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Original article

The gut microbiota as a protective mechanism against tuberculosis in children with household contacts in a TB-endemic environment: A pilot study

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Abstract. *Background and aim:* The gut-lung axis has contributed to the impairment of pulmonary infectious diseases. Gut microbiota appears to play a crucial role in modulating host immunity against tuberculosis (TB) infection in children. We examined the gut microbiota in children with tuberculosis and those who were in close contact and residing in the same household. *Methods:* A cross-sectional study was conducted involving patients aged from 1 month to 18 years who were diagnosed with bacterial confirmation of tuberculosis. Contact investigations were conducted for all patients. The sibling residing in the same household underwent screening for tuberculosis using a tuberculin test. Fecal samples were obtained from two groups upon admission. Next-generation sequencing was utilized to analyze the 16sRNA of the samples. *Results:* This study showed a notable significant decreased in the relative abundance of the phylum *Firmicutes* in children with TB disease compared to exposed TB children. The diversity of microbiota is no significantly altered, as indicated by no significantly different of alpha diversity in children with TB disease. *Conclusions*: The gut microbiota in children with TB disease exhibited differences compared to those exposed to TB, particularly in terms of the relative abundance of *Firmicutes*. This work will provide an understanding of gut microbiota influence on control mechanisms of TB infection in children. (www.actabiomedica.it)

Key words: Tuberculosis, gut microbiota, children, infection, gut-lung axis

Introduction

Tuberculosis (TB) is a major global public health problem generated by the bacillus Mycobacterium tuberculosis (Mtb) (1). The World Health Organization (WHO) indicated that TB was the leading cause of death from a single infectious agent, following 3 years in which it was replaced by coronavirus disease (COVID-19). Globally, an estimated 10.8 million people fell ill with TB. The highest burden is in adult men (aged ≥15 years) with an estimated 6.0 million cases, equivalent to 55% of the estimated total, and 1.3 million cases among children and young adolescents (aged 0-14 years), equivalent to 12% of the estimated total. The persistent increase signifies the enduring repercussions of disruptions to tuberculosis services during the peak years of the COVID-19 pandemic (2,3).

Household contacts are more vulnerable to obtaining TB infection from index cases due to their close proximity. The objective of contact tracing and

screening for TB could lead to the diagnosis of additional cases, emphasizing the impact of case detection and successful treatment (4). Infants and children are more likely to develop severe forms of TB (disseminated and meningitis) due to immature immunological responses (5,6).

The occurrence and development of TB are influenced by numerous factors such as bacterial load and host resistance, but TB can also evade the immune system and can circulate dynamically throughout a spectrum of diseases, depending on susceptibility, and proceed to active TB (7,8). The immunological basis of pathogenesis involves innate and adaptive immunity, defined by the state of immune balance, with coregulation by type 1 T-helper and type 2 T-helper cells. Some evidence suggests that the gut microbiota exhibits numerous significant impacts on immune system processes and protects against pathogenic invasion. The gut microbiota constitutes a complex and dynamic ecology containing over 100 trillion commensal microorganisms, exceeding the quantity of human cells. The important role of gut microbiota in lung infections is now more widely recognized, with a key connection between the gut and lungs known as the gut-lung axis. The advancement of next-generation high-throughput sequencing has facilitated the exploration of the human gut microbiome, yielding insights regarding gut microbiota (9-12). The current study was conducted to investigate the gut microbiota of children afflicted with tuberculosis.

Patients and Methods

Study design

The design of the present study was a cross-sectional study. All patients between 1 month until 18 years diagnosed with TB were included in this study from the pediatric department of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, between May 2024 and September 2024. The diagnosis of TB was identified according to the diagnosis and treatment of the Indonesian National Guideline of Tuberculosis for Children. The diagnosis criteria of TB were made by a combination of the following: Microbiological evidence including positive bacterial confirmation for MTB in sputum, gastric juice, bronchoalveolar lavage fluid, tissue, cerebrospinal fluid, and feces; typical clinical symptoms including cough and fever lasting for more than 2 weeks; and X-rays showed typical signs for pulmonary TB. All patients subjected to contact investigation and have other siblings that lived under the same house. The siblings that were in closely contact with the patient and did not show symptoms of typical TB became the control group as the exposed TB group. All exposed TB children were screened for TB with a tuberculin test.

Prior to sample collection, the parents of each participant signed an informed consent form. The protocol of the present study was approved by the Research and Ethical Committee of Dr. Seotomo Academic General Hospital, Indonesia.

Sample collection and DNA extraction

Stool samples were obtained from the exposed TB group on the day of their hospital examination. We collected fecal samples from TB patients on the day of their admission. All samples were immediately frozen after collection and stored at − 80 °C. The genomic DNA was extracted from each sample using the ZymoBIOMICS™ DNA Microprep Kit (California, USA). The extracted DNA is then subjected to quality control (QC) to measure its concentration using a Qubit 4 fluorometer (Invitrogen) and its purity using a Nanophotometer Implen N50. A minimum DNA concentration of 3 ng/μL is required to proceed to PCR process. Each sample is then barcoded and amplified using 16S primers provided by the 16S Barcoding Kit v14 (SQK-16S114.24, Oxford Nanopore Technologies), which amplifies the entire 16S rRNA gene region, producing an amplicon of approximately 1500 bp. The 16S Barcoding Kit includes barcoded 16S primers with up to 24 barcodes, enabling the differentiation of individual samples. The PCR amplicons are then purified following the protocol of the 16S Barcoding Kit V14. The final library, after library preparation, is sequenced using a MinION R10.4.1 flow cell on a GridION Mk1 sequencer.

Amplification and sequencing of the 16S rRNA encoding gene

The bacterial 16S rRNA gene was amplified with the forward primer 338F (5′-ACTCCTACGG-GAGGCAGCAG-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTA- AT-3′). Sequencing was completed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA), and 300 bp paired-end reads were generated.

Bioinformatic analysis

WF-metagenomics software, version v2.12.1 (https://github.com/epi2me-labs/wf-metagenomics), was used to complete the quality filtering of the readers as well as their taxonomic classification. The quality-filtered readers were clustered into operational taxonomic units (OTUs). Alpha diversity, encompassing bacterial richness and diversity in samples, were assessed using the Chao 1 index, Fisher Alpha index, Shannon index, Simpson index, and Berger-Parker index. The Chao 1 index and Fisher Alpha index reflected the quantity of operational taxonomic units (OTUs) of the microbiota utilized to assessed the richness of the gut microbiota community. The diversity of microbiota was assessed using the Shannon index, Simpson index, and Berger-Parker index, which quantified species richness and their relative abundance in the gut. Utilizing biological evolution data from each sample, the weighted UniFrac metric principal coordinates analysis (PCoA) was employed to determine the beta diversity of the gut microbiota, reflecting community variations between groups.

Statistical analysis

The demographic analysis was completed using Fisher's exact test. For age, weight, height, Chao 1 index, Shannon index, and Simpson index, comparisons were performed by the Mann-Whitney U test. The relative abundance of gut microbiota between groups was compared by the T-test, and p-values < 0.05 were considered statistically significant. Statistical analysis was performed by SPSS software, version 26.0 (IBM Corp., New York, NY, USA).

Results

Demographic data

There were 30 participants in total, comprising 15 children who had bacterial confirmation of tuberculosis and another 15 who were exposed to tuberculosis. Sixty percent of children were diagnosed with pulmonary TB, and 40% of children with extrapulmonary TB (meningoencephalitis TB, lymphadenitis TB, intestinal TB). Three children in exposed TB group were latent condition. Characteristic of study subject described in Table 1.

Diversity of gut microbiota in TB disease and children exposed TB

A total of 30 fecal samples were collected from participants. The current research study found no statistically significant difference in microbiotal richness among the groups. Despite this, although the Shannon and Simpson indices showed no significant difference, a reduction in both indices indicated that the diversity of fecal microbiota in tuberculosis patients was lower than that in children exposed to tuberculosis. The study revealed a significant increase in Berger-Parker index in the TB disease children group compared with the exposed TB children (0,602 (±0,04) vs 0,304 (±0,05), $p = 0.000$) (Table 2).

Community of gut microbiota in TB disease and children exposed TB

A weighted UniFrac PCoA analysis was conducted to assess the differences in fecal microbiota composition between children diagnosed with TB disease and those who were exposed to TB. As reflected in Figure 1, there was no different in cluster between samples of the two groups. Although the whole community structure of gut microbiota in the two groups showed no significant differences, the relative abundance of fecal microbiota in TB-diseased children was distinct from the exposed TB children. According to the sequencing analysis, the community of gut microbiota in TB disease consisted of bacterial phyla:

Table 1. Demographic characteristics of subjects.

	TB disease children (n = 15)	Exposed TB children (n = 15)	p
Age (year)	13.8 (±0.91)	9.6(±1.08)	0.006*
Sex (%) Male Female	 7 (46.7%) 8 (53.3%)	 6 (40%) 9 (60%)	0.713
Weight (kg)	38.73 (±3.36)	34.86 (±5.13)	0.533
Height (cm)	147.7 (±4.87)	133.13 (±6.26)	0.076
Tuberculin test (%) Positive Negative	 13 (86.7%) 2 (13.3%)	 3 (20%) 12 (80%)	0.000*
History of closed contact	5 (33.3%)	15 (100%)	0.000*
BCG Immunization	15 (100%)	15 (100%)	1.000
BCG scar	13 (86.7%)	14 (93.3%)	0.543
Exclusively breastfed	7 (46.7%)	10 (66.7%)	0.269
Mode of delivery Vaginal delivery Sectio cesaria	 12 (80%) 3 (20%)	 11 (73.3%) 4 (26.7%)	0.666

Table 2. Diversity of gut microbiota.

	TB disease children (n = 15)	Exposed TB children (n = 15)	p
Chao	86.26 (±9.33)	82.93 (±10,40)	0.813
Fisher Alpha	11.60 (±1.42)	10.88 (±1,48)	0.727
Shannon	2.67 (±0.22)	2.72 (±1,05)	0.857
Simpson	0.790 (±0.04)	0.804 (±0,05)	0.851
Berger-Parker	0.602 (±0.04)	0.304 (±0,05)	0.000*

Firmicutes, Pseudomonadota, Actinobacteria, Bacteroidetes, and *Lentisphaerota*. In exposed TB children, the most dominant phyla were *Firmicutes, Bacteroidetes, Pseudomonadota,* and *Actinobacteria*. This study demonstrated that phyla *Firmicutes* was significantly decreased in TB disease (p = 0.008). In contrast, phyla *Pseudomonadota* showed a significant increase in children with TB disease (p = 0.034). Phyla *Bacteroidetes* showed a decrease in children with TB disease (Table 3). This result reflected the alteration of gut microbiota in children with TB disease compared to exposed TB children. At the genus level, there were no significant different of genus *Enterococcus, Ruminococcus, Faecalibacterium, Prevotella, Bacteroides* between two groups (Table 4).

Discussions

Host systemic and lung immunity plays an essential role in TB pathogenesis through controlling the clearance, survival, and replication of MTB (13). Upon exposure to MTB, alveolar epithelial cells are the first cell lines that recognize and attach to the outer surface molecules of mycobacteria by various types of PRRs, including C-type lectins and TLRs. Subsequently, several signaling pathways evolve into activated, resulting in the secretion of cytokines and chemokines and prompting the migration of immune cells to the sites of infection (14). Numerous studies reveal the influence of gut and pulmonary microbiota on immune responses in the prevention, progression, and treatment

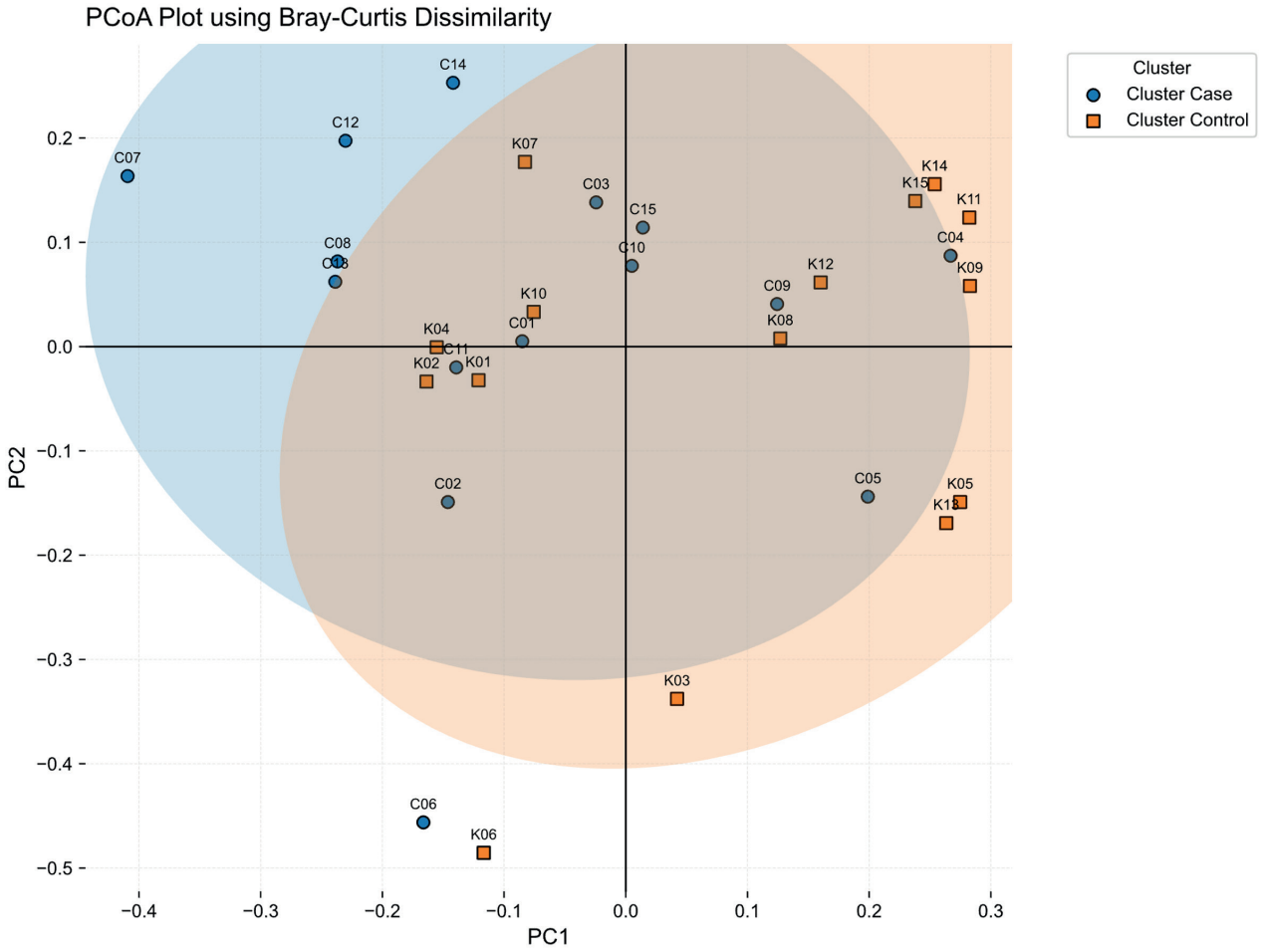

Figure 1. Principal coordinates analysis plots of TB disease children and Exposed TB children. The plots were based on weighted UniFrac distances.

Table 3. Relative abundance of gut microbiota at the phyla level.

Phyla	TB disease children (n = 15)	Exposed TB children (n = 15)	p
Firmicutes	17151.07 (±2471.33)	24949.67 (±4605.65)	0.008*
Bacteroidetes	156.67 (±205.28)	279.93 (±483.33)	0.371
Actinobacteria	122.73 (±366.63)	55.07 (±27.07)	0.498
Pseudomonadota	5909.20 (±9604.12)	352.27 (±681.06)	0.034*

Table 4. Relative abundance of gut microbiota at the genus level.

Genus	TB disease children (n = 15)	Exposed TB children (n = 15)	p
Enterococcus	3995.46 (±1496.35)	3055.40 (±1419.69)	0.518
Ruminococcus	274.33 (±261.63)	246.40 (±198.27)	0.176
Faecalibacterium	1115.67 (±469.17)	1573.20 (±557.51)	0.567
Prevotella	26.73 (±14.65)	20.60 (±18.71)	0.595
Bacteroides	23.33 (±12.95)	35.33 (±13.42)	0.708

of chronic respiratory diseases, addressed as the gut-lung axis (15). The microbiota affects TB prevention, pathogenesis, and treatment primarily by modulating the amount and function of immune cell subsets, producing bacteriocins and bacteriolysins that directly inhibit the growth of M. tuberculosis, and/or by influencing the bioavailability and pharmacokinetics of anti-TB medications (16-21).

This study represents the first attempt to characterize the gut microbiota through next-generation sequencing technology in pediatric TB patients, comparing them with their siblings who are in close contact and reside in the same household. The gut microbiome undergoes continuous changes from birth into adulthood, influenced by multiple variables including the technique of delivery, infant nutrition, exclusive breastfeeding, antibiotic history, and environmental factors like exposure to household pets (22-27). The findings of the study revealed that the gut microbiota patterns in children with TB disease was no differed from those of their siblings who were in close contact with the patient. The gut microbiota dysbiosis in patients with pulmonary TB was notably marked by an increase in the pro-inflammatory bacteria *Prevotella* and the opportunistic pathogen *Enterococcus*, with a deterioration observed after one month of effective anti-tuberculosis treatment (8). In multiple studies, notable decreases were observed in *Prevotella, Bacteroides*, and the order *Clostridiales*, while significant increases were noted in *Escherichia* and *Streptococcus* in TB disease groups compared to healthy controls. Individuals experiencing new TB infections exhibited an increase in *Prevotella*, which showed a positive correlation with elevated CD4+ cell counts (7,21, 28-30).

In our study, the richness of gut microbiota was slightly increased in children with TB disease compared to those exposed to TB but no statistically signicant. Consistent with other studies, the results indicated that both new and recurrent TB patients exhibit an increase in richness when compared to healthy subjects (28). The disparities observed between that study and the current one might derive from the differences in the subjects that were recruited. The study also revealed the significant increase of the Berger-Parker index in the TB disease children group, implying that the uneven distribution or composition of species abundance in the community. Although there was a slight increase in the richness of TB disease in children, the relative abundance of certain bacteria at the phyla level showed significant differences. This study highlights a decrease in the phyla *Firmicutes*, which are identified as pro-inflammatory bacteria, alongside an increase in the phyla *Pseudomonadota*, a proposed group that comprises the type genus *Pseudomonas*, regarded as an opportunistic pathogen.

The current investigation, in alignment with the phylogenetic integration of data from other studies, disclosed shifts in the relative abundances of bacterial lineages associated with the families *Ruminococcaceae* and *Lachnospiraceae*. It is essential to recognize that variations in relative abundance present challenges in integrating data collected from diverse host organisms, control populations, and various study designs. Nonetheless, these two bacterial families belonging to the phylum *Firmicutes* are the most prevalent groups of Gram-positive bacteria found in the healthy human colon (31,32). In another study, the phyla *Fusobacteria* and *Actinobacteria*, which include numerous gram-negative bacteria and opportunistic pathogenic species, showed a significant increase in TB patients when compared to the control group (33). In alignment with the current investigation, there was an increase in *Actinobacteria* within the tuberculosis disease group.

The findings of this study demonstrate a general decrease in the diversity of gut microbiota among children with TB disease, as evidenced by the decline in both the Simpson index and the Shannon index. A study involving animals indicated that mice infected with Mycobacterium tuberculosis exhibited a reduction in fecal microbiota diversity (34). The other study indicated that gut microbiota show a slight reduction in the murine model with TB when compared to healthy controls (35).

This study relies on a cross-sectional analysis. While the fundamental environmental characteristics of the subjects are comparable across groups and stringent inclusion criteria were applied during the selection process, individual differences remain inevitable. Another limitation is the relative small sample size due to only 15 patients for each group were enrolled. It may not be sufficient powered to detect a difference between

the groups and limits the generalizability of our findings. Cohort studies with large sample size would give the potential to meticulously track alterations in the intestinal flora of a subject from a state of health to infection and subsequently to the onset of disease.

Conclusions

This study provides an analysis of the characterization and alteration of gut microbiota in household contact children. Thus, this work will lead to an understanding of the influence of gut microbiota on the control mechanisms of TB infection, potentially uncovering a novel approach for fostering a protective immune response against TB in children.

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Ethic Approval: This study was approved as ethically appropriate by the Research and Ethical Committee of Dr. Seotomo Academic General Hospital, Indonesia (No. 0785/KEPK/IX/2023) released on September 26th 2023.

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