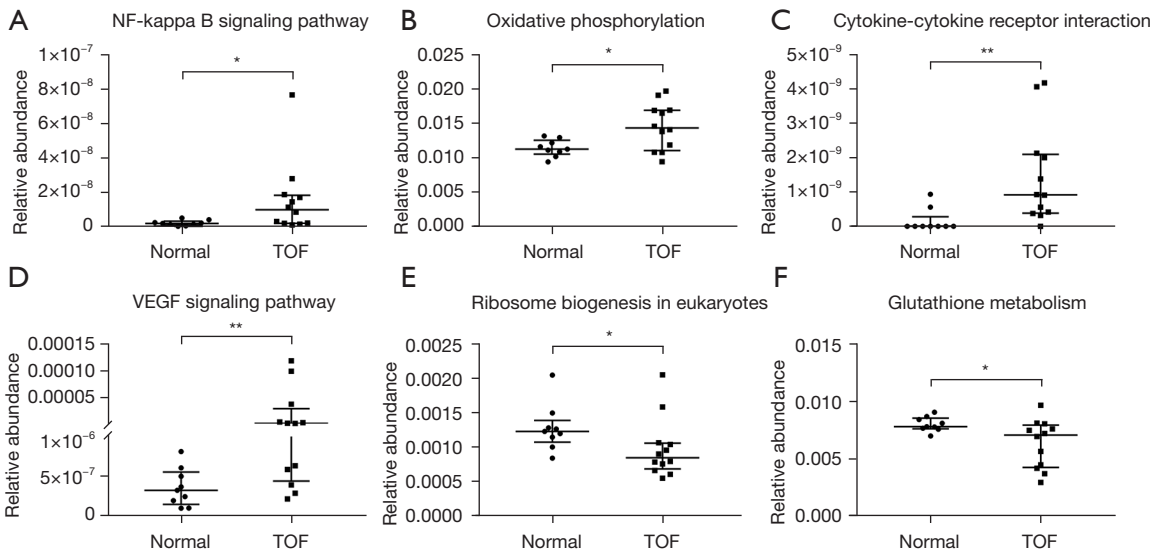

Figure 5 The impaired KEGG pathway annotations at level 3, as determined by Tax4Fun. The functional profile in TOF patients showed a greater abundance of pathways involved in (A) NF-κB signaling pathway, (B) oxidative phosphorylation, (C) cytokine-cytokine receptor interaction, and (D) VEGF signaling pathway. TOF patients showed a fewer abundance of pathways involved in (E) ribosome biogenesis in eukaryotes and (F) glutathione metabolism. *, P<0.05; **, P<0.01. KEGG, Kyoto Encyclopedia of Genes and Genomes; TOF, Tetralogy of Fallot; NF-κB, nuclear factor-kappa B; VEGF, vascular endothelial growth factor.

hypoxia contributed to atherosclerosis by disturbing the gut microbiome and metabolome (24). In our current study, the taxonomic compositions were found to be notably affected in TOF children, and furthermore, the indicator bacterial genera including *Faecalibacterium*, *Akkermansia*, and *Subdoligranulum* were identified as potential biomarkers to distinguish between TOF individuals and healthy children. *Faecalibacterium* has been implicated in inflammation and gut barrier integrity, and its abundance is significantly modified in children with obstructive sleep apnoea syndrome (OSAS) (25,26). In addition, *Akkermansia* and *Subdoligranulum* have been shown to be related to inflammation and metabolic status (27,28). Furthermore, we observed that the intestinal flora in TOF patients was characterized by a significant reduction in the abundance of enzyme EC and KOs. The TOF profile suggested insufficiencies in defense responses to stress (including 3-mercaptopyruvate sulfurtransferase, cyclopropane-fatty-acyl-phospholipid synthase, and DNA-damage-inducible protein J) (29-33), maintenance of metabolic homeostasis (including thiosulfate sulfurtransferase and ornithine carbamoyltransferase) (34,35), synthesis of nucleotides and nucleic acids (including transketolase and transaldolase) (36,37), and ribose transport (including ribose transport system permease protein and ribose transport system ATP-binding protein) (38,39). These results indicated that the functional properties of the gut microbiota were significantly impaired in TOF. It is noteworthy that reduced lysophospholipase activity has also been observed in injured intestinal mucosa caused by ischaemia (40). Our findings are supported by a study showing that children with OSAS had reduced gut microbiota diversity, increased inflammation, and gut barrier disruptors-related strains (26).

To further elucidate the differences in the functional profile between TOF children and healthy subjects, KEGG pathway annotations were performed. It was revealed that, in TOF patients, there was an abundance of gut microbiota associated with immune diseases, while there was a lower abundance of microbiota associated with environmental adaptation, membrane transport, and xenobiotics biodegradation and metabolism. Furthermore, it is worth noting that in the TOF group, the KEGG pathways of NF-κB signaling, oxidative phosphorylation, cytokine-cytokine receptor interaction, and VEGF signaling were significantly enriched, but there were fewer pathways associated with ribosome biogenesis in eukaryotes and glutathione metabolism. There is a close relationship between hypoxia and the NF-κB signaling pathway, as well as cytokine-cytokine receptor interaction and the VEGF signaling pathway (41-44). The NF-κB signaling pathway plays a crucial role in inflammation, immunity, cell proliferation, differentiation, and survival (45), as well as in intestinal homeostasis and diseases (46). Additionally, the NF-κB signaling pathway has been implicated in hypoxic conditions, yet its role may be double-edged. Study has shown that the NF-κB signaling pathway is activated in cyanotic myocardial tissues and hypoxia-stimulated cardiomyocytes, which is inferred as a compensatory response to stress (41). However, another study revealed a pathogenic aspect, in which NF-κB signaling played an important role in chronic intermittent hypoxia-induced atherosclerosis (43). In addition, oxidative phosphorylation is crucial to the synthesis of ATP (47) and the increased enrichment of oxidative phosphorylation in TOF may serve as an adaptive mechanism for a more energy requirement. This inference can be supported by a previous study on TOF patients, where a potential adaptive mechanism was found to enhance ATP output and minimize hypoxic damage (8). However, a reduced relative abundance of ribosome biogenesis in eukaryotes was observed, which suggested that protein production may be impaired in TOF. Interestingly, the metabolism of glutathione, which serves as a major intracellular antioxidant and an indicator of oxidative stress (48,49), was significantly less enriched in TOF patients. These results further indicated that the gut microbiota in TOF patients has been perturbed, resulting in increased levels of inflammatory, oxidative, and immune responses, and decreased levels of adaptation, synthesis, and metabolism.

There were some limitations to this present study. Since the sample size was small, it was difficult to explore the relationship between altered bacterial diversity and clinical parameters such as pressure gradient across the RVOT. In addition, this was an observational study, and future causality studies are warranted to translate the human observations into preclinical validation.

Conclusions

Pediatric patients with unrepaired TOF have intestinal dysbacteriosis that is characterized by altered taxonomic compositions and impaired functional profiles. These findings suggest that the interplay between the host and the gut microbiota has been dysregulated by TOF. However, further causality studies are warranted to progress from human observations to preclinical validation.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-33/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-22-33/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-33/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of the Guangdong Provincial People's Hospital (No. KY-Q-2021-091-01) and all procedures were conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consents were obtained from the parents or guardians of the participants.

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