The Association Between Serum Ferritin and The Gut Microbiome in Patients with Active Tuberculosis Disease in South India

A Thesis

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by

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ABSTRACT

Background: Tuberculosis is one among the primary top ten causes of death and is the leading cause of a single infectious agent. Tuberculosis is a consequence of a rather particular immune failure against *M. tuberculosis*. Thus, evaluating the role of essential factors including serum ferritin and the gut microbiome in modulating the host immune system is vital in providing a better understanding of the complexity of the TB disease process. To our knowledge, no studies have been conducted to evaluate the role of serum ferritin to the gut microbiome in TB disease. The objective of this study was to characterize the composition and diversity of the gut microbiome by the level of serum ferritin in patients with new Active Tuberculosis Disease (ATBD).

Methods: In this cross-sectional study, all participants (n=32) were adult patients with new ATBD who attended an outpatient hospital, Arogyavaram Medical Centre (AMC), in Madanapalle, South India. Active pulmonary Tuberculosis disease was confirmed by Xpert MTB/RIF. Serum ferritin levels in this population were evaluated by Chemiluminescence immunoassay (ng/mL) and categorized as normal (15-250 ng/mL) or high (>250 (ng/mL), and by tertile in this population. Rectal swab samples were collected for DNA extraction, amplification and 16s rRNA sequencing (V3-V4 region; Illumina Miseq). Quantitative Insights into Microbial Ecology (V1.9.0) and GreenGenes 13.8 were used in taxonomy assignment of sequences and analysis sequenced data.

Results: The median of serum ferritin was 350.1 ng/mL (IQR, 179.1-719.15). About 68.75% participants had elevated serum ferritin. The gut microbiome predominantly belonged to five phyla (median (IQR): Firmicutes (46.1% (36.5-51.6)), Bacteroidetes (32.4% (24.5-40.4)), Proteobacteria (9.3% (6.1-28.1)), Actinobacteria (2.1% (1.3-4.2)), and Fusobacteria (0.3% (0.0-3.8). At the genus level, sequences predominantly were: *Prevotella* (11.7% (4.0-24.3)), *Bacteroides* (5.0% (1.2-

14.3)), *Faecalibacterium* (4.8% (1.5-6.5)), *1-68* (0.2% (0.0-2.0)), *Phorphyromonas* (0.4% (0.0-2.2)), and *WAL_1855D* (0.2% (0.0-1.7). The relative abundance of phylum and genus did not differ significantly by the level of serum ferritin (Normal or High, tertiles, $p>0.05$). Alpha diversity (Chao1, Observed-species, Shannon, Simpson) also did not differ by the serum ferritin level ($p>0.05$). The Firmicutes phylum and *Prevotella* genus were found to be negatively associated with serum ferritin ($p<0.05$). *Bacteroides* genus was observed to be positively associated with serum ferritin ($p<0.05$).

Conclusions: The changes of the composition of the gut microbiome such as changes of relative abundance of Firmicutes phylum, *Prevotella* and *Bacteroides* genera could have related to serum ferritin as a part of the immune response against *M. tuberculosis.* The findings from our study might as well related to the characteristic of *Prevotella* and *Bacteroides* that unable to synthesize heme and have the essential requirement of a high amount of iron and its precursors for growth. Further longitudinal studies with multiple time points and larger sample sizes are required to evaluate the role of serum ferritin to the gut microbiome of tuberculosis-infected patients.

BIOGRAPHICAL SKETCH

Pratiwi Ridwan focuses on evaluating micronutrients role in infectious diseases, including tuberculosis, particularly in vulnerable populations. Prior to her studies in the Master of Science program in the Division of Nutritional Sciences at Cornell University, her educational background included a Bachelor of Medical Science from Universitas Padjadjaran in Bandung-Indonesia and a Medical Doctor degree from Hasan Sadikin hospital, Universitas Padjadjaran in Bandung-Indonesia.

In dedication to all my patients in Central Sumba-East Nusa Tenggara

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Introduction

Tuberculosis (TB) remains a significant public health burden worldwide. Presently, TB is one among the primary top ten causes of death and is the leading cause of a single infectious agent. In 2017, TB caused a concerning 1.3 million deaths and 10.0 million new cases all over the world (1).

Tuberculosis is an infectious disease resulting from a specific immune failure delivered through *Mycobacterium tuberculosis* (2). Interaction between *M. tuberculosis* and the host determines the fate of infection and will initiate an inflammatory response by the host. The host's immune response to control *M. tuberculosis* infection involves a strong pro-inflammatory response which is modulated by anti-inflammatory mediators to balance the inflammation (3).

Iron is an essential micronutrient which is directly involved in host-microbiome interaction by altering microbes or viral growth and also by modulating the host immune system (4). Ferritin is a soluble protein found in the macrophage of the Reticuloendothelial System (RES) and parenchymal cells in the liver that facilitate iron storage in the body. Serum ferritin routinely used as an indicator for measuring the status of iron storage and also known as an acute phase protein (APP) which can increase due to inflammatory response, including that caused by *M. tuberculosis* (5, 6). During inflammation, IL-1 and TNF-α, cytokines produced as part of the innate immune response will limit bacterial iron availability by decreasing the amount of iron released from ferritin stores and decreasing iron absorption in the gut (7, 8).

Iron is known to drive the changes of the gut microbiome composition in both animals and humans. Several studies providing the evidence of the role of iron availability in the gastrointestinal tract to the alteration of the gut microbiome (4, 9-12), but there was lack of information about the function of ferritin as an acute phase protein to the changes of the gut microbiome.

Most recently, the gut microbiome was shown to play essential roles in the host biological functions such as nutrient metabolism, development of the host immune system, and metabolic homeostasis (13). It has been suggested that the gut microbiome may affect immune response not only in the gastrointestinal tract but also at the systemic level (14).

Several studies have been conducted both in humans and animals to evaluate the composition and diversity of the gut microbiome in TB disease (13, 15, 16). These studies were conducted to determine whether the composition of the gut microbiome contributes to the effectiveness TB control, including immunomodulation, decreased transmission, the progression from latency and disease severity and drug resistance (17).

Thus, evaluating the role of essential factors including ferritin and the gut microbiome in modulating the host immune system is vital in providing a better understanding of the complexity of the TB disease process. Despite prior evidence which showed that iron is associated with the gut microbiome during the diseased state (4, 9-12), the role of serum ferritin as an acute phase protein with regards to the gut microbiome during disease, both in general and in the New ATBD state, remains unclear. To our knowledge, no studies have been conducted to evaluate the role of serum ferritin in the gut microbiome of tuberculosis patients. Furthermore, the objectives of our study were to:

1) characterize the composition and diversity of the gut microbiome, and; 2) compare the difference between the gut microbiome composition and diversity between the levels of serum ferritin in new ATBD patients.

Literature Review

Tuberculosis

Definition and epidemiology of tuberculosis

Tuberculosis (TB) is an infectious disease caused by the *Mycobacterium tuberculosis*. This bacillus bacteria usually infects via pulmonary pathways, though it may also infect organs besides the lungs such as the pleura, lymph nodes, abdomen, bones, genitourinary tract, skin, joints and meninges (1). Despite being a relatively well-known disease, the etiology of TB was discovered on 24 March 1882, TB remains a global public health problem affects all countries and all age groups. Tuberculosis predominantly affects adults (aged ≥15 years of age), and roughly two thirds of the cases have been found in India (27%), China (9%), Indonesia (8%), Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%), and South Africa (3%) (1, 18).

Immune response in tuberculosis

Innate immune responses:

In a healthy individual, TB is a consequence of a rather particular immune failure against *M. tuberculosis* or other *Mycobacteria*. These bacteria infected hosts through inhaled droplet containing *M. tuberculosis* bacilli. Once the *M. tuberculosis* is inhaled, it travels to the pulmonary alveoli, alveolar macrophages and submucosal dendritic cells, which will begin their battle against the *M. tuberculosis* as part of the innate immune response (2).

Alveolar macrophages recognize *M. tuberculosis* through pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and Nod-like receptors (NLRs) which sense the microbial biochemical components of the pathogen-associated molecular patterns

(PAMPS). These molecules activate the alveolar macrophages, and as soon as the activation process is sufficiently initiated, the alveolar macrophages produce nitric oxide and reactive oxygen species (ROS), which eliminate *M. tuberculosis*, by enabling phagocytic activity. *Mycobacterium tuberculosis* is then entirely engulfed by the phagolysosome, and alveolar macrophage will secrete pro-inflammatory cytokines such as TNF-α (2, 19).

As soon the *M. tuberculosis* is engulfed by the alveolar macrophage, the virulence of *M. tuberculosis* can inhibit macrophage activation and become more resistant by inhibiting phase-lysosome fusion and detoxification of nitrogen and oxygen radicals and dormancy. Impaired infected cell's response to *M. tuberculosis* and the bacilli replication into sufficient number within alveolar macrophage may cause the infected cells to burst and lets *M. tuberculosis* release as well as infect the surrounding cells (19). *Mycobacterium tuberculosis* is also able to modulate macrophage apoptosis and inhibit Interferon gamma (IFN-γ)-mediated macrophage activation (2).

Dendritic cells (DCs) observed to have a pivotal role in generating *M. tuberculosis*-specific T-cell response supporting the findings of dendritic cell's function in priming naive Cells (19). Neutrophils besides play a protective role, not merely as first-line non-specific phagocytes, but also by secreting anti-bacterial proteins. Neutrophils loaded by phagocytized bacteria start to be apoptotic, thereby eliciting macrophage activation. Natural Killer (NK) cells as circulating lymphocytes are drawn to the site of infections and recognizing and remove the pathogen. During the process, NK cells produce IFN-γ and will activate macrophage to secrete cytokines like IL-12, IL-15, and IL-18 which later will activate $CD8^+$ and link to the adaptive immune system (2).

Adaptive immune system

The adaptive immune response to *M. tuberculosis* is primarily dominated by the protective response by TH1type ($CD4^+$ T-cells), although it has been found that more T-cell subsets including $CD8^+$ and TH17 cells as well as B-cells involved with this specific response (2). The adaptive immune system initiated by the signaling and presentation of the microbial peptide by the macrophage and DCs to the $CD4^+$ cells via MHC class II molecule. As the macrophages migrated to the draining lung lymph node, the presentation of mycobacterial antigen occurred in the draining lymph node and followed by the activation of the other T-cells and $CD4^+$ cell. Activation of T-cell will induce secretion of pro-inflammatory cytokines such as IL-1β, IL-6, IL-21, and IL-12p40 (2).

Activated T-cells are going to migrate out of lymph node to the infection site in the lung. During the process, T-cells will secrete IFN-γ which later on will activate microbicidal machinery pot the macrophage and induce macrophage to secrete IL-18 as part of protective TH1 Type response. It also induces the production of toxic NO (2). $CD8^+$ T-cells participate in the adaptive immune response to *M. tuberculosis* infection by recognizing bacterial peptide and lipids bound to MHC/HLA class IA molecules which more will initiate a cytotoxic response toward bacteria as well as to the phagocyte in which they live. The $CD8^+$ T-cell also secrete IFN-γ and TNF-α (2, 20).

Serum ferritin

Definition

Ferritin is a water-soluble protein of iron storage which fulfills the regulation of metabolic function in all cells and also acts as iron sources in macrophages of the Reticuloendothelial System (RES) and parenchymal cells of the liver for the protein and heme synthesis (21, 22).

Metabolism of ferritin

Ferritin synthesis is regulated by intracellular iron availability by modulating initial responses of apoferritin synthesis and regulating translational process which required movement of messenger ribonucleic acid (mRNA) from Ribonucleoprotein fraction (RNP) to the polysomes and therefore will improve the rate of ferritin subunit translation (23, 24). The translational process involves Iron Response Element (IRE) which will be bind to Iron Regulatory Protein (IRP) as in the low iron availability, IRP affinity to IRE will increase as well as inhibit ferritin translation. In contrary, IRP will not bind to IRE in the high amount of iron, and the ferritin mRNA synthesis will increase (24-26).

Ferritin is an iron storage protein which circulates in small portion proportional to the quantity of Fe in the storage. Serum ferritin routinely used as an indicator for measuring the status of iron storage but it is not valid to measure iron storage during condition eliciting an acute phase response (27-29) such as malignancy, infection, and in the liver, renal and autoimmune diseases (30-32).

Serum ferritin in inflammation

Infection and trauma are actually accompanied by an Acute Phase Response (APR), a non-specific process which involves the generation of Acute Phase Proteins (APPs) before the total activation of the immune response (5). Due to APR, monocytes and macrophages will release cytokines IL-1, IL-6, and TNF-α that induce local and systemic immune responses and these cytokines will modulate the production of APPs by hepatocytes. The positive APPs include C-reactive protein (CRP), 1-antichymotrypsin (ACT), α_1-acid glycoprotein (AGP), likewise referred to as

orosomucoid, serum amyloid A (SAA), fibrinogen, haptoglobin, caeruloplasmin, and ferritin (5, 6).

The increase in plasma ferritin concentration paralleled with the expansion of plasma CRP during acute pneumonia, TB, rheumatoid arthritis and neutropenic sepsis, which may increase approximately 130% within 48 hours supporting the evidence of ferritin role as an APP. Nevertheless, the degree to which the ferritin concentration rose was affected by the underlying iron status of the subjects (5, 6).

The regulation of iron metabolism is generally under the control of IRPs that bind to sequences on mRNA and protect mRNA from degradation (7). An acute phase response usually induced by the infection and will lead to the iron sequestration, limited Fe erythropoiesis and anemia (34). During infection, the control of iron metabolism is modulated by IL-1 and TNF-α. as FTH and FTL; the genes responsible for encoded ferritin are sensitive to cytokines (IL-1 and TNF) and paracrine signaling molecules including Nitrogen monoxide (NO), glutathione, and other reactive oxygen species (ROS). The plasma ferritin concentration increases, despite a reduced concentration of serum iron as a result of modulation of ferritin synthesis, is sensitive to cytokines on both translational and transcriptional level in several cells like mesenchymal cells, hepatocytes, monocytes and macrophages (7, 26).

Serum ferritin is an acute phase reactant and also a marker of inflammation and will increase in a wide range of inflammatory conditions, including acute infection. Increases of serum ferritin reflects the rises of total iron storage but the storage was sequestered and not available for

hematopoiesis, which resulted in iron deficiency. These conditions assumed to be part as a defense mechanism in reducing serum iron from utilization by pathogens (35).

It started to be evident that to understand the serum ferritin concentration some degree of the acute phase response was needed (28). Thus, it was recommended that, along with ferritin, an independent biomarker of the acute phase response, for example, AGP or CRP, should be measured (33). The serum concentration of the APR like CRP, AGP, and ACT has been utilized to adjust the effect of the APR on the markers of micronutrient status when evaluating the prevalence of deficiency in the healthy population (34).

Serum ferritin in tuberculosis

Ferritin synthesis is stimulated by the inflammatory process caused by *M. tuberculosis* (39). The increase of serum ferritin in TB patients caused by cytokines produced by monocytes and macrophages (IL-1 and TNF-α) as part of the immune response against *M. tuberculosis* (40).

During the inflammation, increase concentration of IL-6 will upregulated hepcidin concentration that downregulates ferroportin and reduce iron flow into extracellular fluid from all its sources. Hence, recycled iron is retained in the macrophages of the liver and spleen and reduce iron absorption (36).

Macrophages and kidney proximal tubule cells are likely the major cellular sources for serum ferritin. If macrophages are the main source of serum ferritin, it may explain the fact that serum ferritin is increase in inflammation, when increased hepcidin levels inhibit iron recycling from macrophages, causing macrophage iron retention, systemic iron deficiency and anemia (37).

As a phagocytic cell, macrophage plays pivotal role against infection by chemotaxis to the site of infection, and endocytosis, and eliminating infecting microbes. Macrophages are highly metabolically active and rich in various metabolic intermediates that can be utilized by intracellular microbes including Mycobacteria (36).

During infection, increase hepcidin concentration will induce the endocytosis and proteolysis of ferroportin, and hence, retained iron in cytoplasmic ferritin. It is, therefore reasonable to assume that, while high plasma hepcidin concentrations act to sequester iron from extracellular organisms, the increased availability of iron within phagocytes may promote the survival of intracellular microbes (36). Although serum ferritin usually has low iron, the rise of serum ferritin synthesis during inflammation by the macrophage will reduce the effectivity of lymphocytes (35).

Several studies provided clinical evidence which demonstrates disease susceptibility and the response to infection and inflammation worsen with elevated iron stores. Inflammation worsen with elevated iron stores. For tuberculosis, it has been demonstrated that oral iron increase mycobacterial growth and that morbidity and mortality increase in patients receiving iron supplementation. In fact, dietary iron is associated with occurrence and death from tuberculosis (38).

The concentration of serum ferritin is found increases in TB patients. Serum ferritin assessment is regarded as the sensitive technique to determined iron status but, in TB, using ferritin level to determine iron storage must be utilized with caution as serum ferritin levels do not accurately express the quantity of iron in such patients therefore, patients are able to have iron deficiency despite normal level of ferritin (41).

There were several studies provided the evidence of the association between serum ferritin and TB which also showed that *M. tuberculosis* as the most common infectious cause of increased serum ferritin (34, 42, 43). Among TB patients, serum ferritin observed to be 50% higher compared to the patient without TB (34). Although the considerable intense acute phase reactants among TB patients are affecting the immediate measures of storage and iron tissue, there are several plausible biological predictors which active in the variation of the serum ferritin rise (34).

Gut microbiome

Definition

The microbiome is the collection of bacteria, archaea, viruses, and eukaryotes that colonized the human body and formed a mutually beneficial relationship with the host (44). Microbes inhabited several sites in the human body such as gastrointestinal tract, skin, genitourinary and also respiratory tract. The number of microbes in the human body and the number of human cells were of the same order, with the estimated ratio of bacteria/human cells is 1.3:1 (45). As human gastrointestinal tract has a large estimated surface area, it presents a significant surface for microbial colonization. The colon itself contains approximately 70% of all the microbes colonized the human body (44). The human microbiome is also identified by the richness, evenness, and diversity of its microbial communities (46).

The healthy human gastrointestinal tract is colonized by four predominant phyla bacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Aside from the main composition of the core microbiome, the relative proportion and diversity of the species vary across individuals (47). The composition and the stability of the gut microbiome could change depending on the host

and environmental factors including genetics, age, nutrition, probiotics, prebiotics, antibiotics, drugs and diseases (48, 49).

Diversity of the gut microbiome

Alpha diversity is the level of diversity of species within a sample. It can be measured by several metrics, such as observed species, and Chao1 index which measured the number of species found in a sample (species richness). Alpha diversity also can be measured by Shannon and Simpson's index of species richness which combine the information of species richness with abundance data into a single value of evenness (50, 51).

Association of iron and the gut microbiome

Iron association with the gut microbiome and the immune system is well characterized. As iron deficiency will disrupt host immune response, the availability of iron is also able to affect the composition and diversity of the gut microbiome (52).

Association of iron administration and the gut microbiome

Iron administration to the infants and young children showed significantly affecting the composition of the gut microbiome by increasing the abundance of pathogenic bacteria including several taxa from *Enterobacteriaceae* family and low proportion of *Lactobacillus* when compared with the control group (10, 12). In vitro studies also provided supporting evidence of increased iron content causes the decrease of commensal bacteria and increased the abundance of pathogenic bacteria and its virulence (53, 54).

Association of iron deficiency and the gut microbiome

Iron deficiency additionally plays a role that is crucial in the gut microbiome composition which supported by the evidence from in-vitro, human and animal studies. In a low iron condition, there were changes of the gut microbiome composition like the decrease abundance of short-chain fatty acids (SCFAs) producing bacteria such as *Roseburia* members, *[Eubacterium] exhale*, *Clostridium* cluster IV members, and *Bacteroides* (55-59). Low iron conditions also increase the abundance of *Enterobacteriaceae* family as well as *Enterococcus, Lactobacillus* and reduced abundance of the *Coriobacteriaceae* (55-57, 59).

Gut microbiome and immune system

Gastrointestinal tract not only housing the highest amount of microbiome (44) but also contained the highest number of the immune cells in the body (60). Microbiome and host immune system have a bidirectional association, where microbiome plays an essential role in initiating, training and functioning the host immune system and in exchange, the immune system involved in maintaining homeostasis between host and the gut microbiome. Together, the gut microbiome as well as the immune system managing the innate and adaptive immune response in a complicated relationship by selects, calibrate and terminate the responses in the appropriate manner. The disruption of this balance will result in the pathologies including inflammatory disorder, autoimmune and allergies (61).

Intestinal immune system work in two steps in order to restrict the exposure of the pathogenic bacteria to the host tissue including, first by limiting the direct contact between intestinal bacteria and the epithelial cell surface and second, by constraint the pathogenic bacteria to intestinal site and restrict their exposure to the systemic immune compartment (compartmentalization) (62).

Gut-lung axis

Gut-lung axis usually associated with respiratory disease (63, 64). The concept of the gut-lung axis has been studying for years to find out the alternative intervention for respiratory diseases through changes of the gut microbiome. The mechanism of the gut-lung axis may not be clearly understood, but as both gut and lungs are have the same origin of embryonic and have similarities in structure (65), these two compartments also shared the same mechanism against microorganisms. The dysbiosis in gut and lungs is associated with changes in immune response and even to diseases development in the lungs (65).

Gut and lungs compartment can affect each other composition of the microbiome. The animal model study provided evidence of microbiome colonized in the nasal cavity of mice likewise can certainly be discovered in the gut microbiome (66). This evidence showed that gastrointestinal tract usually exposed to the pathogen introduced to the airway (14).

Gastrointestinal tract might expose to bronchus content through aspiration which will introduce live bacteria from bronchus content to the gut. Bacteria from the gastrointestinal tract also can translocate to the airways through aspiration of vomit or esophageal reflux. During the dysbiosis, disruption of the integrity of mucosal epithelial cells may let microbiome pass the mucosal barrier and enter the circulatory system and further on will cause systemic inflammation (65).

The gut microbiome is able to affect the lung microbiome through modulation of the systemic immune response (65). There are several possible mechanisms of cross-talk between the gut microbiome and the lung microbiome through immune responses:

Bacterial Structure

The gut microbiome products including lipopolysaccharide (LPS), lipoteichoic acid, CpG, and peptidoglycan can bind and activate TLRs in the gastrointestinal tract (67). Not only epithelial cells recognize bacteria through TLRs mechanism but also immune cells such as macrophages, dendritic cells, lymphocytes B and neutrophils. Interaction between gut microbiome product and TLRs on immune cells such as macrophage will initiate production of chemokine and cytokines and trigger the inflammatory response. Toll-like receptors signaling also leads to activation of the transcription factor of NF-kB macrophages (68). This activation will modulate the initiation of expression genes involving in innate immune response and inflammation in the lungs (67, 69, 70).

Short Chain Fatty Acids (SCFAs)

Short-chain fatty acids (SCFAs) are produced from the fermentation process of partially and non-digestible polysaccharide by the microbes in the colon (71). These metabolites are important energy sources for the host and the gut microbiome metabolism as well as through SCFA production; microbes increase the efficiency of digestion by extracting the maximum caloric energy from food (72).

There are three predominant SCFAs, acetate, butyrate and propionate. In human gastrointestinal, propionate is mainly produced by the Bacteroidetes phylum. Meanwhile, butyrate produced primarily by the Firmicutes phylum (72, 73). Apart from SCFAs function as the main energy resources for colonocytes, SCFAs are also known for their activity in mediating intestinal immune function. Generally, SCFAs affecting the performance of leucocyte by modulation of the capability of leucocyte migration and inflammatory mediators. SCFAs, particularly propionate and butyrate, have anti-inflammatory activity by suppressing the production of pro-inflammatory mediators like

TNF-α, IL-12, IL-6 and NO by the macrophages as well as monocytes and affecting their capacity to capture antigens and inducing T-cells. Butyrate additionally discovered to decrease Nuclear Factor-κB (NF-kB) activity and able to alter the composition of the mucus layer by inducing mucin synthesis (71, 74). The proliferation and differentiation of T lymphocyte also regulated by SCFAs by inducing the generation of T-reg (75, 76).

Gut microbiome and tuberculosis

The composition of the gut microbiome in tuberculosis patients

Bacteroidetes and Firmicutes are the most predominant phyla in the human gut microbiome. Several studies provided evidence of the gut microbiome changes in new ATBD patients. At the phylum level, the relative abundance of Bacteroidetes phylum observed to be lower in 19 new ATBD patients compared to the healthy control (HC) (44.1% new ATBD vs 58.8% HC, p= 0.020). On the contrary, Firmicutes composition showed no significant difference between new ATBD patients and HC group. Another predominant phylum in the human gut microbiome, Proteobacteria which also contain gram-negative bacteria was discovered to have higher relative abundance in new ATBD patients group compared to HC group (11.7% new ATBD vs 4.0% HC, p=0.017) (13).

A similar result also can be found in a study conducted in a pilot study in new ATBD patients. The relative abundance of Bacteroidetes phylum was lower compared to household control. Firmicutes and Proteobacteria phyla relative abundance were discovered higher in TB patients group compared to the household group (16). In the animal study which follow the changes of the gut microbiome prior *M. tuberculosis* infection, during and after infection showed decreased relative abundance of the gut microbiome with Firmicutes phylum discriminated the two condition before

and after *M. tuberculosis* infection (15). The study resulted in discriminated relative abundance between pre and post-infection sample. All the significant differences of OTUs found more abundant in the pre-infection sample (15).

The composition of the gut microbiome also observed at genera level. In new ATBD patients, there was a higher relative abundance of SCFAs producers such as *Faecalibacterium, Coprococcus, Phascolarctobacterium,* and *Pseudobutyrivibrio* compared to healthy control (16). But the opposite trend observed in other studies which showed the lower relative abundance of SCFAs producing bacteria such as *Roseburia* in new ATBD group compared to the healthy control (0.5% new ATBD vs 1.5% HC, p=0.008). Lower relative abundance of *Coprococcus* also found in new ATBD group compared to healthy control (0.5% new ATBD vs 1.1% HC, p=0.012) (13). It is known that SCFAs have a pivotal role in innate immunity and energy homeostasis (77).

The relative abundance of *Prevotella* genus found to be significantly lower in new ATBD patients compared to the healthy control (6.2% new ATBD vs 22.5% HC, p=0.005) (13, 16). The high relative abundance of *Prevotella* also associated with the initiation of cytokines production and leading to systemic inflammation (78, 79). The significant difference of the gut microbiome community before and after *M. tuberculosis* infection suggested that the relative abundance of the gut microbiome regulated during *M. tuberculosis* infection and as a result of host immune response changes towards *M. tuberculosis* infection (15).

The diversity of the gut microbiome in tuberculosis patients

The alpha diversity of the gut microbiome in TB patients, assessed by the Chao1 diversity index, the Simpson index, and Shannon diversity index. The Chao1 diversity index in TB patients

observed to be moderately higher compared to healthy individuals. There was no significant difference between Simpson and Shannon indexes between TB patients and healthy individual. In the animal study using mice model, there was lower richness and diversity of the gut microbiome measured using ecological beta-diversity measures (13).

The study result suggests that *M. tuberculosis* infection associated with a higher biodiversity of the gut microbiome in TB groups. Alpha diversity assessed by the observed species and Shannon index indicated greater richness and diversity of species in new ATBD group (both p=0.03) compared to healthy control (16). Overall diversity assessed through the ACE diversity index in new ATBD showed moderately higher than healthy control. The chao1 was modestly higher in new ATBD compared to HC (16).

Methods

Patients

In this cross-sectional study, all the participants (n=32) were adult patients (>18 years) with new ATBD who attended an outpatient hospital, Arogyavaram Medical Centre (AMC) in Madanapalle, South India. New ATBD confirmed by Xpert MTB/RIF. Patients who received anti-tuberculosis treatment over for more than four weeks in the past five years, who had other conditions or diseases which required hospitalization, and who were pregnant or lactating were excluded from the study.

Data collection

Serum samples were evaluated for serum ferritin (ng/mL) by Chemiluminescence immunoassay (Advia Centaur XPT immunoassay system; Siemens Healthcare Diagnostics Inc., Washington, DC, US). Sputum samples were assessed by Xpert MTB/RIF (Cepheid, Sunnyvale, California, US) to confirm new ATBD (80). C-reactive protein (CRP) was evaluated by latex-enhanced immunoturbidimetry (mg/dL; Advia 1800 Chemistry Analyzer; Siemens Healthcare Diagnostics Inc., Washington, DC, US). Rectal swab samples were collected and stored initially at 4° C and subsequently at -20°C or -80°C until deoxyribonucleic acid (DNA) extraction.

Gut microbiome: 16s rRNA sequencing and analysis

Rectal swab samples were collected for DNA extraction (QIAMP DNA Mini kits; Qiagen Inc., Valencia, CA, US). DNA concentrations were estimated using NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, US). Genomic DNA (25 ng of each sample) was amplified for 26 cycles using KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Boston, MA, US). The gut microbiome was characterized by targeting V3 and V4 hypervariable regions of the

16s rRNA gene by primers (proprietary; Genotypic Technology Pvt. Ltd.; Bangalore, Karnataka, India).

Amplicons were sequenced by Illumina MiSeq (Illumina, San Diego, CA, US). Quantitative Insights into Microbial Ecology platform (QIIME, version 1.9.0) was used in taxonomy assignment of sequences and analysis sequenced data (81). The query sequences were clustered using the UCLUST method and taxonomy of the clusters were assigned based on ≥ 97% sequences similarity against 16s rRNA database, GreenGenes (82, 83). Prior to alpha diversity calculations, data were rarified with a depth of 50,000 reads. Shannon, Simpson, and Chao diversity indices were calculated through QIIME. Data visualization was conducted using R software (Version 1.1.383; RStudio Inc., Boston, MA, US) (84, 85).

Serum ferritin (ng/mL) was categorized by cut points: normal serum ferritin (15-250 (ng/mL)), high serum ferritin (>250 (ng/mL)) (7) and also into tertiles.

Statistical analysis

Statistical analysis was performed using SAS University Edition (3.8 Basic Edition; SAS Institute Inc., Cary, North Carolina, US). Normality of the data was tested via normal probability plots, skewness, kurtosis estimates, and the Shapiro-Wilk test. Descriptive statistics of study participants characteristic were reported with means (Standard Deviation), n (%) and for the non-normally distributed variables were reported with medians (interquartile range). The differences between subgroups of study participants were compared with parametric (T-test, ANOVA) or non-parametric tests (Wilcoxon, Kruskal-Wallis).

Spearman correlation was used to identify univariate correlations between serum ferritin and the relative abundance and alpha diversity of the gut microbiome. Multiple linear regression models were used to compare the association of serum ferritin to the abundance and diversity of the gut microbiome.

Variables were log transformed to improve normality and homoscedasticity where appropriate. Serum ferritin was converted into μg/mL (multiplied by 0.001). All the statistical tests were 2-sided, and $p<0.05$ was considered statistically significant.

Results

Sociodemographic and biochemical characteristics

This study included 32 participants with a median age of 52 years (IQR 35.5-58.5), and more than three-quarters of the study participants were male (78.1%; Table 1).The median serum ferritin concentration was 350.1 ng/mL (IQR, 179.1-719.15); stratified by the tertiles, the median values were : 152.5 ng/mL (IQR, 133.9-172.3), 333.5 ng/mL (IQR, 266.7-412.0), and 1134.7 ng/mL (IQR, 708.8-2171.8; Table 1). About 31.25% participants had normal serum ferritin levels (15-250 ng/mL), and 68.75% had elevated serum ferritin levels (>250 ng/mL). The median serum CRP was 7.2 mg/dL (IQR, 3.6-11.2; Table 1).

Gut microbiome

In this study population, the gut microbiome predominantly belonged to five phyla (median, IQR): Firmicutes (46.1% (36.5-51.6)), Bacteroidetes (32.4% (24.5-40.4)), Proteobacteria (9.3% (6.1-28.1)), Actinobacteria (2.1% (1.3-4.2)), and Fusobacteria (0.3% (0.0-3.8)). At the genus level, sequences predominantly were: *Prevotella* (11.7% (4.0-24.3)), *Bacteroides* (5.0% (1.2-14.3)), *Faecalibacterium* (4.8% (1.5-6.5)), *1-68* (0.2% (0.0-2.0)), *Phorphyromonas* (0.4% (0.0-2.2)), and *WAL_1855D* (0.2% (0.0-1.7)); Table 1) as well as other unclassified genera.

The mean (SD) values of the alpha diversity indices were: Chao1 (1023.6 (350.5)), Observed species (647.5 (219.3)), Shannon (5.1 (1.1)); the median value of Simpson was 0.9 (IQR, 0.9-1.0; Table 1).

Gut microbiome and serum ferritin

The relative abundance of OTUs at the phylum and genus levels did not differ significantly, based on any of the considered subgroups stratified by the serum ferritin concentration (normal-high and tertiles; all p>0.05; Table 2). Alpha diversity indices (Chao1, Observed-species, Shannon, and Simpson) did not significantly differ by their serum ferritin concentrations (p>0.05). The relative abundance (%) of Bacteroidetes and Firmicutes phyla declined as serum ferritin concentration increased. On the genus level, similar results were observed in the *Porphyromonas* genus which decreased with increasing serum ferritin levels. The same trend was observed in Chao1, observed species, and Shannon alpha diversity index. In contrast, the relative abundance of Fusobacteria increased with increasing serum ferritin concentration, which was also found in the *Bacteroides* genus. None of the results were statistically significant (p>0.05), (Table 2).

Spearman correlation was used to identify associations between the relative abundance of the gut microbiome at phylum and genus level and predictor variables such as serum ferritin, CRP, age and sex. This test revealed a correlation between serum ferritin concentrations and the relative abundance of Proteobacteria (p=0.05; Table 3). At the genus level, serum ferritin was associated with the relative abundance of *Bacteroides* (p=0.03; Table 3). Serum ferritin was furthermore associated with alpha diversity indices: Chao1 (p=0.03; Table 3) and observed species (p=0.05; Table 3). Serum ferritin was also correlated with age (p=0.05; Table 3) and sex (p=0.01). All correlations described have been listed in Table 3.

Using linear regression models, we identified one phylum that was associated with serum ferritin. Serum ferritin was found to be inversely associated with the relative abundance of Firmicutes (ß= -0.3, p=0.02; Table 4). For every increase of 1 µg/mL in serum ferritin, we saw a reduction of

0.3% in the relative abundance of Firmicutes. At the genus level, the relative abundance of *Prevotella* was observed to be inversely correlated with serum ferritin (ß = -0.9, p=0.02; Table 3), for every increase of 1 µg/mL in serum ferritin, we saw a reduction of 0.9% in the relative abundance of *Prevotella.* In contrast, relative abundances of *Bacteroides* were positively correlated with serum ferritin (ß =0.9, p=0.01; Table 3) which showed that for every increase of 1 µg/mL in serum ferritin, we saw a rise of 0.9% in the relative abundance of *Bacteroides*.

Table 1. Characteristics of the population

Characteristics of the Population	**Median (IQR) or n (%)**
Sociodemographic	
Sex (male)	25 (78.1)
Age (years)	52 (35.5-58.5)
Biochemical	
Serum Ferritin (ng/mL)	350.1 (179.1-719.15)
Normal, 15 ng/mL – 250 ng/mL	10 (31.25)
High, >250 ng/mL	22 (68.75)
Tertile 1, ≤ 262.4 ng/mL	152.5 (133.9-172.3)
Tertile 2, >262.4 ng/mL – ≤644.8 ng/mL	333.5 (266.7-412.0)
Tertile 3, > 644.8 ng/mL	1134.7 (708.8-2171.8)
CRP (mg/dL)	7.2 (3.6-11.2)
Alpha diversity[a]	
Chao1	1023.6 (350.5)
Observed species	647.5 (219.3)
Shannon	5.1(1.1)
Simpson[a]	0.9 (0.9-1.0)
Phylum abundance (%)	
Actinobacteria	2.1 (1.3-4.2)

Bacteroidetes	32.4 (24.5-40.4)
Firmicutes	46.1 (36.5-51.6)
Fusobacteria	0.3 (0.0-3.8)
Proteobacteria	9.3 (6.1-28.1)
Genus abundance (%)	
Bacteroides	5.0 (1.2-14.3)
Faecalibacterium	4.8(1.5-6.5)
Porphyromonas	0.4 (0.0-2.2)
Prevotella	11.7 (4.0-24.3)
1-68	0.2 (0.0-2.0)
WAL_1855D	0.2 (0.0-1.7)

[a]Reported in median for non-normally distributed (confirmed by Shapiro-wilk test)

Table 2. Relative Abundance of OTUs and alpha diversity at phylum and genus level, stratified by the level and tertiles of serum ferritin

	Normal Serum Ferritin (15 ng/mL – 250 ng/mL) N=10	**High Serum Ferritin (>250 ng/mL) N=22**	**P value**	**Tertile 1 (≤ 262.4 ng/mL) N=10**	**Tertile 2 (>262.4 - ≤644.8 ng/mL) N=11**	**Tertile 3 (≥644.8 ng/mL) N=11**	**P value**
Phylum(% abundance)	**Median (IQR)**	**Median (IQR)**		**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	
Actinobacteria	2.1 (1.6-5.2)	2.1 (1.0-3.7)	0.6	2.1 (1.6-5.2)	1.7 (1.0-3.7)	2.5 (0.9-4.4)	0.8
Bacteroidetes	37.4 (28.3-45.2)	29.9 (21.0-39.1)	0.08	37.4 (28.3-45.2)	30.5 (5.9-37.7)	29.2 (26.2-40.2)	0.1
Firmicutes	46.6 (40.8-52.4)	43.5 (24.4-51.5)	0.2	46.6(40.8-52.4)	46.2 (24.4-51.8)	41.2 (18.1-51.5)	0.4
Fusobacteria	0.0 (0.0-0.4)	0.8 (0.0-5.4)	0.2	0.0 (0.0-0.4)	0.2 (0.0-4.2)	2.8 (0.6-12.8)	0.1
Proteobacteria	8.1 (2.9-12.7)	14.9 (7.1-50.2)	0.08	8.1 (2.9-12.7)	20.6 (9.0-55.3)	7.1 (5.8-50.2)	0.07
Genus (% abundance)							
Bacteroides	2.8 (0.8-14.0)	5.7 (3.2-16.8)	0.4	2.8 (0.8-14.0)	3.2 (0.9-11.7)	11.4 (5.3-21.9)	0.1
Faecalibacterium	5.0 (3.6-6.4)	3.9 (1.2-6.7)	0.7	5.0 (3.6-6.4)	3.2 (1.8-6.7)	5.3 (0.5-7.3)	0.9
Porphyromonas	0.6 (0.0-2.5)	0.2 (0.0-1.9)	0.6	0.6 (0.0-2.5)	0.3 (0.0-1.7)	0.1 (0.0-2.1)	0.8
Prevotella	23.1 (10.0-29.2)	10.6 (2.2-17.8)	0.08	23.1 (9.9-29.2)	10.1 (3.4-22.9)	11.1 (0.6-17.5)	0.2
1-68	0.2 (0.1-0.4)	0.3 (0.0-3.1)	0.7	0.2 (0.1-0.4)	0.3 (0.1-12.1)	0.1 (0.0-1.6)	0.4
WAL_1855D	1.0 (0.0-4.1)	0.1 (0.0-0.7)	0.2	1.0 (0.0-4.1)	0.1 (0.0-0.7)	0.2 (0.0-1.3)	0.5
Alpha diversity indices							
Chao1	1101.5 (845.4-1287.6)	923.3 (649.0-1324.5)	0.5	1101.5 (845.4-1287.6)	1019.1 (788.8 – 1461.0)	868.9 (618.8-1324.5)	0.7
Observed species	704.5 (527.0-842.0)	600.5 (415.0-838.0)	0.5	704.5 (527.0-842.0)	555.0 (378.0-688.0)	651.0 (415.0-855.0)	0.6
Shannon	5.5 (4.7-6.0)	4.9 (4.3-5.8)	0.4	5.5 (4.7-6.0)	5.0 (4.0-5.5)	4.8 (4.3-6.0)	0.7
Simpson	0.9 (0.9-1.0)	0.9 (0.9-1.0)	0.3	0.9 (0.9-1.0)	0.9 0.8-1.0)	0.9 (0.9-1.0)	0.6

Table 3. Associations between the variables and serum ferritin stratified by level of serum ferritin

Serum ferritin	Normal ferritin Rho (pvalue)	High ferritin Rho (pvalue)
Age	0.4 (0.3)	-0.4 (0.05)
Sex	0.0 (0.9)	0.5 (0.03)
Proteobacteria	0.6 (0.01)	0.2 (0.5)
Bacteroides	0.4 (0.3)	0.5 (0.03)

Table 4. Association between the variables and serum ferritin stratified by tertile of serum ferritin

Serum ferritin	Tertile 1 Rho (pvalue)	Tertile 2 Rho (pvalue)	Tertile 3 Rho (pvalue)
Sex	0.0 (0.9)	0.7 (0.01)	0.3 (0.4)
Proteobacteria	0.5 (0.1)	-0.3 (0.4)	0.6 (0.05)
Bacteroides	0.4 (0.3)	-0.2 (0.6)	0.7 (0.03)
Prevotella	-0.3 (0.4)	0.2 (0.6)	-0.6 (0.06)
Chao1	0.3 (0.4)	0.7 (0.03)	-0.3 (0.4)
Observed Species	0.3 (0.4)	0.6 (0.05)	0.1 (0.8)

Table 5. Multiple linear regression at phylum level (Firmicutes)

	Firmicutes	
Independent variable	**Parameter estimate**	**P value**
Intercept	4.0	<0.01
Serum Ferritin	-0.3	0.02

Table 6. Multiple linear regression at genus level (*Prevotella)*

	Prevotella	
Independent variable	**Parameter estimate**	**P value**
Intercept	3.2	<0.01
Age	-0.0	0.9
Sex	-0.7	0.4
Serum Ferritin	-0.9	0.02

Table 7. Multiple linear regression at genus level (*Bacteroides*)

	Bacteroides	
Independent variable	**Parameter estimate**	**P value**
Intercept	1.9	0.05
Age	-0.0	0.8
Sex	-1.1	0.1
Serum Ferritin	0.9	0.01

Figure 1. Stacked bar graph of relative abundance of the gut microbiome at phylum level

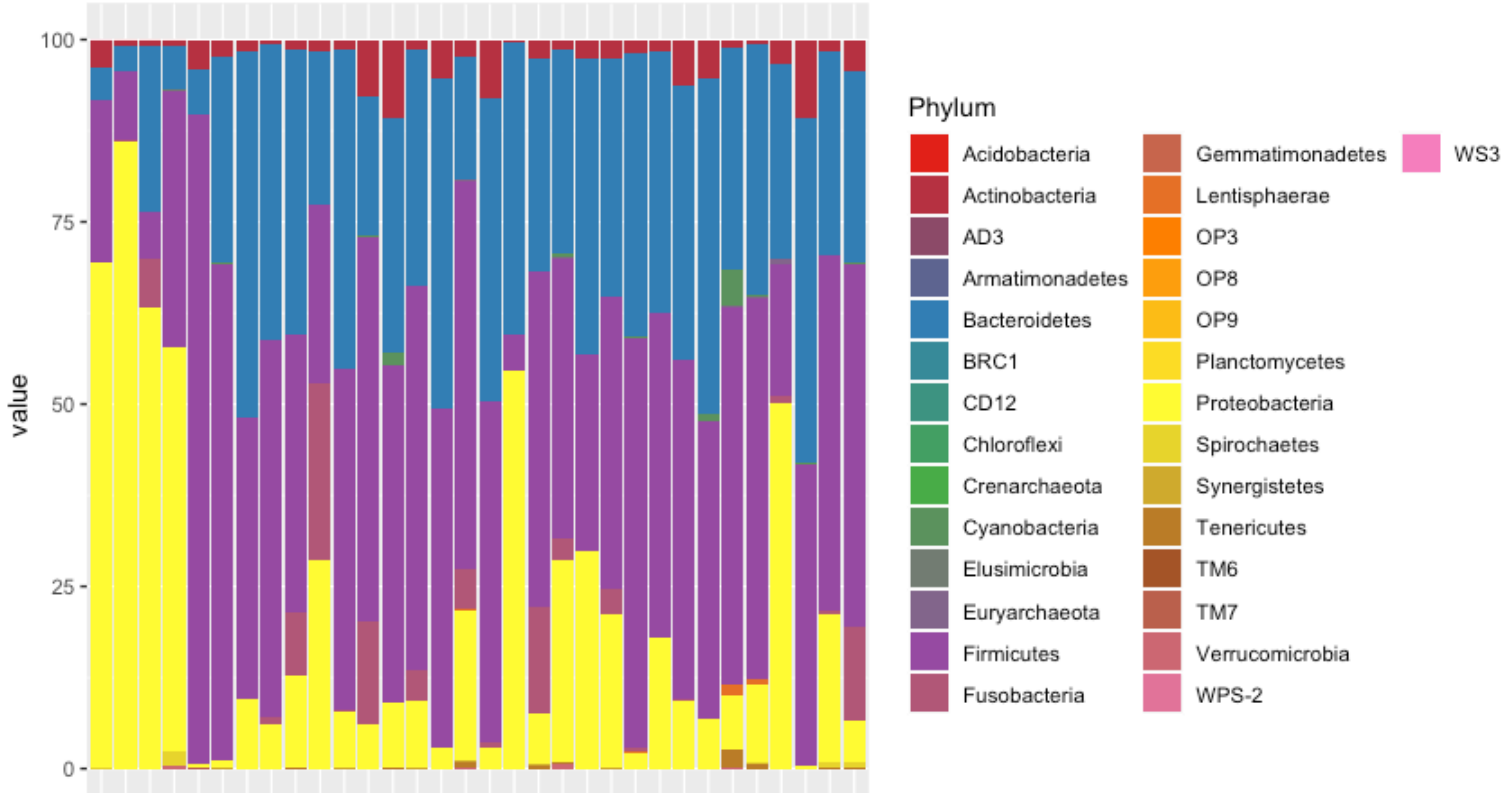


Figure 2. Stacked bar graph of relative abundance of the gut microbiome at genus level

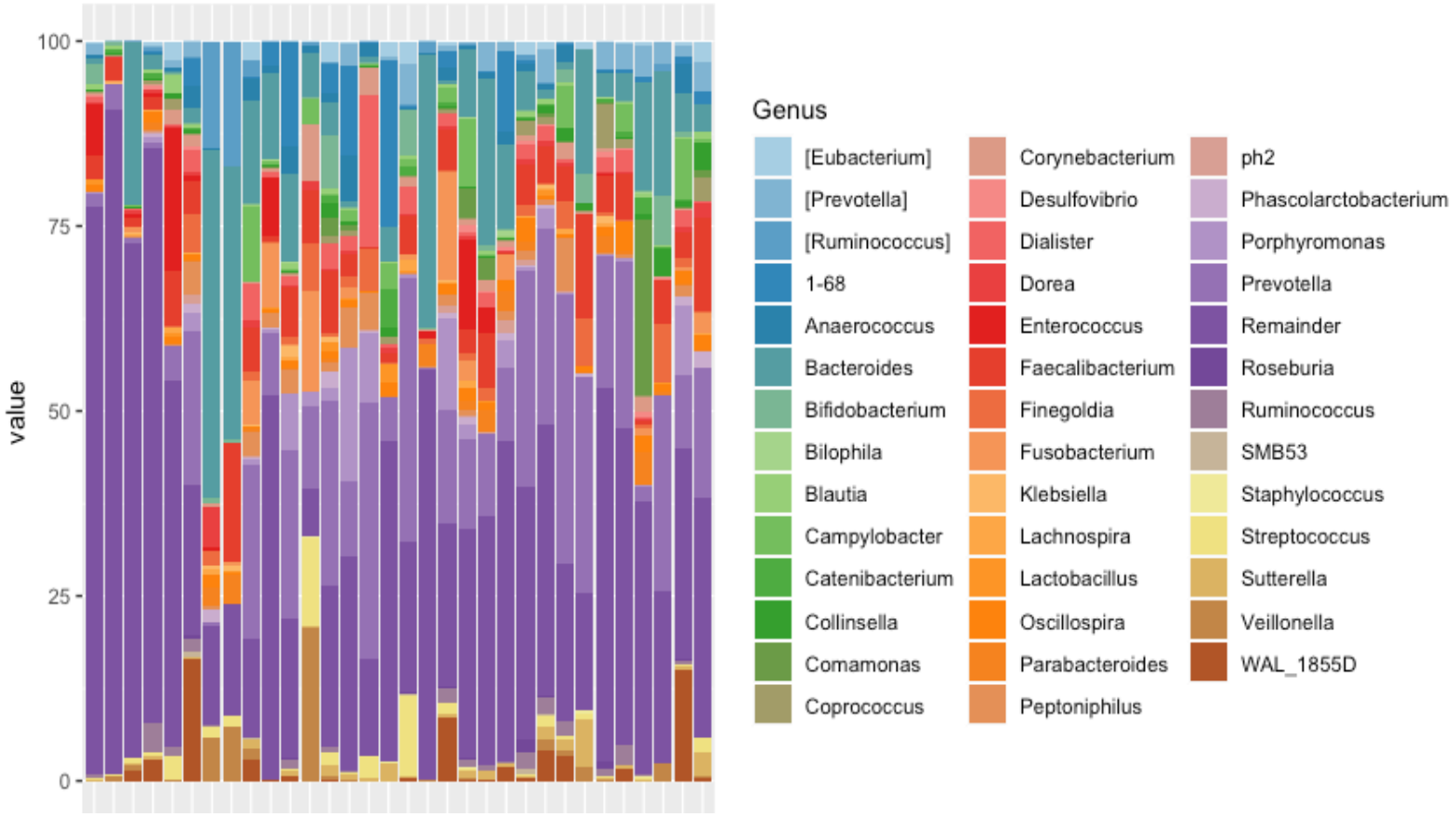


Figure 3. Relative abundance of the gut microbiome at phylum level stratified by the level of serum ferritin

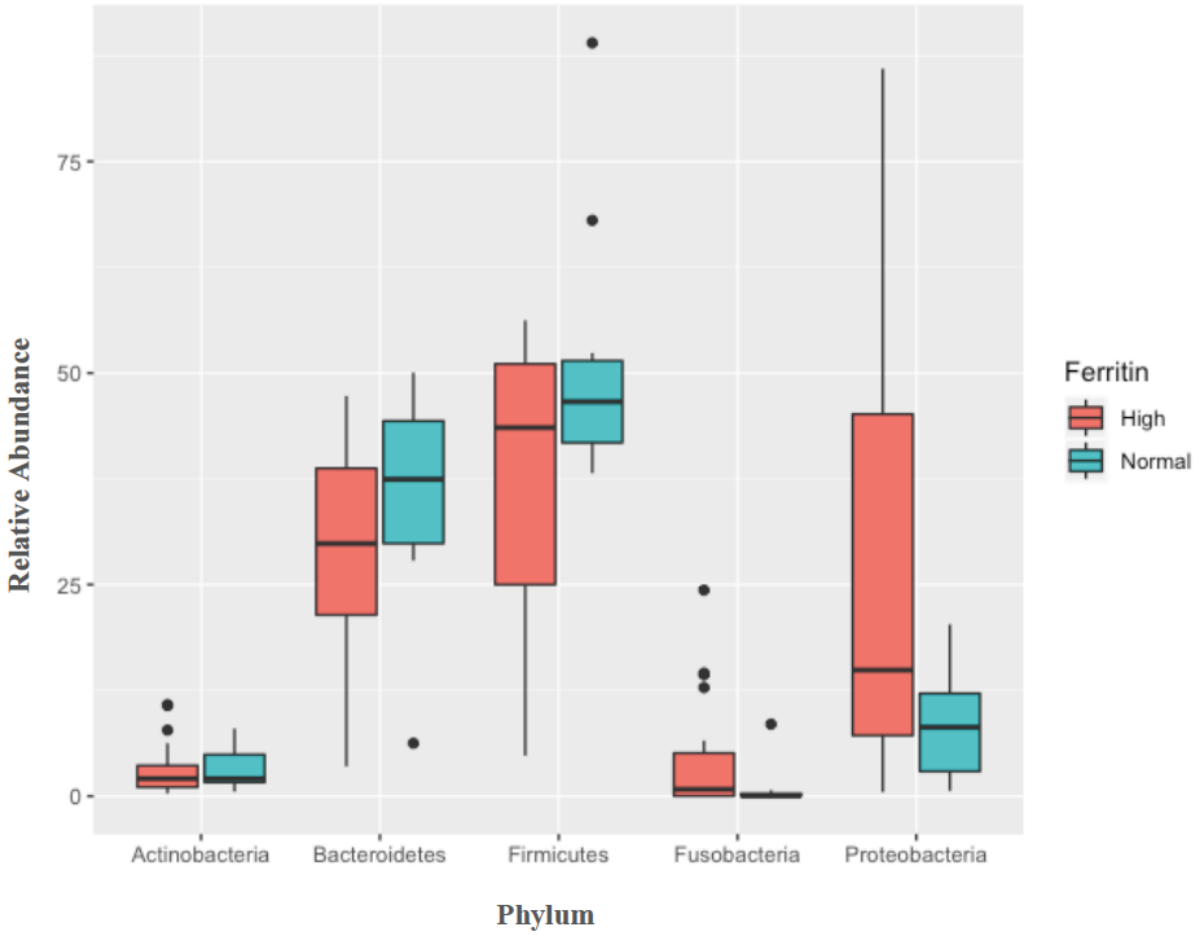


Figure 4. Relative abundance of the gut microbiome at phylum level stratified by tertiles of serum ferritin

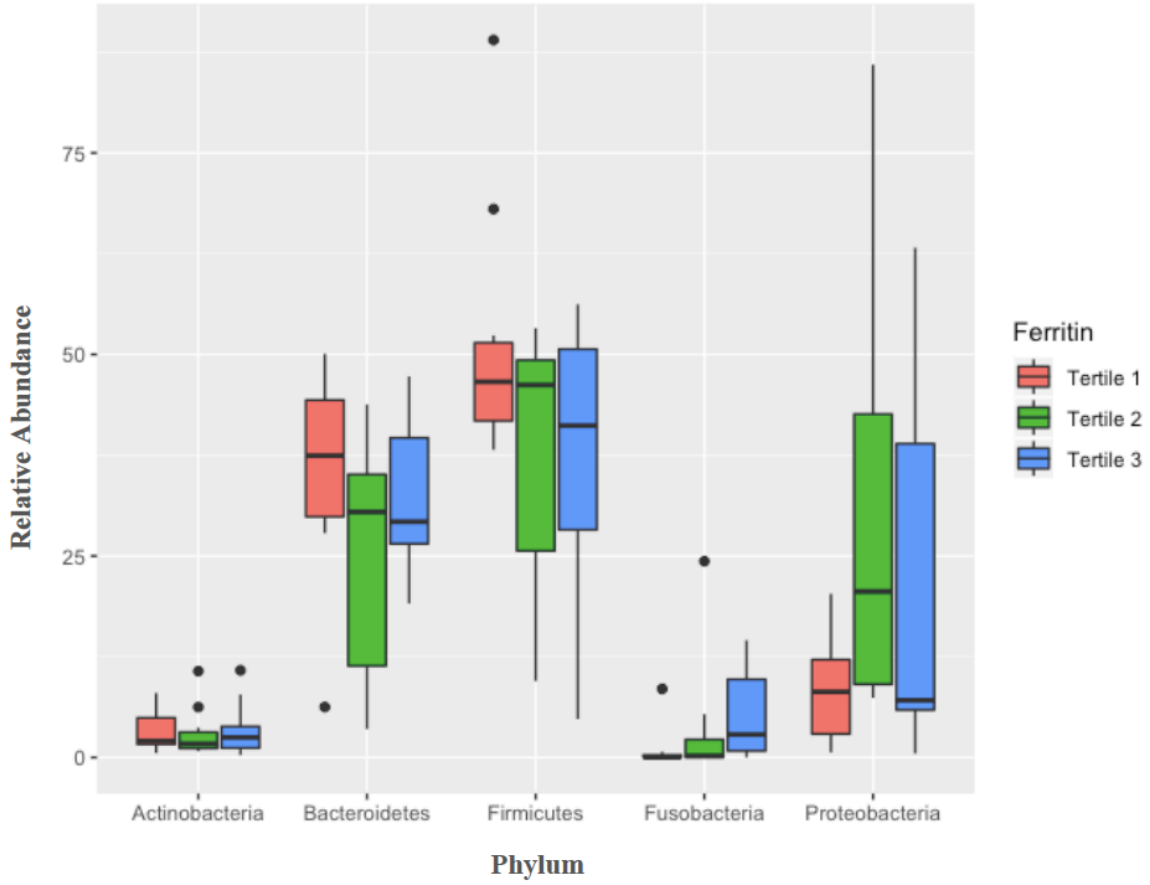


Figure 5. Relative abundance of the gut microbiome at genus level stratified by the level of serum ferritin

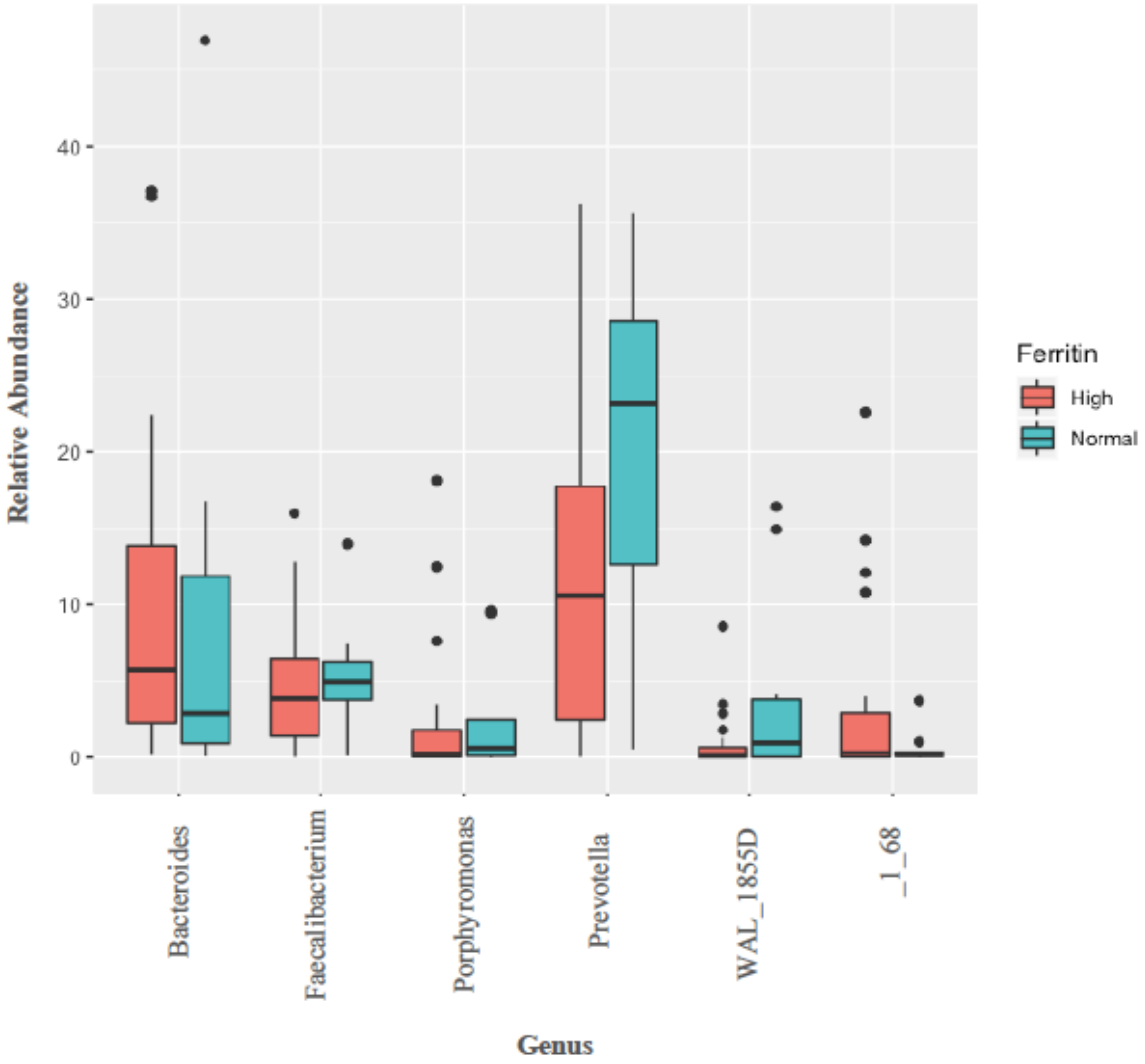


Figure 6. Relative abundance of the gut microbiome at genus level stratified by the tertiles of serum ferritin

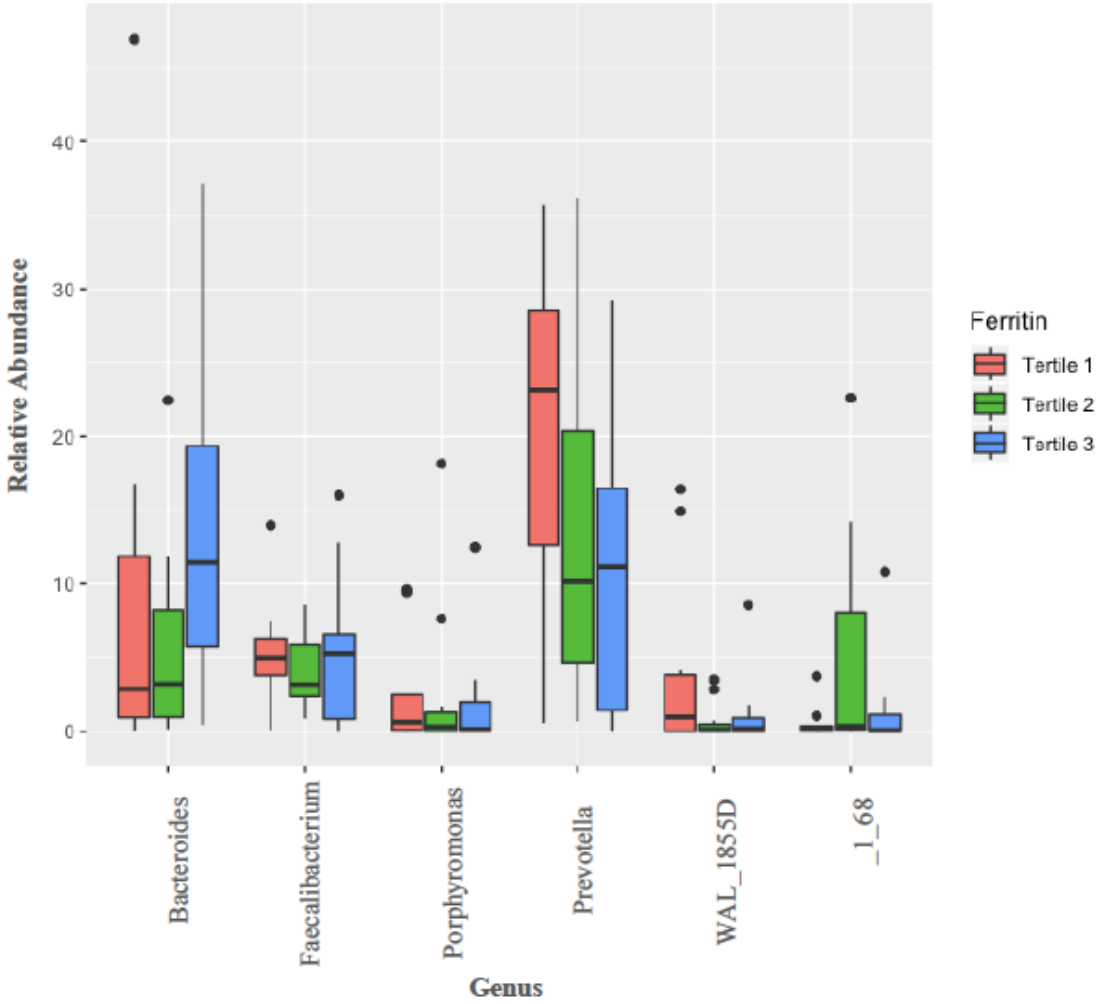


Figure 7. Alpha diversity of the gut microbiome measured by Chao1 index and Observed species stratified by the level of serum ferritin

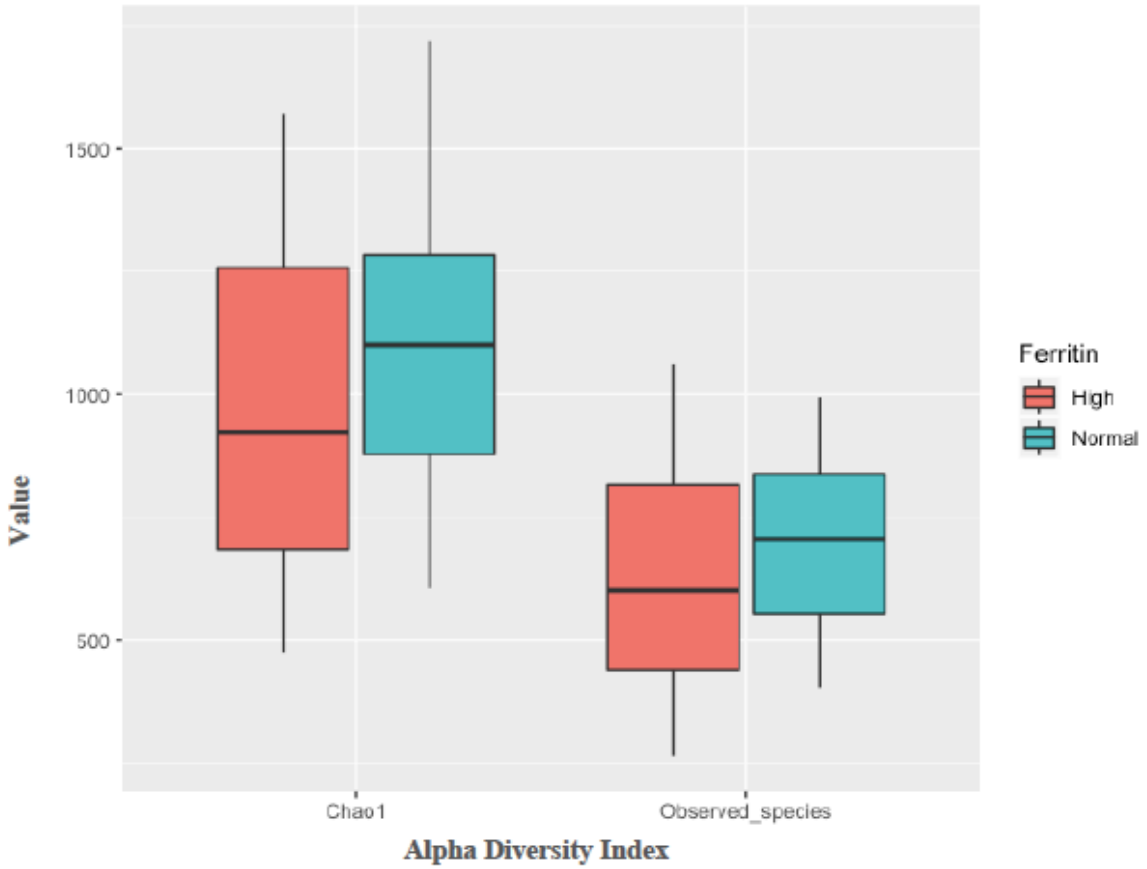


Figure 8. Alpha diversity of the gut microbiome measured by Shannon and Simpson index stratified by the level of serum ferritin

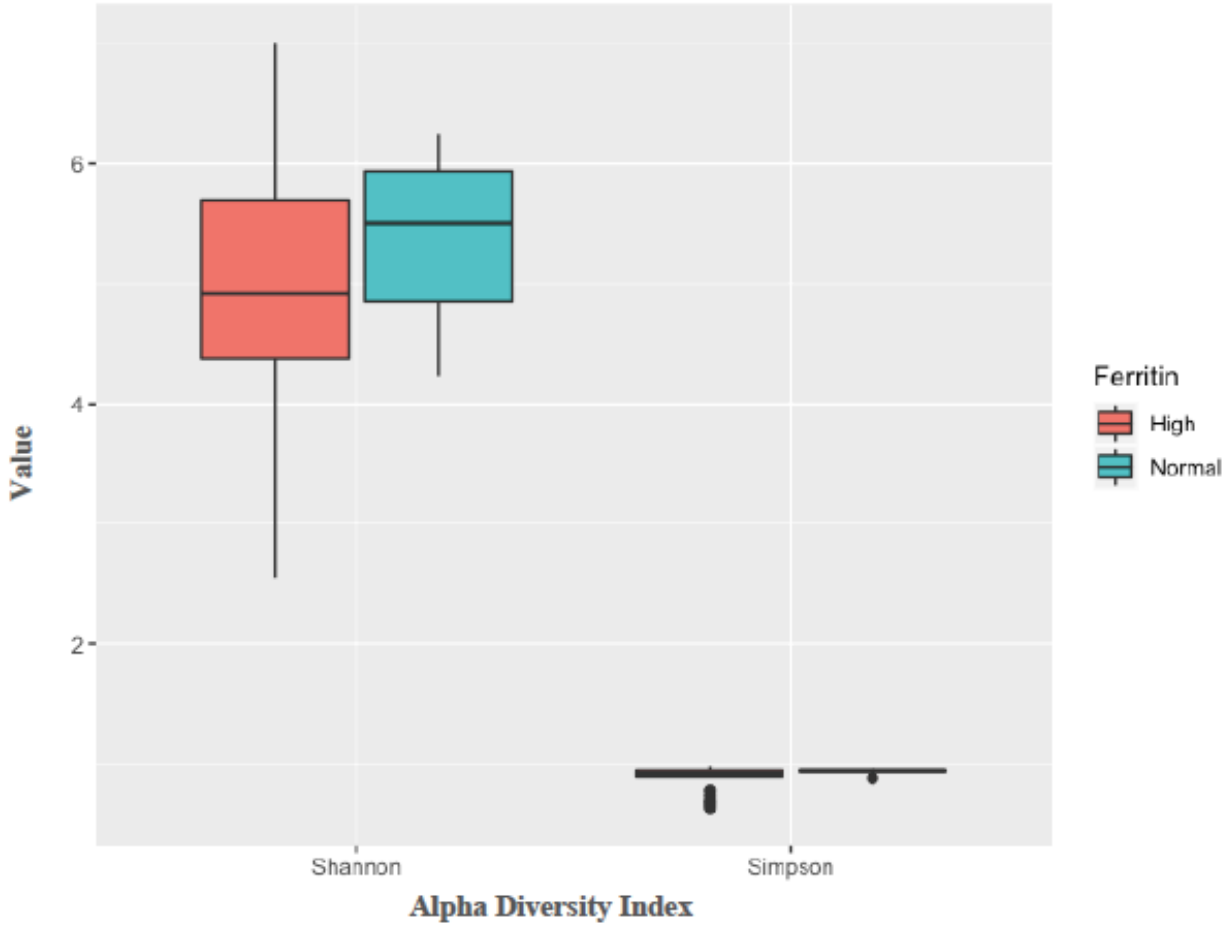


Figure 9. Alpha diversity of the gut microbiome measured by Chao1 index and Observed species stratified by tertiles of serum ferritin

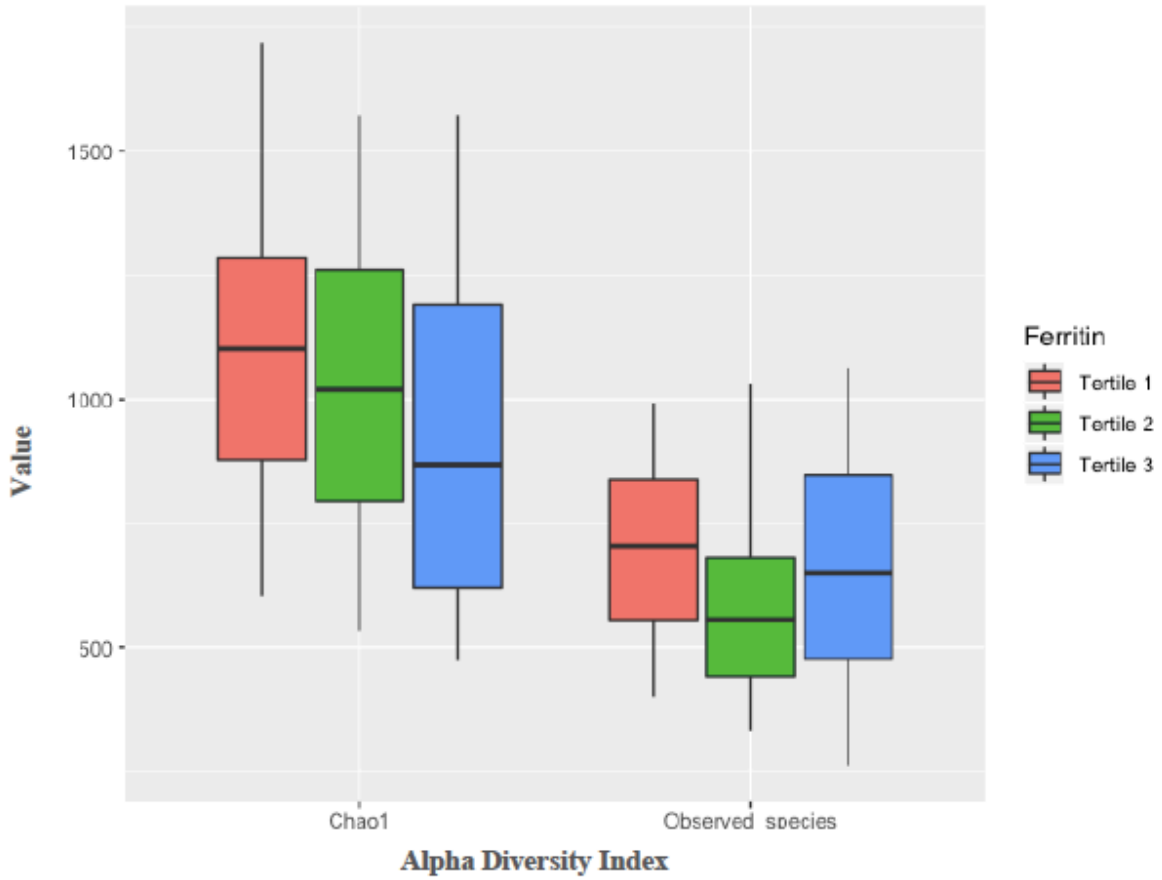
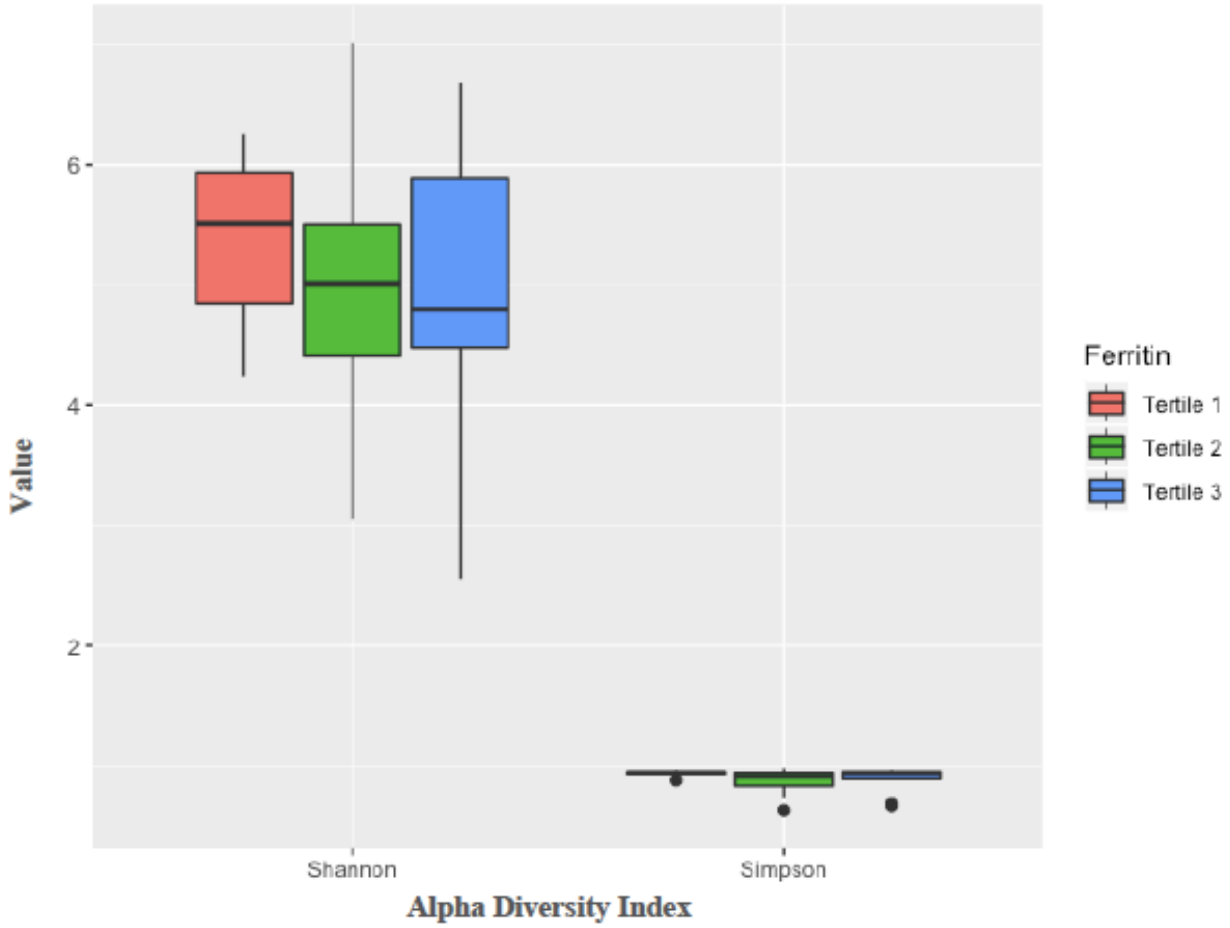


Figure 10. Alpha diversity of the gut microbiome measured by Shannon and Simpson index stratified by tertile of serum ferritin



Discussion

Gut microbiome composition compared to the healthy population

The results showed four predominant phyla in the gut microbiome of new ATBD patients, including Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria consistent with the previous studies conducted in healthy populations (86-89). At the genus level, the gut microbiome of our participants had a high representation of *Prevotella* and *Bacteroides*. These findings are similar to studies conducted among healthy individuals especially in Indian population as OTUs belongs to the genus *Prevotella* are discriminately abundant in non-western population including Indian population which may be attributed to high-carbohydrates dietary intake (88, 90-92).

Among our participants, Bacteroidetes and Firmicutes observed to have lower proportion (78.5%) compared to the other study in healthy individual which observed Bacteroidetes and Firmicutes constitute more than 90% of gut microbiota (93). In a study comparing the gut microbiome in Indian healthy population to the American gut microbiome, the Actinobacteria, Bacteroidetes and Proteobacteria are significantly more abundant in Indian population compared to American population (89). Similar trends also could be seen in another study that observed the gut microbiome of Indian population, which the gut microbiome predominantly comprises by Bacteroidetes (71.5%), Firmicutes (18.7%), Proteobacteria (3.8%) and Actinobacteria (0.6%) which constitute about 95% out of total gut microbiota (91).

The baseline human gut microbiome study also supporting the findings which found the healthy human gut microbiome consists of three predominant phyla. Bacteroidetes (73.13 ± 22.16%), Firmicutes (22.2 ± 18.66%) and Proteobacteria (2.15±10.39%) (94). But the opposite result found

in a study which also involved Indian population, observed that the gut of healthy Indians was dominated by Firmicutes followed by Bacteroidetes, Actinobacteria, and Proteobacteria (95).

Gut microbiome in new active tuberculosis disease

Firmicutes was the most abundant phylum among our participants (46.1%) and followed by Bacteroidetes (32.4%) and Proteobacteria (9.3%). Similar predominant phyla were observed to constitute the gut microbiome of new ATBD patients in previous studies (13, 16). At the genus level, *Prevotella, Bacteroides,* and *Faecalibacterium* were discovered to be the most abundant genera, found to be similar to the result findings of studies conducted in new ATBD patients (13, 16).

Faecalibacterium is known to be one of the most important butyrate-producing bacteria in the human gastrointestinal tract. In our study, *Faecalibacterium* relative abundance was higher in the high level of serum ferritin compared to the normal level of serum ferritin. Butyrate has anti-inflammatory activity by suppressing the production of pro-inflammatory mediators like TNF-α, IL-12, IL-6, and NO by the macrophages as well as monocytes and affecting their capacity to capture antigens and inducing T-cells. Butyrate additionally discovered to decrease nuclear factor-κB (NF-kB) activity (71, 74).

The high abundance of *Prevotella* also associated with the initiation of cytokines production and leading to systemic inflammation (78, 79). The high abundance of *Prevotella* induces production of several pro-inflammatory cytokines such as IL-1β, IL-6, and IL-23 by dendritic cells which can mediate mucosal Th-17 immune response and neutrophil recruitment. Mucosal inflammation

modulated by *Prevotella* leads to systemic dissemination of inflammatory mediators, bacteria and bacterial products and affect systemic disease outcome (78, 79, 96).

In our study, lower relative abundance of *Prevotella* observed in the high level of serum ferritin (tertile 3) compared to normal serum ferritin. Although in our study the differences of the gut microbiome stratified by the level of serum ferritin were not statistically significant, from these findings, we might hypothesize that TB modulated the immune response that associated with alteration in the gut microbiome. Changes in the gut microbiome related to reduced inflammatory mediators which affect the ability of immune response in the elimination of *M. tuberculosis.*

Serum Ferritin in new active tuberculosis disease

In this study population, about 68.75% of participants had high serum ferritin (>250 μg/mL) which may be initiated by cytokines produced by monocytes and macrophages (IL-1, IL-6, and TNF-α) as part of the inflammatory reaction due to *M. tuberculosis* (40). Increases of serum ferritin were also reported in previous studies among new ATBD patients (34, 39, 97). The rise in serum ferritin concentration paralleled with the rise of CRP concentration during TB infection. Serum ferritin could increase about 130% within 48 hours supporting the evidence of ferritin role as an APP. All participants in our study population had high CRP (>0.5 mg/dl) as expected due to TB infection. The rise of serum ferritin and CRP as acute phase proteins support the association between TB and inflammatory responses (39).

As *M. tuberculosis* infection cause rises of serum ferritin, it also increased hepcidin levels which inhibit iron recycling from macrophages, causing macrophage iron retention, systemic iron deficiency and anemia (37). It is, therefore reasonable to assume that, while high plasma hepcidin

concentrations act to sequester iron from extracellular organisms, the increased availability of iron within phagocytes may promote the survival of *M. tuberculosis* (36).

Gut microbiome and serum ferritin

In our study, we found that serum ferritin was associated with the abundance of Firmicutes phylum and at the genus level, *Prevotella,* and *Bacteroides*. Serum ferritin was found to be negatively associated with Firmicutes, and according to our model, together with other covariates including age and CRP, for every increase of 1 µg/mL in serum ferritin, we saw a reduction of 0.3% in the relative abundance of Firmicutes (p=0.02). Firmicutes is one of the most abundant phyla in the gut microbiome, and the majority of the microbes belonged to this phylum are *Clostridium cluster IV* and *Clostridium cluster XIVa*. The findings consistent to the previous studies which observed the association between low iron condition with the decrease abundance of short-chain fatty acids (SCFAs) producing bacteria such as *Roseburia* members, *[Eubacterium] exhale*, and *Clostridium cluster IV* members (55-59).

At the genus level, *Prevotella* was observed to be negatively associated with serum ferritin. Serum ferritin, age, sex, and CRP were found to be associated with *Prevotella*. Every increase of 1 µg/mL serum ferritin, we saw a reduction of 0.9% of *Prevotella* relative abundances (p=0.02). On the contrary, *Bacteroides* showed to be positively associated with serum ferritin (p=0.01). Every increase of 1 µg/mL serum ferritin, we saw an increase of 0.9% of *Bacteroides* relative abundance.

Prevotella and *Bacteroides* are the most abundant genera in the gut microbiome belonged to Bacteroidetes phylum. *Prevotella* and *Bacteroides* suggested competing for the same niche as participants with the high abundance of *Prevotella* usually have a low abundance of *Bacteroides*

(92, 98). The association of *Prevotella* and *Bacteroides* in the gut microbiome with the serum ferritin may not be clearly understood, but some studies found the evidence of these genera association with iron status and also iron availability in the colon. In a study involving infants, the relative abundance of *Prevotella* found higher in non-anemic infants compared to anemic infants (12).

On the contrary, *Bacteroides* positively associated with serum ferritin in our study. This result consistent with the other study result. In a study involving infants given bottle-fed supplemented iron observed to have significantly higher *Bacteroides* compared to infants given bottle-fed without supplemented iron (9). An in-vitro study also found a similar result that showed an association of low abundance of *Bacteroides* genus in a very deficient Fe condition (55).

A few bacteria including *Haemophilus, Bacteroides, Porphyromonas, Prevotella,* and *Bartonella* cannot synthesize heme and have the essential requirement of a high amount of iron and its precursors for growth (99). Although the characteristic of these genera in using heme as iron resources might explain our result findings but the association between iron especially serum ferritin and *Prevotella* and *Bacteroides* in the gut microbiome is still not clearly understood. *Prevotella intermedia,* pathogenic bacteria in the oral microbiome found to be able to utilize hemoglobin as a source of iron (100, 101). Meanwhile, *Bacteroides* observed to required heme and non-heme as their iron resource (102, 103). There was no sufficient information about how *Bacteroides* acquired iron in the lumen colon, but as iron plays a pivotal role in both host metabolism and also microbiome, the availability of iron and the competition to acquire iron could affect the composition of the gut microbiome (12, 57, 104).

Strength and limitations

Our research is the first study to observe the association of serum ferritin and the gut microbiome in new ATBD. Knowing the emerging role of the gut microbiome and iron to the host immune response may contribute to a better understanding of TB disease process, treatment and the severity of the outcome. There is still a limited number of studies investigating the gut microbiome in TB disease especially in a vulnerable population with the high burden of TB in the world, including India.

Although we observed the association between serum ferritin and some microbes at phylum and genus level, the small sample size, cross-sectional study design and no comparison to the healthy control lead to an inability to evaluate the causal relationship between serum ferritin and the gut microbiome. Further study with multiple time points is required to assess the association between serum ferritin with the gut microbiome which will help to reveal the link of ferritin as an acute phase protein, the gut microbiome, and TB. In this context using 16s rRNA sequencing to evaluate the composition and diversity of the gut microbiome is appropriate.

Conclusion

The changes of the composition of the gut microbiome such as relative abundance of Firmicutes, *Prevotella,* and *Bacteroides* genera could have related to serum ferritin as a part of the immune response against *M. tuberculosis* (17). As *M. tuberculosis* infection cause rises of serum ferritin and hepcidin levels which inhibit iron recycling from macrophages, causing macrophage iron retention, systemic iron deficiency and anemia (37). It is, therefore reasonable to assume that, while high plasma hepcidin concentrations act to sequester iron from extracellular organisms, the increased availability of iron within phagocytes may promote the survival of *M. tuberculosis* (36).

The findings from our study might as well related to the characteristic of *Prevotella* and *Bacteroides* that unable to synthesize heme and have the essential requirement of a high amount of iron and its precursors for growth (35). As an increase of hepcidin during infection reduce iron flowed into extracellular and will restrict iron availability to these bacteria (99).

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