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Research Paper

Anaerobe-enriched gut microbiota predicts pro-inflammatory responses in pulmonary tuberculosis

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ABSTRACT

Background: The relationship between tuberculosis (TB), one of the leading infectious causes of death worldwide, and the microbiome, which is critical for health, is poorly understood.
Methods: To identify potential microbiome-host interactions, profiling of the oral, sputum and stool microbiota [n = 58 cases, n = 47 culture-negative symptomatic controls (SCs)] and whole blood transcriptome were done in pre-treatment presumptive pulmonary TB patients. This was a cross-sectional study. Microbiota were also characterised in close contacts of cases (CCCs, n = 73) and close contacts of SCs (CCSCs, n = 82) without active TB.
Findings: Cases and SCs each had similar α- and β-diversities in oral washes and sputum, however, β-diversity differed in stool (PERMANOVA p = 0•035). Cases were enriched with anaerobes in oral washes, sputum (*Paludibacter, Lautropia* in both) and stool (*Erysipelotrichaceae, Blautia, Anaerostipes*) and their stools enriched in microbial genes annotated as amino acid and carbohydrate metabolic pathways. In pairwise comparisons with their CCCs, cases had *Megasphaera*-enriched oral and sputum microbiota and *Bifidobacterium*-, *Roseburia*-, and *Dorea*-depleted stools. Compared to their CCSCs, SCs had reduced α-diversities and many differential taxa per specimen type. Cases differed transcriptionally from SCs in peripheral blood (PERMANOVA p = 0•001). A co-occurrence network analysis showed stool taxa, *Erysipelotrichaceae* and *Blautia,* to negatively co-correlate with enriched "death receptor" and "EIF2 signalling" pathways whereas *Anaerostipes* positively correlated with enriched "interferon signalling", "Nur77 signalling" and "inflammasome" pathways; all of which are host pathways associated with disease severity. In contrast, none of the taxa enriched in SCs correlated with host pathways.
Interpretation: TB-specific microbial relationships were identified in oral washes, induced sputum, and stool from cases before the confounding effects of antibiotics. Specific anaerobes in cases' stool predict upregulation of pro-inflammatory immunological pathways, supporting the gut microbiota's role in TB.
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1. Introduction

Tuberculosis is one of the world's leading causes of death, with 1.4 million fatalities reported in 2019 alone [1]. Understanding the determinants of tuberculosis (TB) pathogenesis is a global research priority

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Research in context

Evidence before this study

The microbiome is crucial for human health and a growing body of research suggests it may have a role in TB. We did a PubMed search to identify studies published before November 2020, using the terms “microbiome”, “microbiota” and “tuberculosis”. Briefly, these studies documented few microbiome differences according to TB status in oral and upper airway specimens such as sputum, whereas the gut microbiome of active tuberculosis cases was reported as perturbed compared to healthy controls. We noted significant limitations in the literature, including how patients often had different treatment statuses, few comparisons of different specimen types were in the same patients, a paucity of data on the gut, comparisons were often between TB cases and healthy controls rather than symptomatic patients without TB, and no published studies linked the microbiome to the host immune system in TB, which is crucial to uncovering putative mechanistic linkages.

Added value of this study

To address these knowledge gaps, we concomitantly analysed the oral, sputum, and gut microbiota and peripheral blood transcriptome in pre-treatment cases compared to symptomatic controls, as well as contacts. Our findings indicate that cases are specifically enriched in anaerobes hitherto unidentified in this context in oral washes and induced sputum (*Paludibacter, Lautropia*), and have a distinct stool microbiome characterised by enriched anaerobes (*Anaerostipes, Blautia, Erysipelotrichaceae*) and precursor inferred pathways (amino acid and carbohydrate metabolic pathways) of short chain fatty acids, which are well known immune system modulators. Furthermore, these enriched gut anaerobes significantly predicted the upregulation of pro-inflammatory pathways associated with disease severity, and these same gut microbiome-transcriptome network interactions were absent in SCs; suggesting they are unique to TB.

Implications of all the available evidence

Collective evidence suggests specific gut anaerobes may modulate the host likely via short chain fatty acids, however, more mechanistic studies are needed. This may represent a new non-*Mycobacterium tuberculosis*-specific microbial target for both therapeutic and diagnostic interventions.

that is required to identify novel vaccination and therapeutic strategies [1]. The human microbiome is a key modulator of host immune responses and has wide-ranging effects on health [2]. The microbiota may be causatively associated with disease. For example, microbial airway commensals protect mice against influenza-mediated death [3] and, in HIV-positive pneumonia patients, pulmonary enrichment with *Prevotellaceae* independently predicts mortality. The microbiota may also influence immunity at distal sites, such as the lungs [4]. For instance, early-life reduction in *Faecalibacterium, Lachnospira, Rothia*, and *Veillonella* in the gut is associated with an increased risk for asthma in humans [5].

Importantly, data supporting a role of the microbiome in TB disease are emerging. Aerosol challenge with *Mycobacterium tuberculosis* modifies murine gut microbial communities six days post-exposure and macaques have increased microbial diversity and more oral taxa in the lung one-month after infection with *M. tuberculosis* [6,7]. Distinct sputum and gut microbial communities are found in cases compared to healthy controls, [8,9] however, most studies do not include TB patients before confounding effects of antibiotics [10–12].

Key knowledge gaps remain that preclude interventions that modify the microbiome to limit TB infection, progression, disease, and/or relapse [8]. First, prior work primarily compared cases to healthy rather than symptomatic controls (SCs; defined as patients with symptoms requiring investigation for TB who later had TB ruled out), which is not representative of clinical scenarios as healthy controls do not routinely present to clinics. Second, the extent to which the composition of microbial communities in TB cases are therefore specific to TB, rather than poor lung health or respiratory infections in general, has not been evaluated. Third, aside from one study, [13] there is no comprehensive analysis of the microbiota across different anatomical sites within the same patients, including respiratory secretions (which contain microbes from the site-of-disease) and the gut (the body's most microbially-rich compartment). Critically, any such evaluations involving TB cases and SCs can identify potential mechanisms by which taxa exert immunomodulatory effects on the host.

Blood transcriptional profiling is one approach to evaluating host immune responses and is an innovative method to derive testable hypotheses by which the microbiome and host influence each other and potentially TB clinical outcomes. Such hypotheses could mirror what is known for other lung diseases. For example, *Veillonella* and *Megasphaera* spp. enriched in the lower airways of lung cancer patients are associated with upregulated host inflammatory pathways (“extracellular signal–regulated kinase”, “phosphoinositide 3-kinase”) [14]. In healthy HIV-infected individuals who later progress to active TB, [15] enriched pulmonary anaerobes (*Prevotella, Veillonella, Haemophilus*) inversely correlate with CD4+ lymphocytes counts, and in active TB patients, interferon (IFN)-inducible (type I and II), myeloid and inflammatory genes are consistently upregulated [16].

We therefore sought to comprehensively characterise the microbiome and host immune system in TB patients from our high burden setting. We evaluated oral, sputum and gut microbiota in TB cases, comparing them to symptomatic controls, and close contacts. We targeted cases before the confounding effects of TB treatment and specifically analysed the relationship between the microbiome and host to elucidate potential mechanisms of interaction to inform the development of interventions.

2. Methods

2.1. Ethics

The study was approved by the Stellenbosch University Human Research Ethics Committee (N14/10/136). Participants provided written informed consent.

2.2. Patient recruitment, consent, and data collection

Presumptive TB patients: Patients with signs and symptoms of pulmonary TB disease (defined as presumptive TB patients) were classified as cases or symptomatic controls (SCs) in an observational TB diagnostic study [17]. Briefly, adult presumptive TB patients ($\geq$ 18 years) self-presenting to a primary healthcare facility in Cape Town, South Africa (12 April 2016–24 July 2017) for investigation and not on TB treatment within the last two months were eligible. Demographic and clinical data [including HIV status and TB symptom score (TBscoreII)] [18] were recorded. Cigarette and alcohol consumption were assessed using the Fagerström Test for Nicotine Dependence (FTND) [19] and Alcohol Use Disorders Identification Test (AUDIT) [20] questionnaires, respectively.

Close contacts: Two adult close contacts cases (CCCs) or close contacts of SCs (CCSCs) who had not taken any antibiotics within three months and preferably from the same household were identified. Contacts with TB were excluded.

2.3. Specimen collection and processing

Each participant provided an oral wash (after gargling with 20 ml sterile water for ~10 s). An aliquot of the sterile water used for the oral wash was stored as a background DNA sampling control in the same type of collection container. Similarly, 20 ml sterile saline (Ysterplaat Medical Supplies, Cape Town, South Africa) was passed through the nebulisation equipment (Ultrasonic Hospital Grade WH-802, Hitech Therapy, Johannesburg, South Africa) and stored before sputum induction (Ysterplaat Medical Supplies). Sputum was induced with 5% saline for 7−10 min. Three sputa were collected for microbiome and TB testing. Stool collection was done at home by participants using an EasySampler (ALPCO, Salem, USA) in a receptacle containing DNA stabilization buffer (Stratec Biomedical, Birkenfeld, Germany). Oral washes, induced sputum and background controls were decontaminated with N-acetyl-L-cysteine (NALC) on the day of collection, pelleted (3217 x *g*), and resuspended in 2 mL phosphate buffer (pH 6.8; BD, Johannesburg, South Africa) before biobanking at −80 °C. Stool samples were typically received the day after collection and frozen at −20 °C. Whole blood was collected in Tempus tubes (Applied Biosystems, Foster City, USA) and stored at −20 °C.

2.4. Mycobacterium tuberculosis *complex testing*

Induced sputum from presumptive TB patients was tested using Xpert MTB/RIF (Xpert; version G4) or Xpert MTB/RIF Ultra (Ultra; version 2) per the manufacturer's instructions. Sputa for culture were first decontaminated with Mycoprep (BD) and inoculated into a Mycobacteria Growth Indicator Tube 960 (MGIT960; BD). *M. tuberculosis* complex-confirmation was done with MTBDR*plus* (Hain Lifesciences, Nehren, Germany). Case definitions are described in the Supplement.

2.5. Nucleic acid extractions

Microbial DNA was isolated from oral washes, induced sputum, and background controls (one specimen-control pair selected for every 10 patients) using the Purelink DNA Microbiome Kit (Invitrogen, Carlsbad, USA) and from stool using the PSP Spin Stool DNA Plus Kit (Stratec Biomedical, Birkenfeld, Germany). Human RNA was isolated from blood using the Tempus Spin RNA Isolation kit (Applied Biosystems). Contaminating genomic DNA was removed using the TURBO DNA-free kit (Ambion, Austin, USA), globin mRNA depleted using the GLOBINclear kit (Ambion, Austin, USA), RNA concentrated using the RNeasy MinElute Cleanup kit (QIAGEN, Hilden, Germany) and its quality assessed using a Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA).

2.6. 16S rRNA gene sequencing and analysis

Bacterial 16S rRNA gene sequencing (V4 region, 150 bp read length, paired-end) was performed on the Illumina MiSeq and resulting sequences pre-processed using Quantitative Insights into Microbial Ecology (QIIME 1.9) as previously described [15]. Sequences are available in the Sequence Read Archive (PRJNA664352). Samples with < 1000 total reads were excluded prior to downstream analyses in R (v.3.5.1). α-diversity (measures the count and abundance of different taxa *within* a sample) and β-diversity (compares microbial composition *between* samples) [21] was calculated using *vegan*, [22] and differential abundance testing done with *DESeq2* [23] (v.1.22.2) (Supplementary Methods). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [24] was used for microbial metagenomic inference from stool 16S rRNA sequences and comparisons made with *DESeq2*.

2.7. Statistics

Non-parametric statistical tests (Mann-Whitney, Kruskal-Wallis; alpha 0•05) were used to compare α-diversity and relative abundances, and permutational multivariate analysis of variance (PERMANOVA) was used for β-diversity.

2.8. RNA sequencing and analysis

Our whole blood RNA sequencing was described previously in detail [25]. These data are on Array Express (accession number E-MTAB-8290). Differential gene expression was assessed using *DESeq2*, where q-values < 0•01 were considered statistically significant. Ingenuity pathway analysis (IPA) was used to identify canonical pathways different in cases compared to SCs, with inputted genes having q-values < 0•05.

2.9. Microbiome and transcriptome co-occurrence network analysis

This was done within each patient group and sample type by first constructing a compositionally-robust co-occurrence network between genus-level taxa using our previously-established *SparCC* pipeline [26]. P-values were obtained via bootstrapping using 500 iterations. Edges with a bootstrapped p-value ≤ 0•05 and correlation strength ≥ 0•15 were retained. After constructing the inter-taxa co-occurrence network, correlations between taxa and transcripts were superimposed upon the *SparCC* network and significant correlations (Spearman $p < 0•05$) were added. The combined network was visualized in Cytoscape (v.3.8.0).

2.10. Role of funding source

Funders had no role in study design, data collection, data analyses, interpretation, or writing of the manuscript.

3. Results

3.1. Study population

We recruited 105 presumptive TB patients (58 cases, 47 SCs) and 155 close contacts (73 CCCs, 82 CCSCs, Fig. 1). Presumptive TB patients were more likely to be male, HIV-positive, have a self-reported co-morbidity, and had a previous TB episode compared to their close contacts (Table 1). Compared to SCs, cases were more likely to be anaemic, of black ethnicity, and have more severe symptoms.

3.2. Background DNA sampling controls show minimal cross-contamination of oral washes and sputum

We compared microbial communities in sampling controls to confirm that taxa in low biomass specimens were likely not contaminants. Water and saline controls had lower α-diversity compared to paired oral wash and induced sputa, respectively (Shannon diversity, $p < 0•001$ for both comparisons). Controls did not cluster in principal coordinate analysis (PCoA; weighted UniFrac PERMANOVA $p = 0•001$ for both comparisons) nor unsupervised hierarchical clustering heatmaps (Figs. S1 and S2).

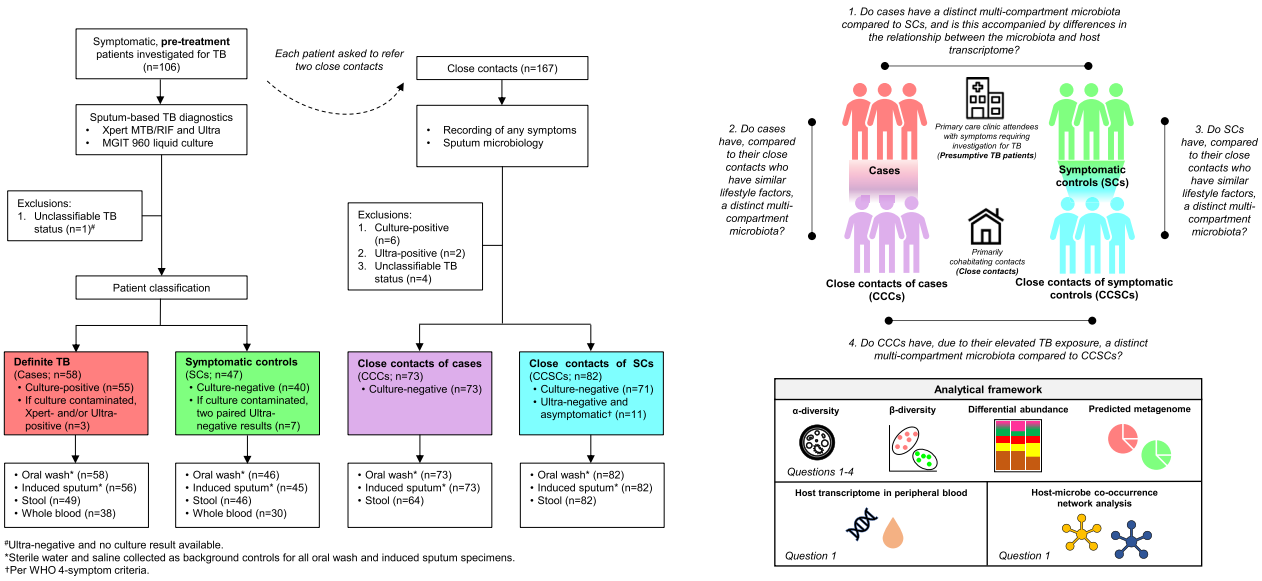

Fig. 1. Study profile. Oral washes, induced sputum and stool were collected from presumptive TB patients (n = 58 cases and 47 SCs) and close contacts of cases (n = 73) or SCs (n = 82) for microbiome analysis. Whole blood was collected from a subset of cases and SCs for transcriptome. SCs: symptomatic controls, CCCs: close contacts of cases, CCSCs: close contacts of symptomatic controls.

Table 1
Demographics and clinical characteristics. Data are median (IQR) or n (%).

Characteristic[¶]	Patients with presumptive TB[*]			Close contacts				
	Cases (n = 58)	SCs (n = 47)	p-value[#]	CCCs (n = 73)	p-value[†]	CCSCs (n = 82)	p-value[‡]	p-value[¥]
Demographic								
Age, years	34 (24–43)	42 (27–50)	0•065	35 (26–48)	0•266	36 (25–48)	0•400	0•996
Men	33 (57)	25 (53)	0•704	17 (23)	< **0•001**	24 (29)	**0•007**	0•399
Ethnicity								
Black	14 (24)	1 (2)	**0•001**	10 (14)	0•125	2 (2)	0•910	**0•009**
Mixed ancestry	44 (76)	46 (98)		63 (86)		80 (98)		
BMI (kg.m^{-2}) category [57]								
Underweight (<18.5)	24 (41)	11 (23)	0•052	9 (12)	< **0•001**	9 (11)	0•061	0•793
Normal (18.5–24.9)	27 (47)	24 (51)	0•646	16 (22)	**0•003**	38 (46)	0•605	**0•001**
Overweight (≥25.0)	7 (12)	12 (25)	0•075	19 (26)	**0•047**	30 (37)	0•197	0•158
Shared living space				59 (81)		69 (84)		0•586
Clinical								
HIV	19 (33)	11 (23)	0•291	6 (8)	< **0•001**	1 (1)	< **0•001**	**0•036**
ARVs	4 (7)	7 (15)	**0•020**	3 (4)	0•481	0	< **0•001**	0•064
CD4 count	282 (137–449)	537 (172–766)	**0.045**	–	–	–	–	–
Previous TB	22 (38)	20 (43)	0•631	9 (12)	< **0•001**	6 (7)	< **0•001**	0•292
Haemoglobin <10 g dL^{-1}	28 (48)	9 (19)	**0•002**	–	–	–	–	–
TBscore II [18]	3 (2–4)	2 (2–3)		0 (0–1)		0 (0–0)		
Mild (0–5)	50 (86)	46 (98)	**0•034**	44 (60)	–	77 (94)	0•303	–
Severe (≥6)	8 (14)	1 (2)		0 (0)		0 (0)		
Self-reported substance abuse								
Fagerström dependence score [19]	5 (0–6)	5 (0–6)		4 (0–6)		5 (0–6)		
Low to moderate (0–4)	23 (40)	17 (36)	0•715	39 (53)	0•117	34 (41)	0•554	0•136
Moderate to high (≥5)	35 (60)	30 (64)		34 (47)		48 (59)		
AUDIT score	0 (0–11)	7 (0–16)		0 (0–10)		0 (0–13)		
Low to excessive (0–15)	47 (81)	35 (74)	0•419	67 (92)	0•069	69 (84)	0•181	0•148
Harmful to dependant (≥16)	11 (19)	12 (26)		6 (8)		13 (16)		
Self-reported narcotic usage	15 (26)	7 (15)	0•170	14 (19)	0•360	14 (17)	0•747	0•734
Self-reported co-morbidities								
Hypertension	2 (3)	5 (11)	0•142	16 (22)	**0•002**	12 (15)	0•519	0•239
Cholesterol	1 (2)	0	0•366	3 (4)	0•431	7 (9)	**0•039**	0•263
Other	1 (2)	3 (6)	0•439	16 (22)	< **0•001**	13 (16)	0•116	0•334

Abbreviations: SCs: symptomatic controls; CCCs: close contacts of cases; CCSCs: close contacts of symptomatic controls.
[*] Symptomatic and undergoing investigation for active pulmonary TB.
[#] Cases vs. SCs.
[†] Cases vs. CCCs.
[‡] SCs vs. CCSCs.
[¥] CCCs vs CCSCs.
[¶] Missing data: BMI (n = 34); HIV (n = 6); haemoglobin (n = 4); TBscoreII (n = 29); narcotics (n = 2).

3.3. Cases have an oral microbiota enriched with anaerobes compared to SCs, and diversity in each group differs compared to their close contacts

Cases vs *SCs*: No α-diversity (p = 0•674) or β-diversity (PERMANOVA p = 0•861) differences were found between cases and SCs in oral washes (Fig. 2a,b). However, cases were enriched with anaerobic bacteria (*Paludibacter, Lautropia, Selenomonas*) and depleted of *Alloscardovia* (Fig. 2c,d).

Cases vs *CCCs*: In paired analyses, α-diversity was similar (p = 0•512; Fig. 2a). β-diversity differed (PERMANOVA p = 0•002; Fig. 2b) and involved the enrichment of *Megasphaera* and *Pasteurellaceae* and depletion of *Methylobacterium* in cases (Fig. 2e).

SCs vs *CCSCs*: SCs had lower α-diversity (Shannon index: 3.7 vs 3.5, p = 0•010; Fig. 2a). Although β-diversity was similar (PERMANOVA p = 0•180; Fig. 2b), differentially abundant taxa were observed: SCs were enriched in *Mannheimia* and *Actinobacillus* but *Methylobacterium* and *Pseudomonas* were depleted (Fig. 2f).

When β-diversity was analysed within households containing a case or SC, the magnitude of β-diversity differences between patient-contact pairs (cases and their CCCs or SCs and their CCSCs) did not differ (PERMANOVA p = 0•935; Fig. 2g). In summary, cases and SCs have few oral taxonomic differences and compared to their contacts, each have a similar magnitude of differences; suggesting that β-diversity differences compared to contacts likely reflect a general illness-induced state of dysbiosis in the oral microbiome and is not a TB-specific phenomenon. Nevertheless, we identify specific oral taxa that differ.

3.4. Like oral washes, cases' sputum microbiota is enriched with anaerobes compared to SCs and overall diversity in each group differs compared to their close contacts

Sputum is readily available from most presumptive TB patients, however, comparative data on the sputum microbiome from SCs are limited, making the specificity of TB-associated microbial communities unclear.

Cases vs *SCs*: Both α-diversity (p = 0•180; Fig. 3a) and β-diversity (PERMANOVA p = 0•412, Fig. 3b) did not differ in sputum. Compared to SCs, cases were enriched in *Alloiococcus* and oral anaerobes *Peptoniphilus, Paludibacter*, and *Lautropia* (latter two also enriched in oral washes) and depleted of *Pseudomonas* and *Leptotrichia* (Fig. 3c,d). These observations suggest that, as in oral washes, cases' sputum microbial communities are enriched with oral anaerobes.

Cases vs *CCCs*: Paired comparisons showed similar α-diversity (p = 0•466, Fig. 3a). β-diversity differed (PERMANOVA p = 0•017; Fig. 3b), with cases enriched in anaerobes *Moraxella* and *Megasphaera*, and depleted of *Methylobacterium* and *Acinetobacter* (Fig. 3e).

SCs vs *CCSCs*: SCs had reduced α-diversity (p = 0•006; Fig. 3a), and borderline altered β-diversity (PERMANOVA p = 0•052, Fig. 3b), with enriched *Moraxella* and *Alloscardovia* (Fig. 3f).

Paired weighted UniFrac distances between cases and CCCs did not differ from those between SCs and CCSCs (PERMANOVA p = 0•841; Fig. 3g). As observed in oral washes, distinct sputum microbiota in sick patients are therefore not TB-specific but likely reflect a general shift away from a healthy state.

3.5. Overlap in oral and sputum microbiota

If the oral microbiota were like the sputum microbiota, study of the respiratory microbiome in presumptive TB patients would be facilitated as sputum is difficult to obtain and typically consumed for diagnostic testing. Pairwise comparisons of oral washes and induced sputum within each patient group (cases, SCs, CCCs, CCSCs) revealed no α-diversity differences (Figure S3a). Likewise, weighted UniFrac distances between paired oral washes and induced sputa in each patient group were similar (PERMANOVA p = 0•851; Figure S3b). These specimen types showed broad similarity in microbial community membership (Figure S3c) with *Veillonella* (14% of total reads in oral washes vs 15% in sputum), *Prevotella* (21% vs 25%) and *Streptococcus* (26% vs 17%) representing the three most abundant taxa in both specimens for each patient group. Moreover, the top two differential genera identified in cases' oral washes (*Paludibacter, Lautropia*) were also in sputum (although not similarly ranked) (Figs. 2d and 3d). Oral washes communities were therefore like sputum.

3.6. Active TB is associated with specific gut microbiota perturbations despite the absence of mycobacterium in stool

It remains unclear whether differences in the gut microbiota reflect generalised illness or are TB-specific [12,27,28].

Cases vs *SCs*: α-diversity in stool was similar in cases and SCs (p = 0•470; Fig. 4a), however, β-diversity differed (PERMANOVA p = 0•035; Fig. 4b). Cases were enriched in the anaerobes *Anaerostipes, Blautia*, and *Erysipelotrichaceae* (Fig. 4c,d) and, unlike in oral and sputum microbiota, this enrichment was extensive enough to result in overall β-diversity differing relative to SCs.

Cases vs *CCCs*: Cases trended towards increased α-diversity (p = 0•063; Fig. 4a) and differences in β-diversity were observed (PERMANOVA p = 0•037; Fig. 4b) and comprised *Porphyromonas* enrichment, and *Megasphaera* and gut commensal depletion (*Bifidobacterium, Roseburia, Dorea*) (Fig. 4e).

SCs vs *CCSCs*: SCs had lower α-diversity (p = 0•006; Fig. 4a) and β-diversity differed (PERMANOVA p = 0•028; Fig. 4b). Furthermore, SCs were enriched with *Treponema* and the methane-producer *Methanobrevibacter* (Fig. 4f). As for other specimen types, paired weighted UniFrac distances between cases and CCCs were not different from those between SCs and CCSCs in stool (PERMANOVA p = 0•193; Fig. 4g).

We next determined whether cases' and SCs' stool microbiota were more dissimilar to each other (within each group) than their corresponding contacts. Based on weighted UniFrac distances, microbiomes of cases were more dissimilar to each other than their CCCs were to each other (PERMANOVA $p < 0•001$, Fig. S4). Similarly, SCs were more dissimilar to each other than CCSCs (PERMANOVA $p < 0•001$). The degree of dissimilarity within cases did not differ to that in SCs (p = 0•419).

3.7. HIV infection does not affect oral, sputum, and gut microbiomes in presumptive TB patients

Reduced diversity and enriched pathobionts have been reported in the gut microbiomes of HIV-infected individuals[29], however, HIV did not impact microbial diversity, composition, or relative abundances in any fluid (Figs. S5−S10) apart from stool comparisons within CCCs (HIV-infected vs. HIV-uninfected PERMANOVA p = 0•025; Fig. S10b).

3.8. Cases' stools are inferred to have enrichment of microbial genes annotated as part of amino acid and carbohydrate metabolic pathways

Functional profiles of the gut microbiota in cases and SCs were inferred using PICRUSt [24]. Stool OTUs were normalised by 16S copy number and metagenomes predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [30]. 34 KEGG pathways differed significantly between cases and SCs. Cases were enriched in metabolism for amino acids (arginine and proline, histidine, lysine), carbohydrates (starch and sucrose, pentose phosphate pathway, fructose, and mannose), and glycerolipids, and depleted of capacities for arachidonic acid metabolism, lipopolysaccharide biosynthesis and cofactors and vitamins metabolism (ubiquinone and other terpenoid-quinone biosynthesis, folate, riboflavin; Fig. 5).

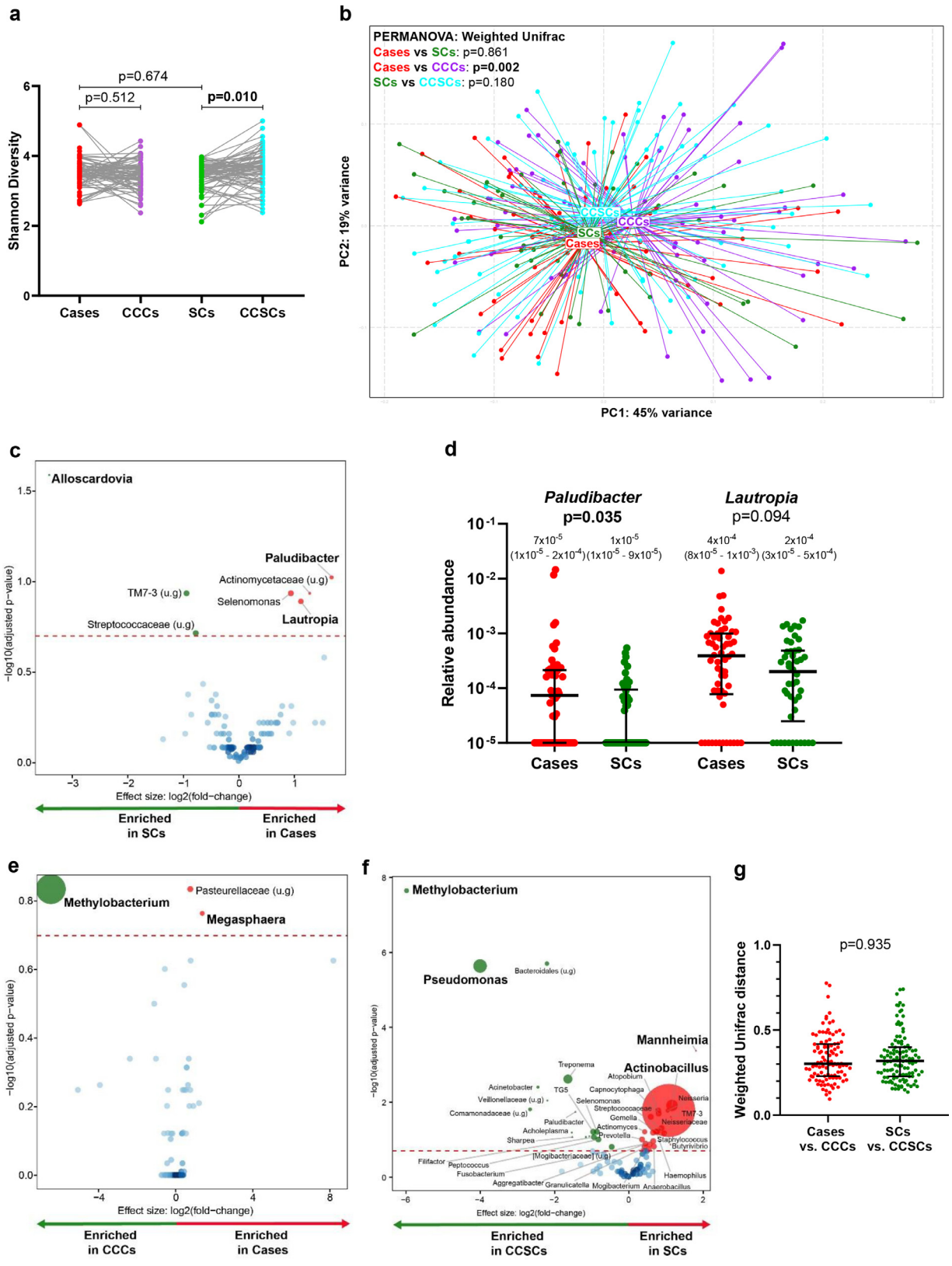

Fig. 2. Oral washes of cases are enriched with the anaerobes *Paludibacter* and *Lautropia*. (**a**) α-diversity based on Shannon diversity. (**b**) Principal coordinate analysis of weighted UniFrac distances between patient groups showing cases (n = 58) and CCCs (n = 73) have distinct oral microbial compositions. **(c)** Volcano plot depicting differentially abundant taxa as determined by *DESeq2* in cases vs SCs (n = 46). Significantly more discriminatory taxa (bolded) appear closer to the left or right and are above the threshold (red dotted line, FDR=0•2). Relative abundance of taxa is indicated by circle size. **(d)** Relative abundance plots with medians and interquartile ranges for most discriminatory taxa in cases i.e., oral anaerobes *Paludibacter* and *Lautropia*. **(e)** Volcano plot depicting differentially abundant taxa (based on paired analysis) in cases vs their CCCs and **(f)** SCs vs their CCSCs (n = 82). Cases are enriched in *Megasphaera* vs. CCCs, whereas multiple taxonomic differences are evident between SCs and their CCSCs. **(g)** Weighted Unifrac distances between groups, where larger distances indicate greater dissimilarity. SCs: symptomatic controls, CCCs: close contacts of cases, CCSCs: close contacts of symptomatic controls. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

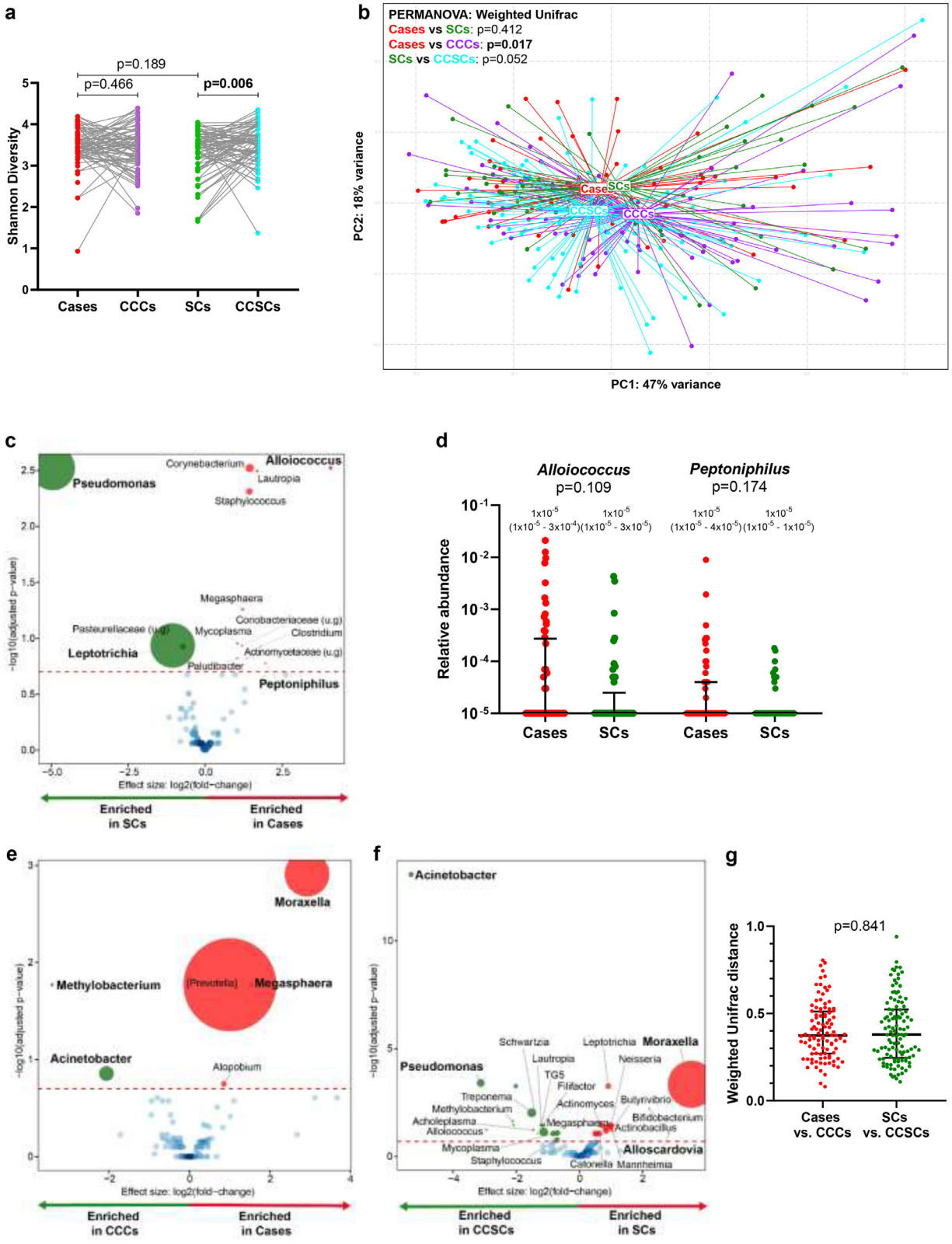

Fig. 3. Sputum of cases are enriched in *Alloiococcus* and *Peptoniphilus* compared to symptomatic controls. (a) α-diversity based on Shannon diversity. **(b)** Principal coordinate analysis showing cases (n = 56) and CCCs (n = 73) have distinct microbial compositions in induced sputum. **(c)** Volcano plot depicting differentially abundant taxa in cases vs SCs (n = 45). Significantly more discriminatory taxa (bolded) appear closer to the left or right, and above the threshold (red dotted line, FDR=0•2). Relative taxa abundance is indicated by circle size. **(d)** Relative abundance plots with medians and interquartile ranges for the most discriminatory taxa in cases (*Alloiococcus, Peptoniphilus*). **(e)** Volcano plot depicting differentially abundant taxa (based on paired analysis) in cases vs. their CCCs and **(f)** SCs vs. their CCSCs (n = 82). *Moraxella* was enriched in both cases (vs. their CCCs) and SCs (vs. their CCSCs). As in oral washes, *Megasphaera* was additionally enriched in cases vs. CCCs. **(g)** Weighted Unifrac distances between groups, where larger distances indicate greater dissimilarity. SCs: symptomatic controls, CCCs: close contacts of cases, CCSCs: close contacts of symptomatic controls. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

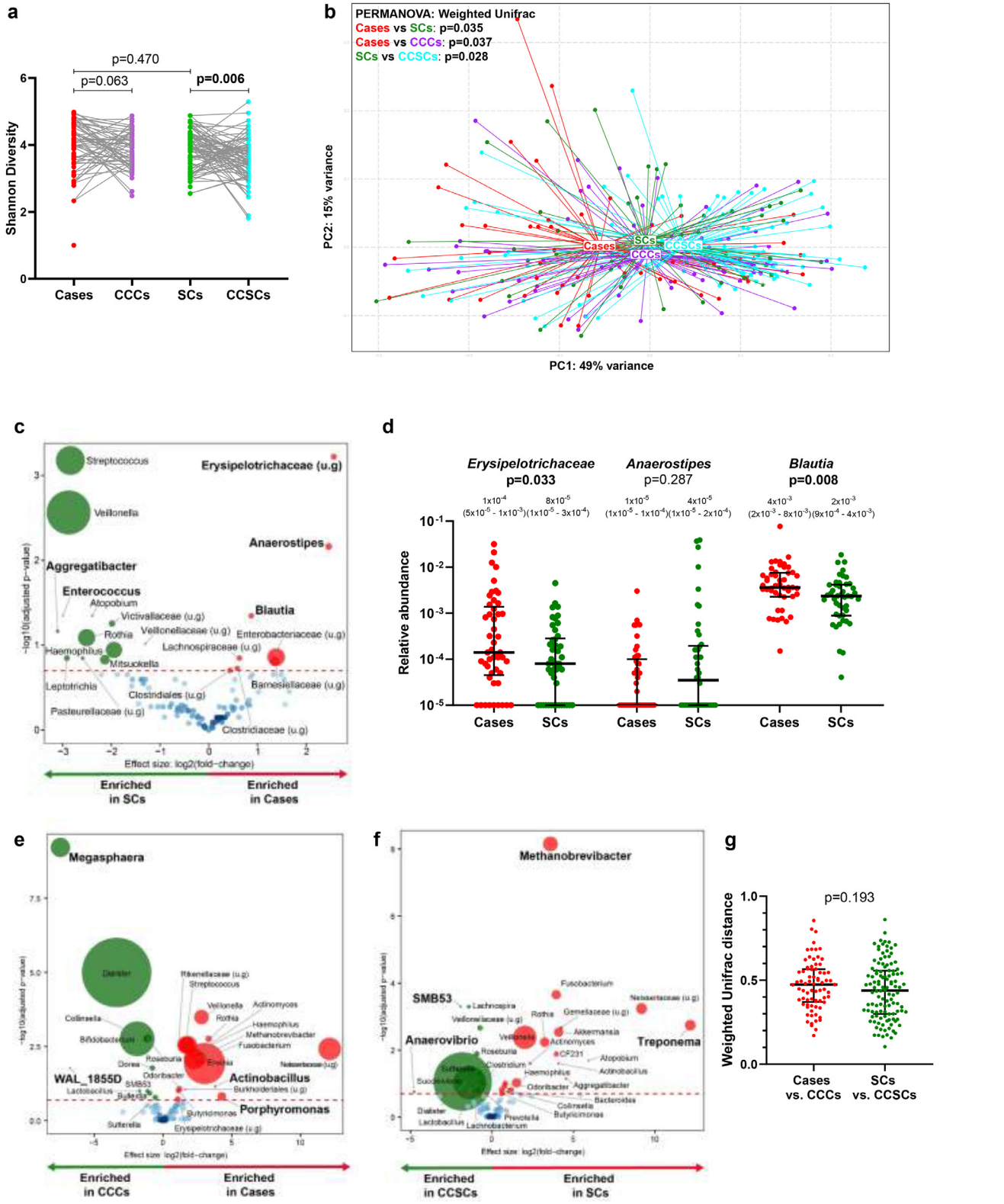

Fig. 4. Active TB is associated with a distinct gut microbiome enriched with *Erysipelotrichaceae, Anaerostipes* and *Blautia*. (**a**) α-diversity based on Shannon diversity. **(b)** Principal coordinate analysis illustrating distinct microbial compositions for cases (n = 49), CCCs (n = 64), SCs (n = 46) and CCSCs (n = 82). **(c)** Volcano plot depicting differentially abundant taxa in cases vs SCs. Significantly more discriminatory taxa (bolded) appear closer to the left or right, and above the threshold (red dotted line, FDR=0•2). Relative abundance of taxa is indicated by circle size. **(d)** Relative abundance plots with medians and interquartile ranges for most discriminatory taxa in cases, including *Erysipelotrichaceae, Anaerostipes*, and *Blautia*. **(e)** Volcano plot depicting differentially abundant taxa (based on paired analysis) in cases vs. their CCCs and **(f)** SCs vs. their CCSCs. Several taxonomic differences were observed in comparisons with cases vs CCCs and SCs vs CCSCs: *Megasphaera*, though enriched in cases (vs. CCCs) in oral washes and induced sputum, were depleted in stool along with many commensals (*Bifidobacterium, Roseburia, Dorea*). **(g)** Weighted Unifrac distances between groups, where larger distances indicate greater dissimilarity. SCs: symptomatic controls, CCCs: close contacts of cases, CCSCs: close contacts of symptomatic controls. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

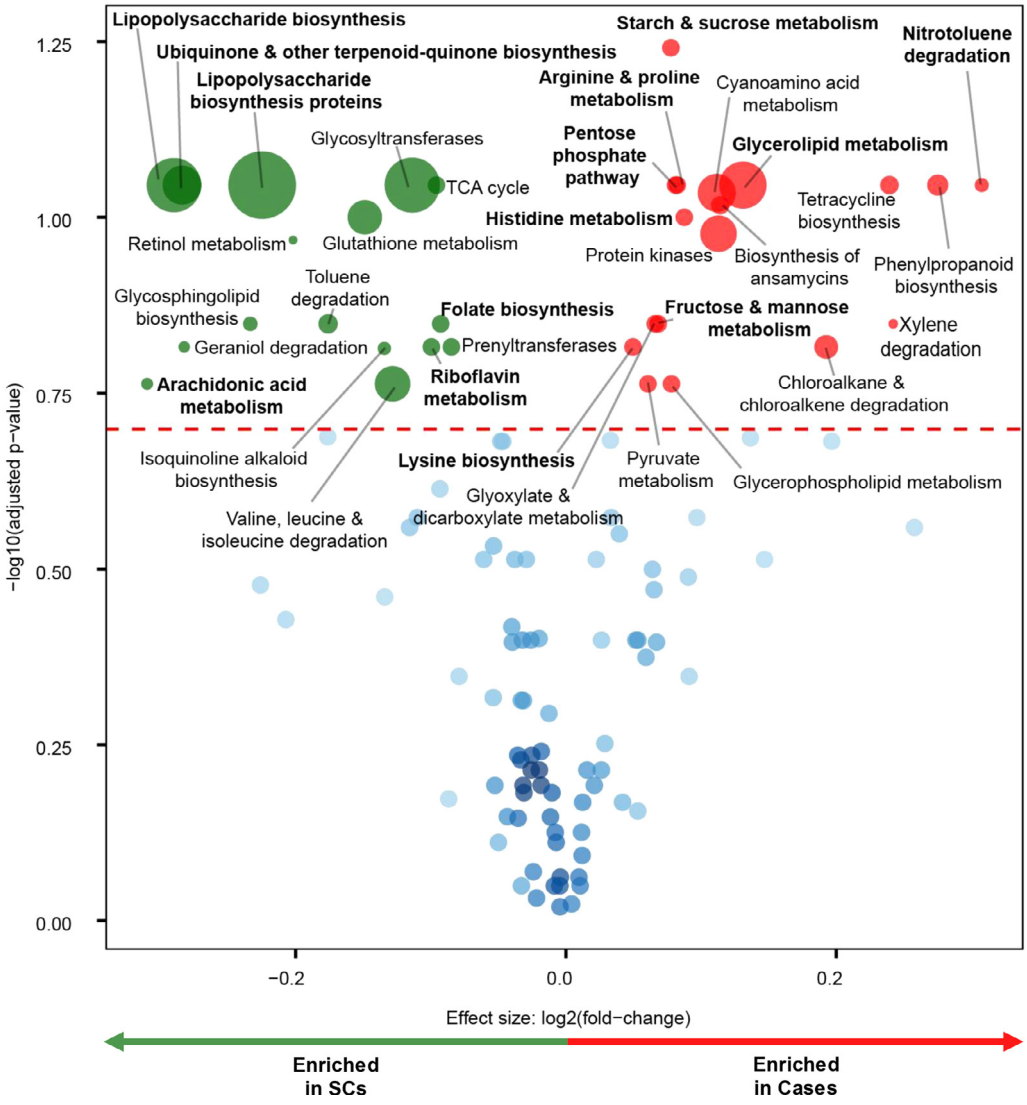

Fig. 5. Cases' stools have increased inferred amino acid and carbohydrate metabolic pathway microbial genes. PICRUSt was used to infer the bacterial metagenome using 16S rRNA sequence data from stool. Thereafter, *DESeq2* analysis was done to compare differentially abundant pathways in cases vs. symptomatic controls (SCs). Significantly more discriminatory pathways appear closer to the left or right, and higher above the threshold (red dotted line, FDR=0•2). Relative gene abundance is indicated by circle size. Key pathways of interest are bolded including precursor pathways for short chain fatty acid production i.e. carbohydrate (starch and sucrose, pentose phosphate pathway, fructose and mannose) and amino acid (arginine and proline, histidine, lysine) metabolism. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

3.9. Multi-omic analysis of host-microbiota interactions reveals specific taxa-pathway relationships

Host transcriptome: Comparison of blood transcriptomic signatures between cases and SCs revealed distinct clustering (PERMANOVA p = 0•001; Fig. 6a), as has been previously reported [25]. Cases had 553 genes upregulated and 648 genes downregulated (Figure S11a). Specific pathways upregulated included Th1, death receptor, and interferon signalling, whereas those downregulated included EIF2, regulation of eIF4 and p70S6K, and mTOR signalling pathways (Figure S11b and Table S1).

Microbiome and transcriptome clustering: A co-occurrence analysis found similar node clustering coefficients between networks of microbiome and transcriptome data in cases and SCs for both oral washes and induced sputa (p = 0•490, p = 0•070) but differences in stool (t-test p < 0•010). In stool, the strength of the correlation between clustering coefficient and degree of clustering was also different between cases and SCs, further demonstrating a distinct network structure (Fisher's r-to-z transformation p = 0•0035; Fig. S12).

Gut microbiome-transcriptome network analysis: None of the taxa we identified as enriched in SCs correlated with transcriptional networks (Fig. 6B). In contrast, gut anaerobes identified as enriched in cases correlated significantly with specific immune pathways in the same individuals: *Erysipelotrichaceae* and *Blautia* co-occur (correlation coefficient of 0•43 for both) and negatively correlate with "death receptor" (−0•45, −0•35) and "EIF2 signalling" (−0•45, −0•35) pathways (Fig. 6c). Furthermore, *Anaerostipes* positivity in cases correlates with "interferon signalling" (0•33), "Nur77 signalling in T lymphocytes" (0•33) and "inflammasome" (0•43) pathways. Overall, these results confirm the presence of a distinct host-microbe phenotype in TB disease and the co-occurrence of specific taxa and host pathways in cases.

4. Discussion

We comprehensively described oral, sputum and gut microbiota in pre-treatment presumptive TB patients and their close contacts, including the host transcriptome in cases. We demonstrate that: (1)

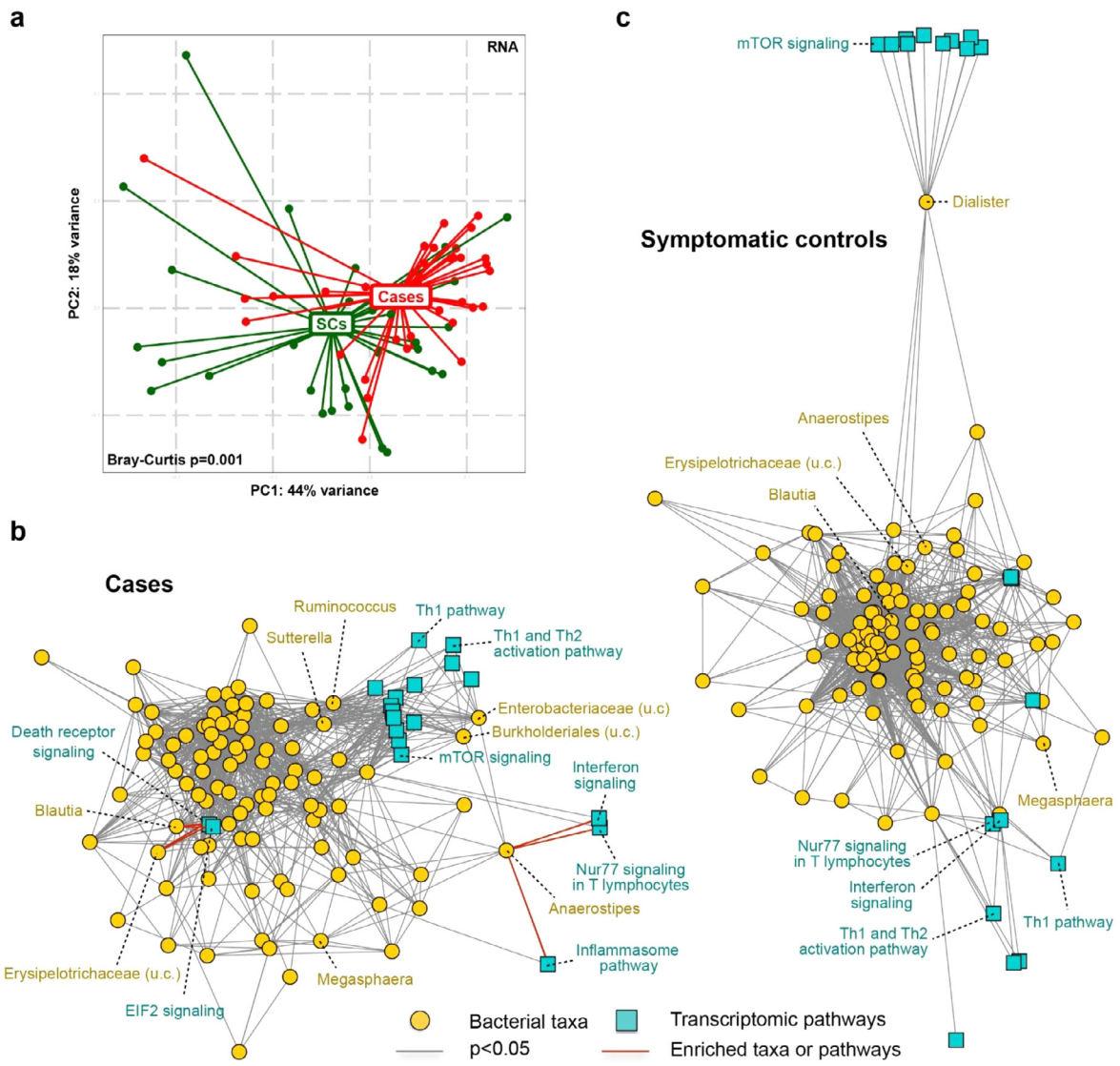

Fig. 6. Unique host transcriptome in cases and co-occurrence network analysis showing certain gut anaerobic taxa that are enriched in cases (red lines, vs. sick controls) correlate with distinct inflammation-modulating pathways: *Blautia* and *Erysipelotrichaceae* co-occur and negatively correlate with "death receptor signalling" and "EIF2 signalling pathways" whereas *Anaerostipes* positively correlates with "interferon signalling", "Nur77 signalling in T lymphocytes" and "inflammasome" pathways. (a) Transcriptomic differences between cases (n = 38) and SCs (n = 30) based on Bray-Curtis dissimilarity index. **(b)** In symptomatic controls, although several taxa were significantly correlated with specific pathways, none of these taxa or pathways were enriched vs. cases. **(c)** In cases, distinct co-occurrence nodes involving "death receptor" and "EIF2 signalling" pathways and *Blautia* and *Erysipelotrichaceae* was observed (left red lines) and "interferon signalling", "Nur77 signalling in *T* lymphocytes" and "inflammasome" pathways and *Anaerostipes* (right red lines). Blue square nodes correspond to transcriptional pathways, while yellow circular nodes correspond to taxa. Edge length is inversely proportional to correlation strength, and the overall structure was obtained using a spring-embedded configuration. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

cases have a distinct gut microbiome compared to SCs and to CCCs, with the anaerobes *Anaerostipes, Blautia,* and *Erysipelotrichaceae* being highly discriminatory and enriched with amino acid and carbohydrate metabolism-related genes; (2) these taxa correlate with specific upregulated pro-inflammatory pathways in cases; (3) the oral wash microbiota in TB approximates that of sputum; and (4) the microbiota of cases and SCs (oral wash or sputum) differ more vs. their respective contacts than to each other suggesting that, unlike what is observed with stool, taxonomic changes at these sites are not TB-specific but rather reflective of poor respiratory health.

Stool taxa differentiating cases from SCs are members of the *Lachnospiraceae* (including *Anaerostipes* and *Blautia*) and *Erysipelotrichaceae* families. *Anaerostipes* has not been described in TB before, however, *Blautia* was previously reported to be enriched in stool versus healthy household contacts [27]. We now show that these taxa are specifically associated with TB disease compared to symptomatic patients without TB, despite *Mycobacterium* not being detectable in stool. These stool compositional differences were not accounted for by taxonomic variation associated with risk factors for TB (i.e. HIV) or factors described elsewhere [31,32].

Certain gut anaerobes ferment substrates (carbohydrates and proteins) thereby producing short chain fatty acids (SCFAs) like butyrate, propionate, and acetate, which influence host immunity [33]. Taxa enriched in cases are butyrate-producers [34–36]. Although we were unable to infer enrichment of microbial genes encoding butyrate metabolism, we did detect an enrichment of genes in cases' stool (vs. SCs) involved in key butyrate precursor pathways (amino acid and carbohydrate metabolism). Butyrate facilitates communication between the microbiota and the immune system [37]. By inhibiting histone deacetylases, butyrate regulates cytokine expression in T cells and macrophages [38–40] and regulatory T cell induction [41]. However, elevated concentrations of butyrate may be detrimental in TB as

it inhibits the proinflammatory responses (TNF-α, IL-1β, IL-17) induced by *Mtb* antigens and subsequent *Mtb* kill [42]. Mechanistic studies are needed to confirm if SCFAs made by the enriched gut anaerobes identified here regulate peripheral immune function and we intend to do such work.

Cases were enriched in many oral anaerobes (*Paludibacter, Lautropia, Alloiococcus, Peptoniphilus*) in oral washes and induced sputum. Previously, in a South African cohort of latently-infected HIV-positive patients, oral anaerobe-enriched lower airways were associated with elevated SCFAs that predicted the later development of active TB [15]. We have now shown such anaerobes have likely proliferated by the time of presentation for active TB disease.

An important strength of this study is the contemporaneous collection of peripheral blood. Critically, the differences between cases and SCs observed independently in the analysis of microbiome and transcriptome data were corroborated by co-occurrence network analysis. For instance, the gut microbiome-transcriptome network differed in cases compared to SCs, as shown by the significantly lower clustering coefficient, which is indicative of a less modular structure and is, in principle, associated with diseased states in general [43]. Further, we observed significant correlations in cases - but not in SCs - between *Anaerostipes, Blautia* and *Erysipelotrichaceae* and defined TB-related host peripheral blood pro-inflammatory responses (interferon signalling, inflammasome), [44,45] cell death signalling, [46] and antibacterial response [47]. This is an important finding in the field of TB and agrees with previous studies which demonstrate immunomodulatory properties for these taxa in other diseases (e.g. *Blautia* and IL-17 [48] and *Erysipelotrichaceae* and IL-1β) [29]. These findings merit further validation and raise the question of whether targeting the gut anaerobes identified here which have inflammatory effects (e.g., using narrow-spectrum antibiotics), would reduce TB pathology.

Another study strength included the pairing of presumptive TB patients and their close contacts. Although a greater resemblance in microbiome signatures exists within households, [49] in our cohort, presumptive TB patients had distinct oral, sputum and gut microbial compositions compared to their close contacts, which is likely as a result of the former group's illness. In cases compared to their CCCs, this difference (in all three compartments) was mainly driven by changes in *Megasphaera* which is considered a biomarker for lung cancer [14,26]. Cases were also depleted of immunomodulatory gut commensals like *Bifidobacterium*, [50] corroborating previous studies [27].

In SCs and their CCSCs, many taxonomic differences were apparent across specimen types (unlike in cases vs CCCs) and likely reflect heterogeneous respiratory illnesses amongst SCs. Furthermore, reduced diversity in comparisons involving SCs vs CCSCs and not in cases vs CCCs (or SCs) suggest that *M. tuberculosis* disease results in fewer microbial perturbations than other bacteria (e.g. *Haemophilus* or *Moraxella*) which predominate during respiratory infections [51]. This agrees with our documentation of *M. tuberculosis* not having high abundance in sputum despite it being the causative pathogen of disease.

Our study has strengths and limitations. Although the lung microbiome is best sampled by bronchoalveolar lavage, [52] this is not feasible in primary healthcare or at large scale. We therefore collected induced sputum, meaning that presumptive TB patients and their contacts who could not expectorate could be included. Mycobacteria could have been underestimated for technical reasons [53] or because sputum contains microbes not just from the pulmonary site of disease (*Mycobacterium* was not a discriminatory taxon in comparisons). Follow up studies using shotgun metagenomics, rather than inferred metagenomics, are warranted and will increase taxonomic and functional resolution of bacteria and other types of microbial populations [54]. Some patients with respiratory symptoms may have received antibiotic treatment (with drugs other than for TB) prior to TB diagnosis and, although this is relatively rare in our setting, we did not collect these data in all presumptive TB patients. An improved understanding of the underlying causes of the spectrum of disease in SCs is especially important to not only the TB field in general [55,56] but especially for microbiome research. Lastly, our objective was to comprehensively characterise the microbiome pre-treatment (before the confounding effects of antibiotics), however, future research on the long-term effects of TB treatment on the microbiome is important, including its link with recurrence.

In summary, TB-specific microbial signatures in oral washes (*Paludibacter, Lautropia*), induced sputum (*Alloiococcus, Peptoniphilus*) and, most notably, the gut (*Anaerostipes, Blautia, Erysipelotrichaceae*) were evident prior to treatment. In the gut, enriched anaerobes correlated with pro-inflammatory host immune pathways known to be associated with TB disease severity. We therefore postulate that the gut microbiota has a role in TB pathogenesis and may regulate lung immunity, however, more research is needed on the types of metabolites produced by these taxa and the mechanisms by which they may influence host immunity. This work therefore lays the foundation for further exploration of the gut microbiome as a potential diagnostic or prognostic marker for TB.

Data sharing

De-identified patient data, the study protocol, informed consent, and datasets generated in this study may be requested from the corresponding author.

Declaration of Competing Interest

GT received in-kind donations (GeneXpert cartridges and machines) from Cepheid and FIND. Cepheid had no role in study design or interpretation of results. BWPR received travel support from Cepheid to attend a conference and present unrelated data. All other authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103374.

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