Mycobacterium tuberculosis, Antimicrobials, Immunity and the Lung-Gut Microbiota Crosstalk: Current Updates and Emerging Advances

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Significance

Mycobacterium tuberculosis remains the major cause of mortality and morbidity from a single infectious agent worldwide, affecting more than 10 million people and killing more than 1.6 million in 2017. Treatment for tuberculosis is relatively long and involves at least four antimicrobials, which have been shown to change the ecology of the gut microbiota. This change in the gut microbiota, further affects the optimal function of the immune system, making it less efficient in fighting the tuberculous bacilli. Moreover, certain microbiota metabolites also prevent an effective function of the immune system, escalating tuberculosis. Hence, it is apparent that an intact gut microbiota, through the immune system and metabolites, protect the host against *M. tuberculosis* infections in the lungs. Subsequently, there is the need to fully understand these interactions to help prevent or treat tuberculosis through the microbiome and immune system. To wit, antimicrobials abuse is a potent risk factor for developing active tuberculosis.

Abstract

Increasingly, gut microbiota distortions are being implicated in the pathogenesis of several infectious and non-infectious diseases. Specifically, in the absence of an eubiotic microbiota, mice are more prone to colonization and infection by *Mycobacterium tuberculosis* (*M.tb*). In this qualitative analysis, the following were observed: (1) antimicrobials cause drastic long-term the gut-microbiota perturbations; (2) *M.tb* cause limited and transient disturbances to the lung-gut microbiota; (3) Pathogens (*H. hepaticus*) affect microbiota integrity and reduce resistance to *M.tb*; (4) Dysbiosis depletes bacterial species regulating proper immune functioning, reducing resistance to *M.tb*; (5) the dysregulated immunity fails to express important pathogen-recognition receptors (macrophage inducible C-type lectin (MINCLE)) and *M.tb*-killing cytokines (IFN-γ, TNF-α, IL-17 etc.), with hampered phagocytic capability; (6) autophagy is central to the immune system's clearance of *M.tb*, control of inflammation and immunity-microbiome balance; (7) microbiota-produced short-chain fatty acids, which are reduced by dysbiosis, affect immune cells and increase *M.tb* proliferation; (8) commensal species (e.g. *Lactobacillus plantarum*) and microbiota metabolites (e.g. indole propionic acid), reduce tuberculosis progression; (9) Faecal transplants mostly restored eubiosis, increased immune resistance to *M.tb*, restricted dissemination of *M.tb* and reduced tuberculosis-associated organ pathologies. Abusing antimicrobials, as shown in mice, is a risk factor for re-activating latent or treated tuberculosis.

Keywords: Autophagy; antimicrobials; metagenomics; microbiome; metatranscriptomics; antimycobacterial; lung-gut microbiota axis.

1. Introduction

The advent of whole-genome sequencing (WGS) and its application in shot-gun metagenomics, are revolutionizing microbiome research [1–3]. Specifically, WGS provides deeper insights into the interconnectivity, composition, relative abundance, diversity, metabolites and biological activities of the microbiome in different luminal surfaces of the human body [1,4–6]. In particular, the lung

microbiome was believed to be sterile until recently when non-culture-based techniques showed the presence of a microbiota in the lung [7]. Furthermore, increasing evidence suggests that the intestinal microbiota directly and/or indirectly modulates the lung microbiota by developing, regulating and inducing the immune system [8,9]. Substantive evidence has been provided to associate gut microbial perturbations with immunity and lung airways' inflammation and inflammatory conditions such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis [8,10]. However, available research findings provide relatively limited evidence on the effect of lung-gut dysbiosis (i.e., changes in the original composition of the microbiome) on *Mycobacterium tuberculosis* (*M. tb*) or vice-versa.

A m ajor cause of dysbiosis in all microbiomes is antimicrobials [1], a cocktail of which remains the main means of treating tuberculosis (TB) [11]. However, first-line Tb antimicrobials such as pyrazinamide and isoniazid are pro-drugs that are only activated inside *M. tb*, raising questions about their dysbiotic effect in the lung-gut microbiota. Furthermore, broad-spectrum antimicrobials such as β-lactams and glycopeptides (vancomycin) that cause very drastic dysbiosis are hardly used in TB patients [12,13], making the importance of antimicrobial (used for treating TB)-induced dysbiosis in TB patients an interesting research question.

In contrast to antimicrobials however, a direct dysbiotic effect of *M. tb* on the lung-gut microbiome axis remains to be firmly established. Furthermore, as *M. tb* can revert to dormancy/latency within the host and later be revived into active disease, some investigators question the effect of latent and recurrent tuberculosis (TB) on the lung-gut microbiome dynamics [4,6,14]. Given the intracellular nature of *M. tb*, it remains to be seen if it can directly cause dysbiosis. It is also not clear if *M. tb* can indirectly cause dysbiosis through immune dysregulation, particularly as a strong association between the microbiota and immune system has been already established [4,5,15–17]. For instance, segmented filamentous bacteria (SFB) in the gut microbiota of mice are implicated in modulating the adaptive immune response in mice to clear infectious agents. Thus, the question remains: can these or related species influence TB pathology in the lungs/airways? In addition, certain diets (prebiotics), such as lipid-rich ones, affect or alter the inflammatory profile i.e., IL-2, -12, and -1β, tumour necrosis factor-alpha (TNFα) etc. through toll-like receptor (TLR) signaling [10,18]; hence,

can such diets exacerbate or control TB? The findings on the microbiota's effect on immunity is increasingly significant, strengthening the hypothesis that the microbiome can hold a key to treating TB. Subsequently, studies of the microbiome dynamics in TB patients cannot be comprehensive without including the innate and adaptive immunity as well as antimicrobials.

How could the microbiome defend against TB through the immunity? The pathogenesis of *M. tb* needs to be briefly recapped to answer this question. On entering the host, *M. tb* is identified by toll-like receptors (TLR) and nucleotide-binding oligomerization domain-containing protein (NOD) signaling. These signals induce macrophages and dendritic cells (DCs) to phagocytose the bacilli, produce cathelicidin (an antimicrobial peptide), and destroy *M. tb* through autophagy [19]. TLR and NOD activate certain autophagy genes (*atg*) to form double-membraned autophagosomes around pathogens. These autophagosomes are then labelled with microtubule-associated protein 1 light chain 3 alpha (LC3) and fused with lysosomes to form single-membraned autolysosomes for degradation [20]. Processes leading to the formations of phagosomes (single-membraned phagophores containing phagocytosed pathogens or unwanted molecules) and LC3-associated phagocytosis (LAP) are fully described elsewhere [20,21]. Within phagosomes of activated macrophages, IFN-γ can mediate the production of nitric oxide (NO) and reactive oxygen species (ROS) to sterilise the pathogens therein [20,21]. IFN-γ also activates autophagy, acidifies phagosomes and traffics autophagosomes to lysosomes for degradation in macrophages [20,21].

By stimulating TLRs to activate DCs and monocytes/macrophages, the microbiota ensures a host-microbiome symbiosis that protects against several pathogenic invasions, including *Salmonella enterica, Helicobacter pylori* and *M. tb* [9,22,23]. Pathogen-activated molecular patterns (PAMPs) that are present on bacterial surfaces, stimulate TLRs and recruit microbicidal macrophages. These PAMPS are ubiquitous on the surfaces of *M. tb* cells. However, *M. tb* expresses another surface lipid called phthiocerol dimycoceroserate (PDIM) that masks the PAMPs to evade microbicidal macrophage recognition. Thus, because commensal bacteria in the upper respiratory tract have PAMPs, *M. tb* cells' survival in this region is relatively lower than in the lower respiratory tract where there are fewer commensals to stimulate TLRs and recruit microbicidal macrophages [22]. The importance of the

microbiome in the host's immune response to *M. tb* can therefore, not be underestimated, and needs thorough investigation.

Thus, important indicator phyla and species such Bacteroidetes (Rickenellaceae, *Bacteroides spp., Prevotella spp.*), Firmicutes (Lachnospiraceae, Lactobacillaceae, Ruminococaceae, *Veillonella spp., Streptococcus spp*.), Proteobacteria (Enterobacteriaceae, *Pseudomonas spp*.), Actinobacteria (Bifidobacteria) and Verrucomicrobia should be traced from healthy to TB patients to determine their role and potential benefits in fighting or slowing/preventing TB pathogenesis.

This review discusses the available scientific evidence on *M. tb*-microbiota-immunity interactions in the context of antimicrobials and other dysbiosis-causing factors. The intricacies and complexities of these interactions are elaborated, while suggesting potential areas for further research. There have been recent reviews on tuberculosis and the microbiome[24–27]; however, these reviews do not incorporate the interactions of the immune system and autophagy with the microbiota, antimicrobials and *M. tb,* a gap this review seeks to fill.

Search strategy.

Pubmed was searched for English manuscripts published up to October 2019. Search words used included "*M. tb* AND microbiome", "*M. tb* AND microbiota", "*M. tb* AND autophagy AND antimicrobials", "*M. tb* AND immun* AND antimicrobials", "*M. tb* AND microbio* AND antimicrobials", and "*M. tb* AND autophagy AND antimicrobials". Duplicates were removed with the help of Mendeley reference manager. The relationships between factors disrupting the microbiota and the lung-gut microbiota feed-back as well as their effects on immunity and *M. tb* were drawn after the qualitative analyses (Figure 1). This relationship was used to design a table (Table 1) and inform the format of the review.

Table 1. Factors causing dysbiosis and their resultant effect on *Mtb* pathogenesis

Factors causing dysbiosis	Types	Host (Human, animal)	Anatomical site (lung, gut etc.)	Country|TB state (n[1])	Effect on microbiota	Effect on immunity (autophagy)	Effect on *M. tuberculosis* pathogenesis	Associated histopathologies	References
Antibiotics	Isoniazid (INH) Rifampicin (RIF) & Pyrazinamide (PZA)	Mice	GIT (gastro-intestinal tract)		↑ Erysipelato-clostridium (Erysipelotrichaceae, genus Eggerthia), Akkermansia, Bacteroidetes (Barnesiella, Para-prevotella, Bifidobacterium & Porphyromonas) Proteobacteria (Enterobacteriaceae). ↓Firmicutes (class Clostridia: Acetivibrio, Robin-soniella, Alkaliphilus, Stomatobaculum, Butyricicoccus, Acetanaerobacterium, Tyzzerella, Ruminococcus, and Peptococcus)	ND (not determined or found)	ND	ND	[4]
	Vancomycin (VAN), ampicillin,	Mice	GIT		Similar to above except ↑ Proteobacteria				

[1] Sample size

neomycin, and metronidazole							
VAN, polymixin B carbenicillin, trimethoprim, clindamycin & amphotericin B	Mice	GIT, lungs, spleen & liver	↓↓growth & diversity of the gut microbiota ↑↑Enterococcus ↓↓Bifidobacterium, Lactobacillus, Campylobacter, & Bacteroides FT partially reversed dysbiosis	↓↓ Expression of IFN-γ and TNF-α; ↑ frequency of Tregs. FTP reversed these effects.	↑↑ Mtb in the lungs, spleen and liver. Were reduced by FTP	Enlarged caecum; larger and greater number of granulomas in lungs & distorted ileum microvilli structure (all these were reversed by FTP)	[32]
RIF	Mice	GIT	↑ Bacteroidetes (Bacteroides) & Verrucomicrobia (Verrucomicrobiaceae) ↓ Firmicutes & Lachnospiraceae	No Δ in CD8 + T cells or ESAT-6-specific CD4 T + cells, Treg were observed	No Δ observed in Mtb burden	ND	[33]
INH & PZA			↑ Bacteroidetes ↓ Clostridiaceae	Impaired metabolism of alveolar macrophages (↓ MHCII, TNFα & IL − 1β) & ineffective bactericidal activity. No Δ in CD8 + T cells or ESAT-6-specific	↑↑ Mtb (reversible by FTP)		

					CD4 T + cells & Treg.			
	Colistin, gentamicin, kanamycin, metronidazole & vancomycin	Mice	GIT		↑ Desulfovibrio (DSV) & Bacteroidetes ↓Firmicutes	↑↑ *Atg5, Atg16l1,* *Lyz, Reg IIIγ Crypt 4 & Irgm1 genes* (indirectly through dysbiosis & ↑DSV)	ND	ND	[39]
	INH, RIF, PZA & Ethambutol (EMB)	Human	GIT	Haiti| 1. TB-positive (19), 2. TB-negative (50), 3. Latent TB (25) 4. TB cured (19)	Little or no Δ in diversity. ↑Erysipelato-clostridium, Fusobacterium, & Prevotella. ↓Blautia, Lacto-bacillus, Eubacterium, Coprococcus, Ruminococcus, & Bifidobacterium (Actinobacteria)	ND	ND	ND	[5]
	INH, RIF, PZA & EMB	Human	GIT	China|1.TB-pos[2] (28), 2. TB-Neg[3] (13) 3. LTBI[4] (10),	↑↑ Bacteroidetes (Bacteroides, Bacteroides, *B. fragilis, B. plebeius*, *B. caccae*, *B. coprophilus*, & Parabacteroides distasonis),	ND	ND	ND	[6]

[2] TB-positive, not yet on drugs

[3] TB-Negative

[4] Latent TB infection

			4. TB-cured (10) 5. TB on drugs (23)	Erysipelotrichaceae & Enterococcus ↓↓ Firmicutes (Clostridiales: Ruminococcus sp. 39BFAA, Ruminococcus gnavus, & Faecalibacterium, *Erysipelatoclostridium ramosum*				
INH & PZA	*M*acrophages	*Ex-vivo*		ND	↑ Autophagy activation, ROS* & phagosomal maturation	↓↓ Mtb	ND	[40]
INH & RIF	*M*acrophages	*Ex-vivo*		ND	↑↑↑Phagolysosome acidity (BDQ & INH). RIF & LNZ pretreatment = no Δ in phagolysosome acidity. ↑autophagy: INH pretretment ↑↑↑autophagy: BDQ & LNZ pretreatment	↓ Mtb	ND	[41]
Linezolid (LNZ) & bedaquiline (BDQ)				ND		↓↓↓ Mtb	ND	
Antimicrobial peptides: lactoferricin	Macrophages	*Ex-vivo*		ND	↑↑EMB cidal activity, autophagy, phagosomal maturation, IL-6 & TNF-α	↓↓*M. avium* growth ↑↑ *M. avium* killing &	ND	[49]

						lysozyme formation		
	VAN, neomycin sulfate, & metronidazole	Mice	GIT & lungs		↑↑↑↑↑ Dysbiosys (Proteobacteria, Enterococcus), which was restored by TDB & *Lactobacillus plantarum*. ↓↓↓ Lactobacillus, Firmicutes & Bacteroides. Reversed by TDB & *Lactobacillus plantarum*	↓↓ MINCLE*, CD4+ T cells (including effector & memory T cells), phagocytic ability, MHC-II & CD86 expression, IFN-γ, IL-6, 1L-12 & IL-17 ↑↑IL-10, FoxP3+ CD4 Tregs, PD-1+ CD4 T cells, $CCR7^{hi}$ $CD44^{hi}$ TDB* & *L. plantarum* reversed these.	↑↑*M. tb* growth M. tb presence was reduced upon (DC cells) treatment with TDB & *Lactobacillus plantarum*	ND	[12]
Antibiotics and *Mycobacterium tuberculosis* complex	*M. tb* & anti-TB drugs (RIF, INH, PZA & EMB)	Human	GIT	China|1. TB-positive (37): new (19), relapsed (18); 2. TB-neg (20)	↑Actinobacteria (Collinsella) & Proteobacteria (Enterobacteriaceae : Escherichia), Cyano-bacteria, Verruco-microbia, Bacilli–Lactobacillales-Lactobacillaceae ↓Bacteroidetes (Prevotella), Lachnospira, Streptococcus,	↓ CD4+ in new TB cases; ↑ CD4+ recurrent cases	ND	ND	[14]

					Firmicutes (Roseburia Coprococcus,			
	M. tb & antibiotics (ampicillin, neomycin sulfate, metronidazole, & VAN)	Mice	GIT & lungs		↑↑ β-proteobacteria ↓↓ Bacteroidetes & Firmicutes FT reversed some of these dysbiosis	↓MAIT cells (within the 1st 7 days); this was reversed with FT ↓IL-17A (from MAIT cells within the 1st 7 days)	↑↑ *M. tb* colonization within the 1st 7 days	Increased caecum size; reversed with FT	[13]
***Mycobacterium tuberculosis* complex**	*M. tb*	Mice	GIT		No significant Δ; Minor differential abundance in Clostridiales (Firmicutes), Bacteroidetes and Tenericutes	ND	ND	ND	[25]
		Human	GIT	Same as reference 6 above	A minor decrease in α diversity was observed	ND	ND	ND	[6]
	M. tb	Macaques	Lung airways		Slight ↑ in SR1, Aggregatibacter, Leptotrichia, Prevotella, Streptococcus, Staphylococcus, & Campylobacter ↓ Lachnospiraceae	ND	ND	ND	[15]
	M. tb	Mice	GIT		↓Clostridiales (Lachnospiraceae, Ruminococcaceae: Clostridium,	ND	ND	ND	[9]

				Catabacteriaceae, Oscillospira)				
M. tb	Humans	GIT & blood	Taiwan|1. TB-positive (25), 2. TB-neg (23), 3. LTBI (32)	↑Bacteroidetes	↑IL-6, IL-1B, & PMN Firmicutes↔CD4+ & CD8+ PMN∴Proteobacteria ↓ lymphocytes	ND	ND	[65]
M. tb	Rhesus Macaques	GIT		↑ Lachnospiraceae (*Roseburia intestinalis*) Clostridiaceae, & Ruminococcaceae, *Succinivibrio dextrinosolvens, Weissella (Leuconostocaceae)* ↓ Streptococcaceae (*Streptococcus equinus*), Bacteroidales RF16 & Clostridiales vadin B660 groups, Erysipelotrichaceae	ND	ND	ND	[35]
M. tb complex	Pigs	mandibular lymph node		Temporal ↑Babesia, Theileria & Pestivirus Temporal ↓Ascogregarina & Chlorella	ND	ND	ND	[60]

	M. tb exposure (contacts of *M. tb* patients)	Humans	GIT & blood (PBMCs)	Haiti|1. TB negative controls (45), 2. TB contacts (31), 3. LTBI (47)	↑ γ-proteobacteria (*S. dextrinosolvens*) ↓↓ Bacteroidetes (*B. ovatus*) &*P. merdae*	Granzyme B inducibility of MAIT* cells≡ *B. ovatus* & *P. merdae* CD4+ MAIT cell abundance≡*S. dextrinosolvens* Clostridia, Erysipelotrichia Negativicutes & Verrucomicrobiae≡immune phenotypes	ND	ND	[31]
	M. tb	Humans	GIT	China|1. TB-pos (46), 2. TB-neg (31)	↑ Coprobacillus & *Clostridium bolteae* ↓ *Roseburia inulinivorans, R. hominis, R. intestinalis, Eubacterium rectale,* & *Coprococcus comes; Bifidobacterium adolescentis* & *B. longum; Ruminococcus obeum & Akkermansia muciniphila*	ND	ND	ND	[30]
Pathogens	*Helicobacter hepaticus*	Mice	GIT		↑ Bacteroidaceae and other unclassified bacteroidales; ↓↓ in Clostridiales,	↑↑ hypercytokinemia & chemokine production; ↑↑	↑ *M. tb* growth	lung tissue destruction; caseous granuloma;	[45]

					Ruminococcaceae, Lachnospiraceae,and Prevotellaceae	activated lung T cells		morbidity/mortality.	
					ND	↓↓85A-specific CD8T cell IFN-γ responses in immunised mice previously infected; ↑↑ IL-10 in GIT	↑ *M. tb* growth; abolished protective effect of Ad85A vaccine	Same pathologies in vaccinated mice as unvaccinated ones	44
Commensals	*Lactobacillus spp.*: *L. plantarum* & *L. casei*	Wild boar (*sus scrofa)*	Blood and *in-vitro*		ND	↑↑ opsonization *L. plantarum* ↓ BCG intake whilst *L. casei* ↑ it.	↓↓ *Mycobacterium bovis* BCG	ND	36
HIV & anti-retrovirals-mediated dysbiosis (increased anaerobes and SCFA production)	Short chain fatty acids (SCFA): butyrate & propionate	Humans	Blood and lungs	South Africa (212) & USA (20)|1. HIV±TB positive (232), 2. HIV/TB-neg (50)	↑↑pulmonary SCFA = ↑↑anaerobes in the lung: Prevotella, Veillonella & Haemophilus ↑Psychrobacter, Pseudomonas & Sphingomonas	↑Treg ↓IFN- γ & IL-17A	↑*M. tb* susceptibility	ND	17
Gut microbiota	Butyrate	Macrophages	*Ex-vivo*		ND	ND	↑*M. tb* susceptibility and growth	ND	47
		Humans	*Ex-vivo:* Peripheral blood mononuclear cells (PBMCs)	Netherlands| Not stated.	ND	↑IL-10 ↓Th17 proliferation	↓Mtb-induced cytokine (TNF-α,IL-1β, and IL-17) response	ND	54

	Indole propionic acid (IPA)	Mice, bacterial cultures	GIT, *in-vitro*	ND	ND	↓*M. tb* growth	ND	[52,53]
	Indole	Dendritic cells	*Ex-vivo*	ND	↓reduced inflammasome activity	↑intracellular bacterial killing	ND	[46]
Immunity (Autophagy)	Autophagy: $Atg5^{-/-}$ (absence of autophagy)	Mice	GIT	↑*Candidatus athromitus*, Pasteur-ellaceae, Pseudomo-nas, Aggregatibacter, Klebsiella, Mannheimia, Gemella & Streptococcus ↓*Akkermansia muciniphila*, Lachnospiraceae, Acinetobacter, Akkermansia, Ochrobactrum, Brevibacterium, Ruminococcus, Sphingomonas and Meiothermus ↓↓Verrucomicrobia	↑ *Muc-2,* RORC TBX21, CD5, CD6, CD7, and CD96 ↓Defa5,	ND	ND	[38]
	Autophagy: $ATG16L1^{HM}$, $LC3b^{-/-}$, $Atg4b^{-/-}$ (absence of autophagy)	Mice	GIT	↓↓↓ *C.rodentium*	↑↑IFN-I response to the microbiota	ND	↓↓ Colonic crypt hyperplasia; ↓↓ *C.rodentium* dissemination to the liver	[48]

Diet	Apple polysaccharide	Mice	GIT		↑↑eubiosis (↑ ↑Bacteroidetes & Lactobacillus; ↓↓ Firmicutes & Fusobacteium), ↑ ↑SCFA (acetic & isobutyric acids)	↑ Occludin, autophagy (goblet cells) TNF-α, MCP-1, CXCL-1, IL-1β,	ND	↓↓colonic patholo-gical & mucosal damage ↓↓gut permeability & chronic inflammation,	[67]
	Vitamin A: all- trans retinoic acid (atRA)	Human and mice	*Ex-vivo*: macrophages *In-vivo:* lungs of mice	Ireland|Not applicable	ND	↑↑autophagy, TNF-α, macrophage cell fusion=multinucleated cells, Inos** ↓IL-10, IL-6	↓↓Mtb and *Bordetella pertussis* growth in macrophages	↓↓pulmonary pathology	[56,57]
	Vitamin D: 1,25-dihydroxyvitamin D_3	Human	*Ex-vivo:* PBMCs	India| 1. TB-positive (40), 2. TB-neg (40)	ND	↑↑autophagy (ATG5 & BECN1), CD206 ↑↑ phagocytosis & cathelicidin ↓↓DC-SIGN, CD209 expression	↓↓Mtb burden	ND	[68]
Smoking	Cigarettte: nicotine	Human and mice	*Ex-vivo* (macrophages) and *in-vivo* (mice)	USA| Not applicable	ND	Inhibits autophagy, impairs macrophages, activates NF-kB & Tregs	↑↑*Mtb* burden	ND	[66]

*↑ increased abundance or richness; ↑↑ strong increase in abundance or richness; ↓ reduced abundance or richness; ↓↓ strong reduction in abundance or richness

↔ directly associated with; ∵ indirectly or negatively associated with; ≡ correlated with. **ND**, not determined. Δ, change. **FTP**, faecal transplant. **PMN**, Polymorphonuclear Neutrophils; ROS, reaction oxygen series. ****iNOS,** inducible nitric oxide synthase; **MAIT,** mucosal-associated invariant T cells; **MINCLE,** macrophage inducible C-type lectin; **TDB**, trehalose-dibehenate (TDB)

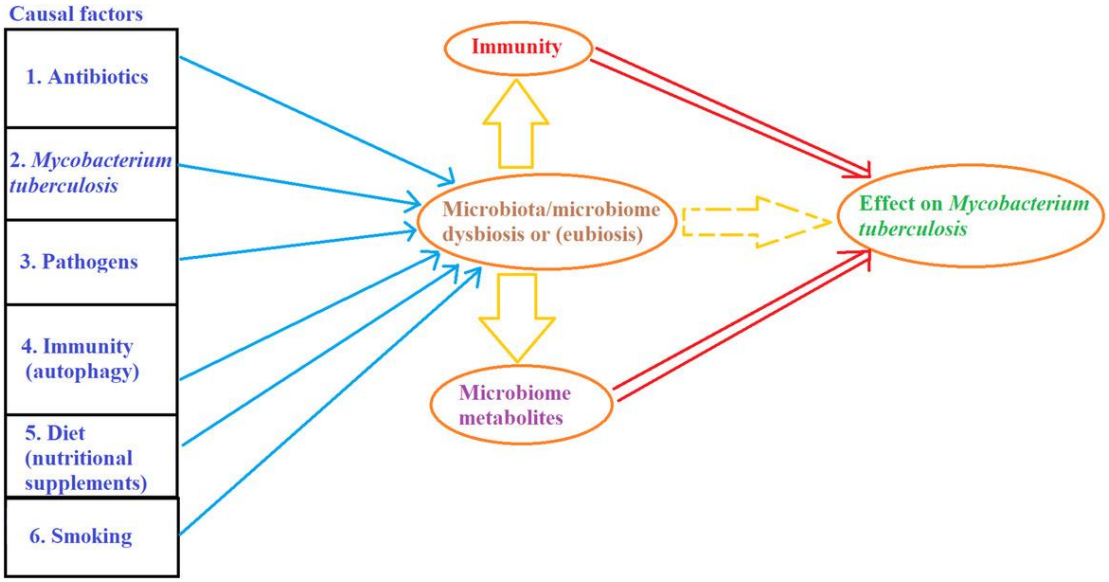

Figure 1. Factors affecting the gut microbiome and their indirect effect on *Mtb* pathogenesis.

2. Antimicrobials, microbiome, immunity, and *M. tb*

The interactions between antimicrobials, the microbiota, the immune system and *M. tb* can generally be explained by identifying the factors that cause perturbations in the microbiota, the subsequent effect of those factors and their direct or indirect effect on *M. tb.* From this premise, it will generally be agreed that the microbiome will hardly undergo any drastic change unless an outside force disrupts it. Owing to the delicate balance between the microbiome and the immune system, it is expected that any disorientation in the microbiome will necessarily trigger an immune response [25,28]. As well, such microbiome disturbances will also affect the functional processes of the microbiota that provides the host with essential nutrients and health benefits. Subsequently, the changes orchestrated by any dysbiosis in the microbiome will put the body at a disadvantage; particularly, when faced with an intractable pathogen such as *M. tb.* It is thus not surprising that although one-third of the world's population is infected with latent TB, only 10 million develop active TB infections yearly [29]. Evidently, the status of the microbiome will be important factor in explaining why TB becomes latent in some persons but develops into active disease in others.

The factors identified to cause dysbiosis in the microbiota are mainly antimicrobials, pathogens, smoking, diet, immune system and *M. tb* (Figure 1). The dysbiosis caused by these factors

in turn modulate the immune system negatively and affects the microbiota's biosynthetic pathways, and subsequently influences the type and abundance of metabolites produced. The modulated immunity and unbalanced metabolites production also affect the progression of *M. tb* in the lungs and throughout the body (Figure 1; Table 1). Thus, factors affecting microbiome stability indirectly influence susceptibility to *M. tb,* such that a restoration of eubiosis reverses *M. tb's* pathogenesis, granulomas and dissemination [12,13,30].

Therefore, the microbiome-immunity-*M. tb* interactions are discussed under seven main sections, according to the dysbiotic factors: 1. Antimicrobials; 2. Immunity; 3. Pathogens; 4. *M. tb;* 5. Smoking; 6. Diet; and 7. Current challenges and future perspectives.

Antimicrobials: the major enemy of the microbiome

To date, antimicrobials remain the major and most potent factor causing dysbiosis, not only in plants, animals and humans, but also in the environment as they indiscriminately destroy all bacteria, both useful and harmful [1]. Their effects on the microbiome are such that the dysbiosis can persist for more than a year [4,5]. In animal models, they drastically disorient the microbiome within the first seven days, allowing drug-resistant microbial species such as Proteobacteria and Enterococcus to subsequently proliferate. Hence, affecting the diversity and relative abundance of the gut microbiome [12,13,31]. Due to the importance of the microbiome in food metabolism in the intestines, their disruption affects the digestive processes as well as the functional and biosynthetic pathways, leading to reduced biosynthesis of important metabolites and nutrients, enlarged caeca and distorted microvilli structures [12,32]. Although faecal transplants (FTs) were able to restore much of the original diversity of the gut microbiota in animals exposed to antimicrobials, the reversal was not complete [12,13,32].

The effects of broad-spectrum antibiotics on the gut microbiome are more drastic than narrow-spectrum antibiotics, which includes first-line anti-TB drugs such as isoniazid (INH/H), pyrazinamide (PZA/Z) and ethambutol (EMB/E); rifampicin (RIF/R) is the only broad-spectrum first-line anti-TB antibiotic [4,5]. Moreover, H and Z are pro-drugs, meaning they are only active once inside the *M. tb* host. Yet, Z and H reduced the relative abundance of the gut (mice) microbiome species significantly, and the affected species were not mycobacteria (Table 1; Figure 1) [4,33]. Expectedly, R,

which is not a prodrug, produced higher gut dysbiotic changes (in terms of abundance and diversity), evincing that the type of TB antimicrobials administered influenced the extent of dysbiosis (Table 1). For instance, PZA-RIF combination had a higher dysbiotic effect than HRZ, owing to the presence of INH that might have a dampening effect on PZA and RIF's dysbiotic effect [4]. Subsequently, investigations into the individual dysbiotic effect of other antimicrobials besides INH will be necessary to inform appropriate antimicrobial combinations.

The effect of oral antimicrobials on the lung microbiota is not fully clear, although one study found no microbiota in the lung post antimicrobial administration [13]. As well, current studies reporting on the microbiota effect of anti-TB antibiotics focus on the gut instead of the lung microbiome. Thus, concomitant changes in the lung microbiome, alongside the gut microbiome during antimicrobial therapy, remain sketchy. This is therefore an important gap that needs immediate attention.

Most studies describing microbiome changes in TB patients (or TB-infected mice) on chemotherapy mainly focus on differences in species abundance and diversity vis-a-vis healthy controls. Yet, the findings have been conflicting, albeit some are corroborative. In particular, two studies carried out in Haiti among TB and non-TB cohorts produced similar results, albeit one was in humans (on HRZ) and another was in mice (on HRZE) [4,5]. In both studies, species associated with immune regulation reduced following antimicrobials exposure in TB patients or mice. Specifically, species of the order Costridiales, known to be associated with altered Treg function, were decreased while those belonging to Erysipelotrichaceae, associated with metabolic and inflammatory disturbances, and Proteobacteria, which contains several pathogenic species, increased in abundance (Table 1). Nevertheless, the amount of dysbiosis caused by vancomycin far exceeded that of the four TB drugs, explaining the lesser colonization of TB patients with enteric pathogens, although they take these medications for at least 6 months [4].

Nevertheless, the anti-TB drugs caused dysbiosis extending beyond a year to 3 years post drug-administration [5]. Yet, TB treatment had no effect on the overall diversity of the microbiome, albeit specific species that are important immunologically (e.g., *Bacteroides* modulates host inflammatory response in mice; *Ruminococcus* and *Coprococcus* regulate expression of peripheral

cytokines such as IL-1 and IFNγ; *Bifidobacterium* induce a Th17 reaction in mice) were drastically depleted, with the perturbation lasting beyond 1.2 years. Again, persons with latent TB (IGRA+), both cured and on treatment, had no intestinal microbiome differences compared with persons without TB, suggesting that latent TB (LTBI) had no effect on the GIT microbiome. This sharply contrasts with the effect observed after administering broad-spectrum antimicrobials (β-lactams, aminoglycosides, fluoroquinolones, azoles) [5,34]. However, close examination showed distinct changes at the species level between the treated, uninfected and LTBI groups (Table 1).

The findings above nevertheless, differed from three studies conducted in China (Table 1)[6,14,30]. Particularly, the studies in China observed changes in species diversity and abundance between healthy and TB patients. Unfortunately, the antimicrobials used by the cohorts in China, except for one study that used HRZE [30], were not delineated as was the case in Haiti (Table 1). Hence, it remains to be seen if the observations made were as a result of *M. tb* infection or the antimicrobials taken by the patients. This is particularly important as Wipperman et al. (2017) showed that antitubercular drugs-mediated gut dysbiosis can persist for more than a year [5]. Strikingly, there was a general reduction in abundance in beneficial commensals belonging to the Bacteriodetes and Firmicutes, such as Roseburia that engenders several SCFAs (short-chain fatty acids), and an increase in Actinobacteria and Proteobacteria, which contain many pathogenic species (*Escherichia, Salmonella, Vibrio, Helicobacter, Yersinia, Legionellales* etc.) (Table 1) [6,14]. Notwithstanding, HRZE exposure reduced genera belonging to Clostridiales such as *Ruminococcus sp*., *Ruminococcus gnavus*, and Faecalibacterium while Bacteriodes members such as *Bacteroides, Bacteroides fragilis, Bacteroides plebeius, Bacteroides caccae, Bacteroides coprophilus*, and *Parabacteroides distasonis* were significantly enriched [6].

These conflicting inter-study and inter-individual microbiome differences before and after antimicrobials administration hampers the effective description of the general effect of TB antimicrobials on the microbiota. Specifically, it causes no changes in species diversity in some studies while the opposite was reported in others. It is however worth noting that the distinct lifestyles of individuals shape their microbiomes differently, making it difficult to get differences that can be

applied to all persons. Subsequently, Namasivayam et al. (2019) argued that it is best to assess microbiome dynamics on the individual level by comparing changes to the baseline instead of between individuals, which we fully agree with [35]. Nevertheless, Hu et al. (2019), in two separate studies, have tried to identify distinct microbiome signatures using species and single-nucleotide polymorphisms (SNPs) in specific genes involved in carbohydrate metabolism [6,30]. These signatures, which were identified using only patients from China, have been suggested as a TB diagnostic index. Notably, *Enterobacter cloacae, Phascolarctobacterium succinatutens, Methanobrevibacter smithii, Bilophila, and* Parabacteroides were distinct in cured patients whilst *Haemophilus parainfluenzae, Roseburia inulinivorans, and Roseburia hominis* were abundant in TB patients [6,30]. SNPs in carbohydrate biosynthesis genes were also suggested as distinct biomarkers [30]. Furthermore, Vorkas et al. (2018) observed a strong correlation between certain gut microbiota species and immune phenotypes in TB contacts. For example, the depletion of some species directly correlated with reduced Granzyme B inducibility of MAIT (mucosal-associated invariant T) cells while $CD4^+$ MAIT cells increased in TB contacts [31]. In addition, *Lactobacillus plantarum* and *Lactobacillus casei* were able to reverse antibiotics-mediated dysbiosis, immune dysregulation and *M. tb* pathologies in mice. In *in vitro* experiments, *Lactobacillus spp.* isolated from pig faeces expressed bacteriocins that inhibited the growth of *Mycobacterium bovis BCG;* they also increased opsonization of *BCG* by phagocytes [36] (Table 1). As revealing as these findings are, they would need to be tested on a larger population to ensure reproducibility worldwide.

Another grave concern regarding antimicrobial-microbiota interactions is the shift in metabolic pathways that affects the biosynthesis of important vitamins. Specifically, SCFAs fermentation, biosynthesis of conjugated bile acids etc. decreased while fatty acid oxidation and biosynthesis of six vitamins viz., B6, thiamine, folate, pantothenate and flavin, reduced in treated patients, evincing the functional/metabolic effect of anti-TB drugs on the gut microbiome [5,6,30]. These revelations suggest a relationship between gut functional metabolism dynamics and TB pathogenesis as the depletion of important nutrients can deprive the host of necessary ingredients to defend itself against TB.

Moreover, there is the need to investigate the effect(s) of metabolized antimicrobials on the microbiome and TB. This is because certain gut microbiota can use antibiotics as a carbon source, engendering different metabolites that can cause unknown effects [37]. In particular, several soil bacteria (of the Phylum Proteobacteria i.e., Orders Burkholderiales, Xanthomonadales, Pasteurellales, Enterobacteriales, Pseudomonadales, Sphingomonadales, Rhodospirillales and Rhizoiales; Phylum Actinobacteria and Order Actinomycetales; and Phylum Bacteriodetes with the orders Sphingobacteriales and Flavobacteriales) with close phylogeny to clinical pathogens metabolized antimicrobials (d-cycloserine, amikacin, gentamicin, kanamycin, sisomicin, chloramphenicol, thiamphenicol, carbenicillin, dicloxacillin penicillin G, vancomycin, ciprofloxacin, levofloxacin, nalidixic acid, mafenide, sulfamethizole, sulfisoxazole, trimethoprim) into ineffective molecules [37]. As can be seen, most of these bacterial orders are of close phylogenetic relationship to known intestinal commensals and clinical pathogens.

Antimicrobials affects immunity & enhances autophagy

By depleting or altering the fine microbiota balance, antimicrobials do not only affect the functional state of the microbiome, but also affect the immune system, predisposing the host to a barrage of infections. In particular, mice whose gut microbiota were depleted with antimicrobials had higher proliferation of *M. tb* in their lungs, spreading to the spleen and liver (Table 1). As well, there were larger and more numerous granulomas in the lungs of mice with gut dysbiosis, coupled with reduced expression of IFN-γ, TNF-α, MHCII, CD86, MINCLE (macrophage inducible C-type lectin), IL-17A, IL-12, and IL-6 cytokines. Further, there were reduced migration, abundance and proliferation of MAIT cells, DCs, CD4 cells, and effector and memory T cells while Treg, IL-10, and exhausted T cells increased; the phagocytic capacity of CD4 cells also reduced substantially (Table 1). These gut-dysbiosis mediated immune changes were observed in the lungs, mediated by MINCLE and reversible by FT and *L. plantarum* gavage. It is worth mentioning that most of these dysbiosis-mediated effects on the immune system were mainly mediated by MINCLE through DCs, and in to a lesser degree, was also observed in MAIT cells [12,13,32]. The reversal of these dysbiosis-mediated effects on the immune system also resulted in the partial or substantial restoration of gut eubiosis and reduction in

M. tb proliferation and associated pathologies (Table 1), confirming the importance of the gut microbiome in *M. tb* prevention.

Contrary to the above, Khan et al. (2019) recently showed that prior exposure to INH and PZA, which are both prodrugs, but not RIF, in mice, resulted in higher *M. tb* burden after infection compared to mice not previously exposed. Notably, INH-PZA affected the innate immunity, but not the adaptive immunity through gut microbiome changes, whilst RIF had no such effect [33]. Although these effects were also reversible by FT, it suggests that different anti-TB drugs affect the microbiome and immunity differently.

Nevertheless, these findings strongly confirm the strong association between the gut microbiota and *M. tb* pathogenesis, mediated by the immune system, including the importance of certain bacterial species and FT in fighting *M. tb.* A strong correlation between the abundance of certain species in the gut and peripheral CD^{4+} T cell counts has been reported [14]. CD^{4+} positively and strongly correlated with Prevotella in new TB cases but negatively correlated with CD4+ in recurrent TB cases [14]. The distinct gut microbiome and metabolic pathways changes, which could last, on average, for 1.2 years, affected bacteria such as *Bacteroides, Bifidobacterium Ruminococcus* and *Coprococcus,* which are known to be beneficial to the peripheral immune system including IL-1, IFN-γ and Th17 modulation (Table 1). Such changes make it easier for cured TB patients to be re-infected with the disease or with other infections, particularly as the dysbiosis can prolong beyond a year [5]. Hence, the impact of antitubercular drugs-mediated dysbiosis on other diseases should be investigated.

In addition to causing dysbiosis and functional metabolic changes, antimicrobials also directly and indirectly interact with the innate immunity through autophagy to influence *M. tb* pathogenesis. Particularly, the depletion of the gut microbiome induces autophagy [38]. Notably, direct treatment of macrophages with antimicrobials have produced conflicting results, depending on the antibiotic used. Whereas some studies saw no direct effect of antimicrobials on autophagy, others did [4,39] (Table 1). In one instance, autophagy-deficient macrophages were unable to clear *M. tb* even in the presence of antimicrobials [40]. Again, autophagy-deficient Drosophila flies succumbed quickly to *M. marinum*

infection while wild-type flies survived longer with lesser bacterial load [40]. Subsequently, the role of the microbiota in influencing autophagy and affecting *M. tb* pathogenesis requires closer investigation. Thus, notwithstanding these conflicts, one thing is clear: autophagy plays a central role in the antimicrobials-microbiota-immunity interactions (Figure 2).

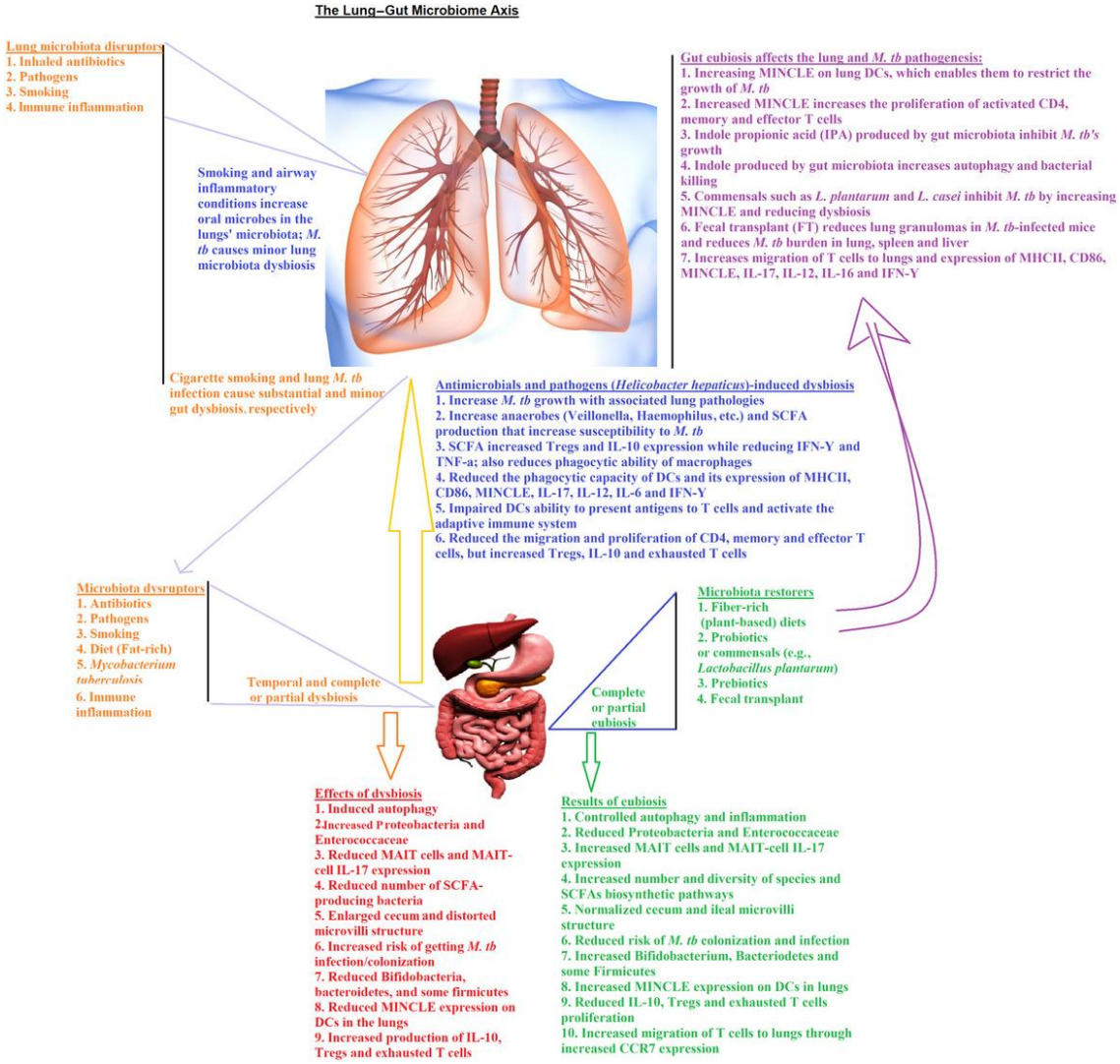

Figure 2. The lung–gut microbiome crosstalk. Microbiota disturbances in the gut affect immune function in the lungs, increasing susceptibility to *Mtb*. Restoration of gut eubiosis, however, increases host resistance to *Mtb*.

For instance, Genestet et al. (2018) showed that INH, RIF, Linezolid (LNZ) and bedaquiline (BDQ) treatment affected *M. tb* pathogenesis in macrophages to various degrees with regards to phagosome escape, autolysosyme formation, autophagy activation and *M. tb* clearance; BDQ and INH substantially inhibited *M. tb*'s phagosome escape. RIF only increased autolysosome formation while BDQ and LNZ enhanced autophagy activation and efficacy, suggesting the superiority of BDQ, LNZ

and INH in *M. tb* destruction in macrophages. Specifically, BDQ and LNZ pre-treatment drastically reduced intracellular *M. tb* survival while INH and RIF hardly had an effect [41]. Similarly, Kim et al. (2012) observed that H and Z activated autophagy in macrophages and Drosophila such that the antimicrobials were ineffective without autophagy genes [40]. HZ treatment enhanced phagosome-lysosomes fusion to form autophagolysosomes and induced production of ROS by mitochondria and host cells that enhanced bacterial killing (Fig. 1) [40].

Cellular and mitochondrial ROS production, induced by these antimicrobials, trigger the autophagy pathway with assistance from host NADPH oxide. Antimycobacterial activity and autophagy were attenuated in the absence of NOX (NADPH oxide)-2 and ROS in macrophages [40,42]. As well, antioxidants inhibited autophagy in *M. tb*-infected cells, confirming that ROS mediates autophagy in cells. ROS destroys DNA, RNA and proteins; hence, even in immunocompromised mice, arginine (an antioxidant)-deficient mutants were quickly sterilised from the host [42]. Moreover, INH, PZA and *M. tb* increased production of cytokines such as TNF-α, IL-6, IL-1b etc. in *atg5*-deficient macrophages [40]. Therefore, not only does antimicrobials directly kill microbes, but also induces autophagy, inflammation and ROS production, which simultaneously act in synergy to destroy bacteria and cause dysbiosis (Fig. 2) [40,42]. Nevertheless, macrophages infected with drug-resistant *M. tb* strains did not induce autophagy or ROS production upon drug treatment, explaining why drug-resistant strains are able to survive both immune and antimicrobial action. Nevertheless, this observation requires thorough investigation [40].

Yang et al. (2017) observed that CIP, a 2nd-line TB drug, generates metabolites in local and systemic tissues at infection sites, which reduces the efficacy of CIP as well as boost macrophage's phagocytic activity [43]. Notably, metabolites generated locally by *E. coli* during infections also affected CIP and immune activity while CIP-treated macrophages could not engulph pathogens as much as those treated with CIP metabolites alone or untreated ones [43]. In the presence of both CIP and its metabolites however, the positive effect of the CIP metabolites on immunity were overshadowed by the negative effect of CIP. Thus, the overall effect of CIP on host immunity seems negative, which can be worsened by dysbiosis. Moreover, metabolites generated by CIP at infection sites enhanced the

biosynthesis of polyamines, which serve as antioxidants to reduce CIP-induced ROS (that can kill microbes), induce protective stress responses and protect pathogens from CIP effects by inhibiting drug uptake [43].

Interestingly, these CIP effects were not influenced by the microbiome as the same effect was seen in germ-free mice [43]. While the microbiome and its metabolites can enhance or dysregulate the immunity [44,45], CIP directly decreases immune strength and cause dysbiosis while its metabolites enhances phagocytosis (macrophages) and reduce antimicrobial action [42,43]. These complex relationships require further studies. Although CIP and its metabolites' effects on macrophages and CIP efficacy are microbiome-independent [43], it is notable that dysbiosis caused by CIP can negatively affect immunity by increasing inflammation [8].

Immunity (autophagy)-microbiome interactions

It is increasingly being realized that the immune system can also cause dysbiosis in the absence of antimicrobials. Specifically, autophagy shapes the contents of the microbiome, which in turn also modulates immune responses [38,46]. These suggest that other pathologies such as HIV, which negatively affects the immune system and autophagy can indirectly affect *M. tb* through the microbiome [17,47].

Yang et al., (2018) observed a significant alteration and reduced diversity in the microbiota of ATG5-knockout mice such that inflammation-reducing species such as *Akkermansia muciniphila* and members of the Lachnospiraceae family diminished while pro-inflammatory (*Candidatus athromitus*) and pathogenic (Pasteurellaceae) bacteria flourished (Figure 1)[38]. The absence of other *atg* genes viz., *atg5, atg7, atg16l1*, have been associated with impaired intestinal barrier functions in Paneth cells, which ultimately affects both the release of AMPs and the microbiota's ecology [38]. In the absence of *atg5* genes, intestinal Paneth cells were morphologically abnormal, making some bacterial families and species flourish while seven genera reduced (Table 1) [38]. Infectious disease pathways and potential pathogens (Pasteurellaceae) were enriched in the small intestines of ATG-deficient mice, suggesting that host cells are more susceptible to infections in the absence of autophagy. Moreover, cytoplasmic lysozymal levels reduced and protective molecules such as MUC2 were highly expressed

by Goblet cells as an indication of the altered inner intestinal layer resulting from over-activated immune response in the absence of ATG [38,46]. From previous studies, the absence of autophagy increased the inflammatory response, essentially altering the microbiome and intestinal layer [40,48]; however, this is yet to be demonstrated in the airway microbiota-*M. tb* interactions.

Autophagy-mediated dysbiosis further stimulated epithelial cells to secrete CCL5 chemokines that recruit immune cells including neutrophils, which cause chronic inflammatory responses (Figure 2)[38]. Furthermore, Lachnospiraceae and Ruminococcaceae, were reduced in the colon and duodenum, respectively, in autophagy-deficient mice; they produce butyrate (SCFA) that are important immunomodulators, including Treg cell differentiation. Intestinal autophagy deficiency resulted in imbalanced Th17/Treg balance, which is necessary to control the strongly pro-inflammatory and anti-pathogenic Th17 cells from destroying host tissues (immunopathology) and causing autoimmunity [38].

Singh et al. (2017) confirmed that antimicrobial-induced dysbiosis not only altered the gut microbial density, but also increased autophagy and AMPs (antimicrobial peptides) production as a result; obviously, this is a reactive measure by the host to restore the dysbiotic state to normalcy (Figure 2) [39]. An oral gavage of mice with Desulfovibrio, a bacterium that blooms in antimicrobial-treated microbiome, also resulted in similar effects. The increased autophagy in the intestinal cells were however, not as a result of the direct effect of the antimicrobials but due to the altered microbial density/dysbiosis. Lysozyme, an AMP, was increased as a result of the dysbiosis (Table 1) [39]. Thus, not only antimicrobials (as recently reported [40,41]) but also dysbiosis induced autophagy, which in turn increased the production of AMPs (Figures 1-3). The presence of LPS (lipopolysaccharides) in Desulfovibrio was potentially responsible for the increased autophagy as LPS in Gram-negative bacteria is known to induce autophagy [39]. The subsequent production of AMP by the host to suppress the overgrown LPS-producing cells and restore normalcy/eubiosis seems a plausible explanation for

AMPs are known for their potent antimicrobial and immunomodulatory effects, although they are yet to be used clinically for treating *M. tb* infections. Bovine lactoferricin peptide, shortened to amino acids 17-30 (LFcin17-30), and its D enantiomer, D-LFcin17-30), have important antimycobacterial properties (Fig. 1)[49]. In both *in-vivo* and *in-vitro* assays, D-lactoferricin and

lactoferricin increased macrophagic killing of *M. avium*, as well as increased the production of TNF-α and IL-6, both of which activate macrophages for intracellular killing. Notably, the AMP did not localize to the *M. avium* phagosomes, but rather increased the formation of lysosomes and autophagosomes, which might be the result of the increased TNF-α and IL-6 expression. Furthermore, the lactoferricin increased the activity of ethambutol (EMB) within the macrophages. Interestingly, the D-LFcin17-30 was very stable *in vitro* and resisted degradation for a long period, unlike other AMPs, explaining its potent antimicrobial and immunomodulatory effect. Nevertheless, it did not increase nitric oxide (NO) or ROS production; hence its killing effect is not associated with these. These results show that the shortened D-lactoferricin primes macrophages for intracellular killing of mycobacteria via enhanced autophagy (Fig. 1).

Similar to Desulfovibrio above, culture medium supernatants of four types of Bifidobacteria and the LPS of enteropathogenic *E. coli* (EPEC) O127:B8 induced autophagy in intestinal epithelial cells (IEC), albeit the EPEC-LPS induction was stronger [50]. Unlike the LPS in Gram-negative bacteria, PDIM (phthiocerol dimycocerosates), a cell wall lipid in *M. tb*, has been shown by Quigley et al. (2017) to be central to the escape of *M. tb* from macrophages' phagosomes into the cytosol to induce cell necrosis and/or escape from the macrophages and cause macroautophagy [51].Certainly, PDIM does not induce autophagy as LPS, further showing that *M. tb* has little effect on the microbiome.

In the absence/inhibition of autophagy, gut microbiota induced the production of type I interferon (IFN-I) in autophagy-deficient mice. In effect, autophagy inhibited the production of IFN-I in the GIT to control inflammation and protect intestinal microbiota, which can trigger IFN-I production (Fig. 2). Notably, autophagy-deficient mice were resistant to the pathogen *Citrobacter rodentium*, while infection with murine norovirus (MNV) resulted in IBD (inflammatory bowel disease)-like inflammations. In the case of *C. rodentium*, autophagy-competent monocytes attracted to the infection site aided in the resolution of the infection [48]. In effect, autophagy influences the composition of the intestinal microbiota through IFN-I inhibition.

Metabolites-immunity interactions

Besides direct interactions with immune cell receptors such as MINCLE, TLR and NOD, to regulate the immune system, the microbiota also produce metabolites that influence the immunity. Recently, indole and indole propionic acid (IPA), both metabolites of the microbiome produced by tryptophan deamination, reportedly increased autophagy (at levels comparable to rapamycin, with increased LC3-II expression, bacterial killing and reduced inflammasome production) and inhibited *M. tb* more than PZA respectively [46,52,53]. Subsequently, IPA remains the first microbiota-derived metabolite found to be more effective against *M. tb* than a current anti-TB drug just as intestinal *Lactobacillus spp.* were active against TB (Table 1 & Figure 3)[12,36,52,53]. (IPA) had adequate pharmacokinetic properties and was well-tolerated in mice, reducing the mycobacterial load by 7-fold in the spleen [52]. IPA is produced in the gut by commensals such as *Clostridium sporogenes*, *Peptostreptococcus anaerobius* and *Clostridium cadaveris.* It is found naturally in the host's blood, with 0.5 to 1ug/mL being found in the blood of untreated mice [52]. Notwithstanding, IPA was able to lower *M. tb*'s CFU only in the spleen, a mystery that is yet to be unravelled.

IPA's medicinal importance is not new as earlier studies found it to be neuroprotective, antioxidant and antiamyloid [52]. Although produced endogenously in the gut of humans, its inability to prevent TB could be due to its lower concentration in the lungs and blood. Nevertheless, it is possible it plays an essential role in preventing active TB in a large population of humans, a suggestion that needs to be experimentally ascertained. As well, its effects on the immune system is yet to be determined, although this preliminary finding shows that not all microbiota metabolites are detrimental to the host's immunity and the fight against TB.

The most common immune-regulating microbiota metabolites are the SCFAs, which include butyrate, propionate and citrate. Particularly, butyrates reduced the phagocytic and mycobactericidal (including non-tuberculous mycobacteria, NTMs) ability of macrophages, increased the production of IL-10 and decreased H37Rv-induced production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-17 in a dose-dependent manner, increasing the proliferation of mycobacteria in macrophages (Figures 2-3; Table 1) [47,54].

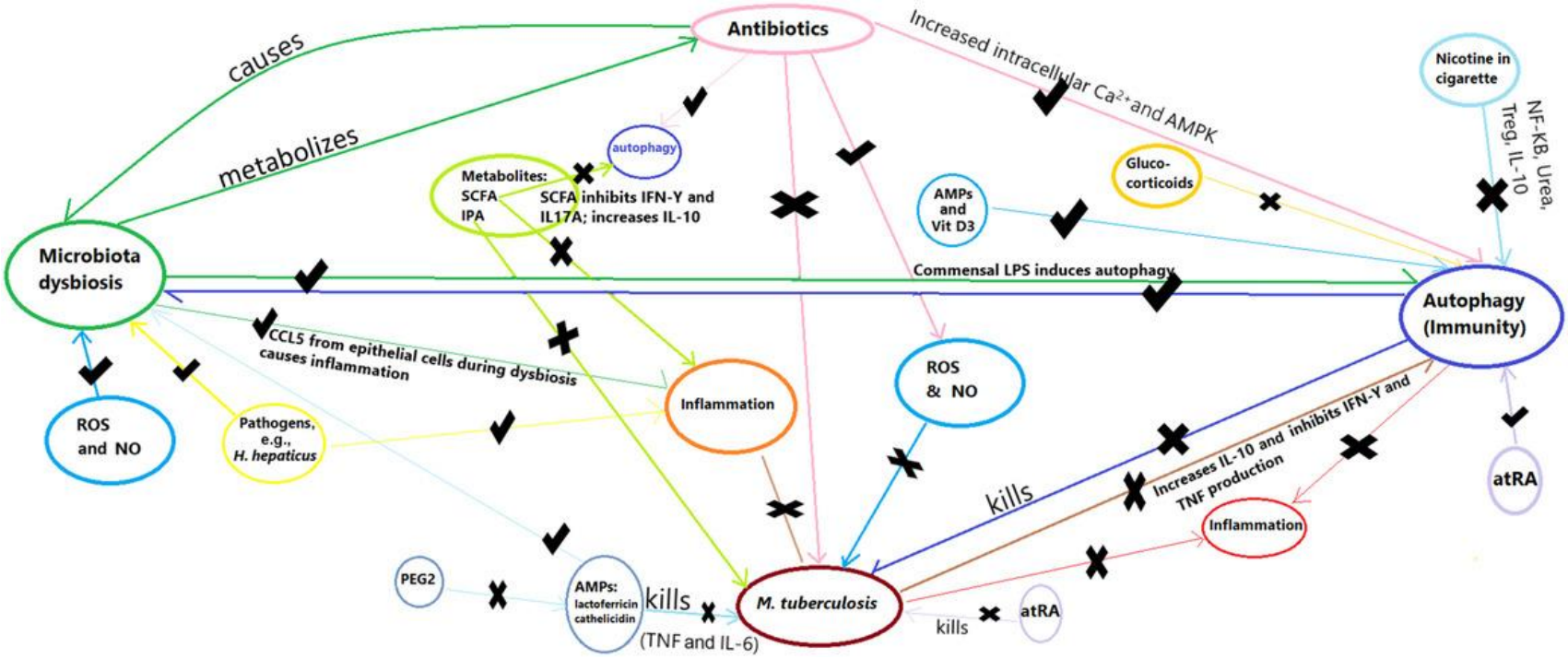

Figure 3. A diagrammatic representation of *Mtb*–microbiota–antimicrobials–immunity (autophagy) crosstalk. Colored lines show the interconnections or crosstalk between antimicrobials, autophagy (immunity), the microbiota (in a dysbiotic state), and *Mtb*, with each color chosen to represent each determinant. Intermediary inhibitors and inducers have also been shown around these four major players in this complex interaction. The cross sign (**X**) represents inhibition, killing, or attenuation of *Mtb*, or a physiological process or condition. The check mark (✓) represents the inducement, activation, increment, production, or enhancement of a process or physiological condition. ROS, reactive oxygen species; NO, nitric oxide; AMPs, antimicrobial peptides; PEG2, prostaglandin E2; atRA, all-trans retinoic acid; SCFAs, short-chain fatty acids; IPA, indole propionic acid; TNF, tumor necrosis factor; IL-10, interleukin 10; IFN-γ, interferon gamma.

Yet, its effect on H37Rv-induced IL-6, IFN-γ, and IL-22 production were non-significant. Thus, these SCFAs are not beneficial to the immune system in terms of TB, but beneficial to diabetes as IL-10 reduces insulin resistance and obesity. In the absence of IL-10, the SCFAs, particularly butyrate, were not able to reduce H37Rv-induced increment of TNF-α, IL-1β, and IL-17 production (these cytokines rather increased in the absence of IL-10), suggesting that IL-10 mediates SCFA activity on macrophages. Furthermore, SCFAs, specifically propionates and butyrates but not citrates, reduced LPS-induced production of TNF-α and IL-6, albeit all three SCFAs decreased IL-1β. Overall, butyrate was more potent in dose-dependently reducing H37Rv and LPS-induced cytokines production (Fig.2-3)[54].

This finding has been corroborated by Segal et al. (2017)'s report on the effect of SCFAs on CD^{4+}, CD^{8+} and Treg lymphocytes in ART (anti-retroviral therapy)-treated HIV patients with latent TB (Table 1)[17]. SCFAs inhibited IFN-γ and IL-17A production in Peripheral blood mononuclear cells (PBMCs) upon induction by TB antigens and increased Treg production from CD^{4+} and CD^{8+} via Foxp1 mediation (Fig. 1). Moreover, higher SCFAs detection/levels corresponded with the presence/abundance of anaerobes such as Prevotella, Veillonella and Haemophilus, whose activities generate SCFAs, suggesting that anaerobic commensals in the airways are a threat to TB eradication as their metabolites inhibit or dampen the effect of anti-TB cytokines [17,55]. Treatment of CD^{4+} and CD^{8+} with SCFAs resulted in drastic reduction in PPD (purified protein derivative)-stimulated IFN-γ (87%) and IL-17A (21%). Butyrate further directly inhibited CD^{4+} and CD^{8+} cytokine induction of Th1 and Th17, incapacitating the host against TB. From these findings, it is obvious that the lung microbiome's activities might rather advance TB than fight against it such that IFN-γ and IL-17A were very low in those who progressed to TB, but was high in those without TB [17].

HIV-infected persons had more blood SCFAs than non-HIV patients while Psychrobacter, Pseudomonas and Sphingomonas species were found in patients with non-detectable SCFAs in their BALs (bronchoalveolar lavage) [17]. Genes involved in the SCFAs metabolic pathway were found reduced in the microbiome of persons with detectable SCFAs, which could mean that the SCFAs catabolic genes were rather downregulated to keep SCFAs concentrations at high levels. In addition,

the level of SCFAs/anaerobes (Prevotella or Veillonella) were not positively correlating i.e. inversely correlated with the CD4+ concentrations in the oral and lower respiratory tract, which could be due to the destruction of these anaerobes at mucosal sites, making them unable to reach the blood. Nevertheless, a highly positive correlation existed between these regions (oral and lower respiratory tract) and blood CD^{4+} and CD^{8+} concentration, an association also observed by Luo et al. (2017) in new TB cases in China, albeit between the gut Prevotella and blood CD^{4+} concentrations [14].

Physiological concentrations of SCFAs (butyrate) drastically reduced the production of TB-induced IFN-γ and IL-17A, increasing patients' susceptibility/risk to TB and, directly increased Foxp1 expression in CD^{4+} and CD^{8+} cells (Fig. 2). Foxp1 is a repressor that represses lymphocyte development and inhibits the activation of B cells, CD^{4+} and CD^{8+} lymphocytes[17]. Per these findings, it is obvious that anaerobes and SCFAs are inimical to the immune system's fight against *M. tb*.

Coleman et al. (2018) recently established the autophagy-enhancing effect of all-trans retinoic acid (atRA), a metabolite of retinol (vitamin A) produced from retinal (first metabolite of vitamin A produced by retinol dehydrogenase mediated oxidation) by an enzyme called retinaldehyde (Fig. 2) [56]. atRA aided co-localization of *M. tb* to autophagosomes and lysosomes for autophagic destruction. This effect was observed in *M. tb* and *Bordetella pertussis*, but not in BCG, because BCG lacks the ESX secretory system that produces ESAT-6, which is detected in the cytosol by the STING/TBK1/IRF3 axis [56]. Physiological concentrations of atRA worked in both mice and human macrophages *ex-vivo*, although vitamin A supplementation has been found to be non-beneficial in preventing TB. Efficient intracellular *M. tb* annihilation was also observed after treating macrophages with retinal and retinol, suggesting that the enzymes to oxidise these into atRA exists within the macrophages [56].

Following up on the work by Coleman and colleagues (2018), O'Connor et al. (2018) designed atRA microcapsules using PLGA microparticulate carrier system (MP), and found that it reduced the lung pathology of *M. tb*-infected BALB/c mice as well as the *M. tb* burden/CFUs in the lung significantly [57]. This effect was also observed *in vitro*, where atRA reduced IL-10 production and stimulated the production of multi-nucleated giant cells (MNGCs) both in the presence and absence of

M. tb. MNGCs are important for forming granulomas that aid in enclosing *M. tb* from further spread throughout the lungs. The atRA-micro particles (MP) were administered intra-tracheally and were found to be as effective alone as when co-administered with rifampicin, suggesting that it can be used alone to mitigate *M. tb* infections. IL-10 prevents lysosome-autophagosome fusion and acidification; thus, its inhibition by atRA might account for the reduced *M. tb* burden in macrophages. atRA solution was also as efficient as the atRA-MP *in vitro*, albeit the atRA-MP targeted the atRA to the macrophages directly. Other authors have shown that atRA reduces cholesterol levels in macrophages as a means to starve the *M. tb* of essential nutrients, or increased ROS and induced autophagy in decimating phagocytosed *M. tb* [57].

Opposite of atRA, prostaglandin E2 (PGE2), an arachidonic acid-derived lipid mediator, promoted the growth of *M. tb* in macrophages by inhibiting the production of cathelicidin and vitamin D-mediated autophagy [58]. PGE2 inhibits vitamin D3-mediated increase in cathelicidin, vitamin D receptor expression, and autophagy in macrophages (Fig. 2)[58]. Arachidonic acid, the precursor of PGE2, is a polyunsaturated omega-6 fatty acid found in animal products or produced from linoleic acid in humans. It is associated with inflammatory mediators in humans, although its effect on TB is yet to be established.

Pathogens-immunity-M. tb interactions

The activities of certain pathogens cause dysbiosis, which subsequently predispose mice to *M. tb* infections. Specifically, mice pre-colonized with *Helicobacter hepaticus* experienced gut dysbiosis that made them susceptible to *M. tb.* Symptoms observed in such mice included subclinical inflammation, serious lung pathologies (lung tissue destruction), higher *M. tb* burden in lungs, higher inflammation and increased accumulation of activated T cells, caseous granuloma, disoriented innate immunity as shown in immune signatures in the lungs and increased morbidity/mortality (Table 1). These observations in *H. hepaticus*-infected mice are evidently indicative of more progressive *M. tb* infection than in non-colonized mice challenged with TB (Figure 1) [44,45].

Furthermore, mice infected with *H. hepaticus* could not be protected by Ad85A subunit vaccine immunisation while their littermates without *H. hepaticus* infection were protected by it. *H.*

hepaticus-mediated abolition of Ad85a immunisation's effect was due to increased IL-10 expression, without which the effect of *H. hepaticus* was silenced [44]. Among mice with *H. hepaticus* infection, there was reduced Ad85A antigen-specific CD^{8+} lung T cells than in non-infected mice, albeit the actual mechanism is unknown. Notably, while parenteral administration of environmental mycobacteria (EM) and BCG did not affect Ad85A protection, concurrent oral administration of EM and BCG reduced parenteral Ad85A protection, which could be due to intestinal microbiota mediation [44].

In addition, a shift to pro-inflammatory bacterial species (of the Bacteroidetes phylum with a concomitant reduction in Firmicutes), which might also play a role in the higher inflammation and reduced immune inhibition of *M. tb* in *H. hepaticus*-colonized mice, has been observed [45]. In sum, the presence of certain bacterial species in the gut can cause dysbiosis and subsequent immune dysregulation that can ultimately affect the immune system's capacity to defend the body against TB..

M. tb-microbiome interactions

Given the interrelationship between the microbiota and immunity, it would be expected that *M. tb* should effect dysbiosis. Specifically, through *M. tb's* immune dysregulation and anti-inflammatory effects when it blocks autophagy, IFN-γ and TNF-α production while increasing IL-10 production [59]. Yet, such an effect on the microbiota of TB patients remain to be seen as there is little evidence to show that *M. tb* results in substantial or significant chronic dysbiosis [4,5,35], which is contrary to that seen with antimicrobials and pathogens (*H. hepaticus*). This observation cuts across mice, pigs (wild boar), macaques and human microbiota, being confirmed by Chao1 and Shannon indices as well as un/weighted UniFrac analyses. *M. tb*'s effect on the gut-lung microbiome is relatively minor, insignificant and temporal [4–6,35,60]. Contrarily, administration of HRZ(E) had persistent effect on the gut microbiome several months after cessation of therapy [4].

The inability of *M. tb* to effect gut or lung dysbiosis is intriguing as other intracellular organisms such as *Haemophilus influenzae* and influenza A virus, which also have little or no interactions with the lung microbiome, can result in dysbiosis [61,62]. *M. tb*'s non-dysbiotic effect is

contrary to what was observed with pathogens such as *H. hepaticus* and diarrhoea-causing enterotoxigenic *E. coli* (ETEC) (Figure 1)[45,63].

Using macaques to study *M. tb*'s effect on the lower airway's (lung) microbiota, Cadena et al. (2018) compared *M. tb*-infected macaques with uninfected controls and studied them prospectively for four months without administering antimicrobials [15]. They observed an initial increase in the lungs microbial diversity one-month post infection, which returned to normal after five months. Moreover, the lung's microbiota diversity and abundance change were fairly constant except in selected macaques and among certain taxa (species) for which there were changes after 4 months of infection. For instance, SR1, Aggregatibacter, Leptotrichia, Prevotella, and Campylobacter increased in abundance while Lachnospiraceae reduced post *M. tb*-infection. *M. tb* infection further led to an overall reduction in microbiota connections while Actinomycetales, the order of *M. tb*, increased from three to nine connections, suggesting that upon infection with *M. tb*, there was an acute microbiota perturbation to allow for the establishment of the *M. tb* infection, after which the microbiota normalized. Notably, no specific species correlated with inflammation during the course of the infection, although important pathogens within Gammaproteobacteria such as Pseudomonas, Yersinia, Salmonella, Vibrio, and *Escherichia coli* became established with six new taxonomic interactions post *M. tb*-infection [15].

The authors suggested that the slow growing nature of *M. tb*, its presence in the lung parenchyma instead of the airways, its growth in granulomas that compartmentalise it, and the relatively lesser abundance and diversity of the lung microbiota might account for the little dysbiosis seen with *M. tb* infection. Oral microbes such as Aggregatibacter, Streptococcus, and Staphylococcus genera, which were enriched in the lungs post *M. tb* infection, might suggest their potential and increased movement from the oral to the lower respiratory microbiome although the underlying mechanism is still enigmatic [15].

Queiros et al. (2019) used metaproteomics to study the lymph node microbiota of wild boars (pigs)'s and found temporal reduction in the microbiome diversity in pigs having TB. Finding similar minor microbiota differences between healthy macaques and TB-infected ones, Namasivayam et al.

(2019) argued that it is best to rather study the microbiome at the individual level instead of between individuals. Particularly, this was due to the higher differences observed within individuals, by comparing their baseline microbiomes to their TB-infected ones, than that observed between individual Rhesus macaques [35]. This suggestion is laudable, particularly as individuals have different microbiomes, which might react differently to external perturbations. Possibly, studying the TB-microbiome at the individual level might produce better results than across individuals. In their work, they found a closer association between disease severity and microbiome structure than between individual monkeys or disease onset, suggesting that the structure of the microbiome in/directly affects disease outcome. Thus, the microbiota could be (1) a biomarker of disease outcome or (2) that disease(s) directly affect host factors, which indirectly affect the microbiome structure [35].

In a meta-analysis involving several studies of *M. tb*-infected lung microbiomes, Hong et al. (2018) identified distinct taxa associated with TB, which were however not the most dominant species in the microbiota. Taxa and species such as *Tumebacillus ginsengisoli*, *Propionibacterium acnes*, and *Haemophilus parahaemolyticus* were differentially abundant in healthy controls. *Deinococcus phoenicis*, *Kurthia gibsonii*, *Brevibacillus borstelensis*, *Caulobacter henricii*, *Actinomyces graevenitzii*, *Rothia mucilaginosa*, and *M. tb* were distinct signatures with increased abundance in *M. tb*-infected persons [64]. On the other hand, Clostridiales, *T. ginsengisoli*, *Pelomonas aquatica*, *P. acnes* and *H. parahaemolyticus* were reduced in *M. tb*-infected lung microbiota. Positively and strongly correlated with the presence of *M. tb* was *Rothia mucilaginosa*, a commensal of the upper respiratory tract. This species is known to opportunistically cause bacteraemia and pneumonia in immunocompromised patients, which could help facilitate *M. tb* establishment in the lungs or vice versa. The different studies had different results in terms of *M. tb*-mediated microbiome changes, which could be due to the different specimens and patients used, geographical location of the participants, sequencing platform and depth used etc. Evidently, this meta-analysis highlights the observation that *M. tb* causes limited species-level microbiota perturbations instead of substantial large-scale dysbiosis [64].

Nakhaee et al. (2018) have established a relationship between the incidence of TB, presence of TB symptoms such as night sweats, weight loss, fever, chest X-ray (CXR) rate, smear rate, BMI, and the type of lung microbiota species and Th1/Th2 immune response. The presence and abundance of *Streptococcus spp*. positively correlated with TB incidence than with non-TB patients, and the presence of *Neisseria spp.* and *Haemophilus spp.* could also affect Th1 response in TB patients due to their positive correlation with that group than among healthy persons. Meanwhile, a lower prevalence of Streptococcus and Neisseria among healthy patients were associated with a Th1-response. Furthermore, it was observed that fever was associated with the abundance of Neisseria and Veillonella in the lung microbiota while lung Th1/Th2 responses were affected by chest X-ray. TB patients with reduced weight had higher Th1 responses [55].

Studies such as that by Nakhaee et al. (2018), and Segal et a. (2018) try to establish the effect of the lung microbiota and metabolite composition on the outcome of TB infections as well as on the reaction of the immune system to TB (Table 1, Fig. 2-3) [17,47,55]. In an earlier study, Winglee et al. (2014) showed that pulmonary *M. tb* infections affected the gut microbiota in mice, albeit fewer or no *M. tb* cells were found in the gut [9]. The dysbiosis was thus shown to be more as a result of the immune response to the *M. tb* infection than to the presence of *M. tb* in the gut, indicating the close relationship between the immune system and the lung-gut microbiome. The authors suggested that direct immune signaling from the lung to the gut might have resulted in significant differences in the relative abundance of Lachnospiraceae and Ruminococcaceae families (Clostridiales) as well as of Bacteroidales in uninfected and pre-infected mice compared to infected mice. Notably, some members of the Clostridiales and Bacteroidales families, such as *Clostridium spp.* and *Bacteriodes spp*. respectively, are known to induce regulatory T (Treg) cells and activate the NF-kB pathway, induce IL-10-producing T cells and modulate the T-helper type 1/2 (Th1/Th2) balance [9].

Huang et al. (2019) also recently reported on the impact of active TB on the gut microbiota and systemic inflammation in non-HIV patients: dysbiosis favouring higher bacteriodetes and lower abundance of firmicutes was associated with higher systemic pro-inflammatory mediators [65].

Although not yet established, the ability of the microbiome to force *M. tb* into latency in newly exposed individuals deserve especial attention and investigation.

Smoking-microbiome-M. tb interactions

Smoking has been identified as a causal factor of dysbiosis in the oral, lung and gut microbiota [28], with cigarette smoke and cigarette smoking being found to escalate TB [66]. Besides the direct inhibition of macrophagic killing of *M. tb* by pure nicotine, the destruction of *M. tb* in macrophages is also impaired. It does this by directly inhibiting autophagy through NF-KB activation and increasing production of both IL-10 and urea by Tregs; thus, increasing intracellular *M. tb* concentrations and orienting macrophages to the M2 phenotype. Nicotine also inhibits apoptosis to prevent *M. tb* destruction (Fig. 2) [66]. Notwithstanding, nicotine failed to inhibit macrophagic killing of *M. tb in* the presence of nicotinic receptor inhibitors and in macrophages lacking nicotinic receptor components. THP-1 and Treg cells exposed to nicotine respectively inhibited autophagy and greatly suppressed macrophage-mediated *M. tb* killing than unexposed ones [66].

Diet-microbiome-M. tb interactions

Finally, whereas the negative effect of antimicrobials on the microbiome and immunity has been widely described, the beneficial role of certain food components on the microbiome and immunity are also being realized; a finding that can be harnessed to correct pathogen and antimicrobial-induced dysbiosis. Wang et al. (2017) showed that apple polysaccharides (AP) corrected dysbiosis caused by a high-fat diet, with a subsequent reduction in Firmicutes, Fusobacterium, and inflammatory mediators such as TNF-α, monocyte chemotactic protein 1 (MCP-1), chemokine ligand 1 (CXCL-1) and interleukin 1 beta (IL-1β) [67]. Moreover, the AP also increased SCFAs production and enriched Bacteriodetes and Lactobacillus, resulting in reduced gut permeability through enhanced autophagy in goblet cells and controlled/reduced chronic inflammation [67]. Besides SCFAs that are known to be inimical to the immune system's fight against *M. tb* [54], the AP effects are indicative of the benefits certain foods have on the health of the microbiota and gut immunity.

The influence of diet on the microbiome can thus have an indirect effect on *M. tb* susceptibility. Particularly, vitamins and minerals such as vitamin D and calcium have been associated

with resistance to *M. tb* [56,68]. Specifically, Ca^{2+} activates autophagy while INH and PZA increased intracellular Ca^{2+} in both *M. tb*-infected and non-infected macrophages; in the absence of Ca^{2+} however, autophagy reduced [40]. These suggest that Ca^{2+} is essential for antimicrobial-mediated autophagy in macrophages (Fig. 2). The role of Ca^{2+} in autophagy also corroborates the role of Vitamin D, which helps the body absorb Ca^{2+}, in autophagy and further necessitates the need to investigate diet-microbiome interactions and effects on TB [40,59,69].

PBMCs from TB patients with and without cavitary disease as well as healthy controls were exposed to 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]. These cohorts were assessed for their expression of mannose receptor (CD206) and DC-SIGN (CD209), as well as autophagy proteins such as ATG5 and Beclin-1 (BECN1). Whereas DC-SIGN receptors were downregulated in all infected macrophages/monocytes, mannose receptors and autophagy genes i.e. BECN1 and ATG5 were upregulated. Notwithstanding, the upregulated CD206 mannose receptor and autophagy proteins were comparatively lower in PBMCs from TB patients than in those from healthy controls. Nevertheless, this upregulation was impaired in cells from TB patients with cavitary disease. The hyper-expression of autophagy proteins, ATG5 and BECN1, highly correlated with phagocytosis and expression of cathelicidin in all PBMCs [68]. This finding thus suggests that vitamin D supplementation could be of benefit to TB patients without cavitary disease due to its direct effect on autophagy, phagocytosis and AMPs production.

3. Challenges and future perspectives

Lung and GIT microbiome studies of TB patients are relatively few. This is particularly true for lung microbiome studies due to the invasive nature of collecting lung (BAL) samples. Moreover, most available TB microbiome works are undertaken using 16S sequencing, which is biased towards already known species, instead of shot-gun metagenomics. In addition, meta-transcriptomics analysis of TB patients' or animal models' lung-gut microbiomes are relatively few. These limit the number of pertinent studies needed to form impeccable conclusions on the *M. tb*-lung-gut-microbiota and immunity interactions as well as the microbiota's metabolic dynamics. Coupled with these challenges is the limitation of available technology that can resolve the microbiota to the species and strain level

after metagenomic sequencing. Moreover, available bioinformatic tools have their own inherent challenges with binning and species identification from metagenomic data [1].

While short read sequencers have higher throughput than long-read sequencers, they are unable to provide species-level and strain-level resolution of the microbiota, which further limits downstream analysis and identification of specific species or strain biomarkers associated with *M. tb* infections in the lung and GIT. Long read sequencers such as PacBio's SMRT II and Oxford Nanopore's MinIon can provide species-level and strain level microbiota resolution, but their low throughput increases their error rates, which makes hybrid sequencing the method of choice for comprehensive microbiome analysis [70,71].

Hybrid sequencing, involving the combination of long-read and short-read sequencing, has been shown to produce better metagenomic reads length and coverage/depth than using single sequencing platforms [71,72]. Although expensive, hybrid sequencing yields better metagenomic and transcriptomic sequence reads for a better resolution of the sputum/BAL and gut microbiome species as well as gene expression profiles to elucidate the interactions between *M. tb*, the immune system and commensals.

One major area of interest is the interaction of the *M. tb* bacilli with lung and gut microbiota before it is phagocytosed by macrophages or DCs. Until recently, *M. tb* was not known to benefit from horizontal gene transfer [73]. Nevertheless, Mehra et al. (2016) recently identified drug resistance ABC (efflux) transporters and important virulence factors/genes such as the ESX-1 transport machinery, including ESAT-6 and CFP-10, and ESX-5 regions of the type-VII secretion system (T7SS) on a genomic island in *M. tb* genomes, suggesting their acquisition from other bacteria[73]. Thus, by using hybrid sequencing to obtain high resolution metagenomic data, there is the possibility of determining the potential exchange of genetic material between *M. tb* and the microbiota before phagocytosis, including the potential roles those acquired genes play in pathogenicity, persistence/dormancy, and resistance.

More advanced studies incorporating hybrid sequencing and Cappable-seq [74,75] approaches in both metagenomic and metatranscriptomic sequencing of TB patients' microbiota are needed to bring clarity to many unanswered questions surrounding the lung-gut microbiome, antimicrobial and immunity cross-talks in TB patients. Moreover, there is the need to also include blood specimens of such cohorts to enable further analysis of immune cells and inflammatory mediators' dynamics during TB infection.

A greater challenge to TB microbiome work however, is efficient sampling of the lung microbiome as available methods are either non-representative or too invasive with potential risk for the patient. For example, the use of sputum or saliva has been shown to be non-representative of the lower respiratory tract or can be contaminated with oral microbiome. The use of BAL fluid and/or bronchoscopial brush specimen is highly invasive and could be also contaminated with pharyngeal or oral microbiota, albeit it is the most representative specimen of the lung microbiota [10]. As well, mice models used to study TB-microbiome interactions have been suggested to be an inefficient model [35].

The need for microbiota-M. tb dormancy and persistence interaction research

Another important area of research in tuberculosis is dormancy and persistence, particularly as most TB-exposed or –treated persons develop latent TB, which can be revived into active disease during old age or immunocompromised conditions. Although a direct effect of the microbiome on dormancy in *M. tb* has not been described, it is not impossible as the microbiome is known to increase macrophage killing and ROS and NO production through NOD-1 and TLR signalling [8,19]. Thus, the ability of NO and ROS to cause dormancy through DosR (DevR) activation in *M. tb* should incite further research into this possibility of the microbiome influencing *M. tb*'s activation of dormancy [38,76]. Furthermore, the ability of nutrient starvation and hypoxia to cause dormancy reins in autophagy and ROS as these two processes are triggered by or cause nutrient starvation, respectively [76,77]. VapBC, a critical component of the Toxin-Antitoxin system (TAS) involved in dormancy state activation, is triggered by IFN or hypoxia, which associates VapBC with ROS and Autophagy [76]. The ability of antimicrobials to also induce MazEF, another component of the TAS that activates

dormancy, and TAC (toxin-antitoxin-chaperones) in *M. tb* within macrophages, ropes in antimicrobials treatment as a potential factor in activating dormancy [76].

We thus hypothesize that the microbiome could indirectly activate dormancy in *M. tb* through NOD-1/TLR signalling, ROS, NO and IFN production as well as autophagy activation (Figures 2-3). This hypothesis needs to be tested experimentally to establish this connection and open new areas of research into microbiota-*M. tb* interactions. Towards this goal, the use of metatranscriptomics (RNA-seq) instead of microarrays as a microbiomics tool cannot be overlooked. Several studies comparing the transcriptional profiles of *M. tb* in sputum [78–80], blood of HIV^- and HIV^+ patients [81], human type II alveolar epithelial cell lines [82] etc. have been conducted, mainly with microarrays, to obtain differential gene expressions of *M. tb* under different environments. In all these studies, it has been established that different genes and transcription factors are either hyper-expressed or repressed to enable *M. tb* either establish an overt infection or revert into dormancy and persistence. However, these differential expressions of dormancy or persistence genes in the context of microbiota interactions is yet to be resolved.

For instance, Chatterjee et al. (2013) studied the transcriptional profiles of the same *M. tb* strains or lineages in patients undergoing DOTS. Although the patients were compliant, they developed MDR-TB, which was found from the transcriptional profiling to be due to the upregulation of drug efflux pumps, ABC transporters, trans-membrane proteins and stress response transcriptional factors (*whiB*) [80]. Meanwhile, there was downregulation of transcription factors (*sig, rpoB*), cell wall biosynthesis (*emb*), protein synthesis (*rpl*) and additional central metabolic pathways (*ppdK, pknH, pfkB*) genes compared to the wild-type controls. Thus, instead of the expected mutations in known antitubercular drug-resistance-conferring genes, the MDR-TB in this study used efflux pumps, ABC transporters, and transmembrane proteins hyper expression, and lower expression of several central metabolic pathway as well as of DNA repair and stability genes such as *rec, uvr, ruv* and *lig*. While the efflux and other transporter proteins exuded the antimicrobials, the lower metabolism activity ensured persistence and dormancy. Further, the repression of DNA repair genes allowed for the selection of a mutated and resistant phenotype [80].

A similar finding by Sharma et al. (2017) in expectorated sputum from TB patients was recently reported. The authors found lower expression rates of ATP synthase, ribosomal proteins, virulence-associated genes encoding proteins such as PDIM, PGL, ESAT-6 and CFP-10, as well as protein export genes such as the type VII secretion system components including certain ESX-1, -3 and -5 genes. Interestingly though, the dormancy-associated genes, *dosR* and *dosS,* were not entirely repressed while the persistence-associated gene, *mprA*, was highly expressed [83]. In another study, a higher expression of aerobic respiration, protein synthesis, energy production and ESAT-6-like genes, and a significant repression of the DevR (DosR) regulon, hypoxia-induced genes and genes involved in nitrate reduction and transport (non-aerobic respiration) were found in *M. tb* cells growing in type II alveolar epithelial cells [82]. In all these, the association between the *M. tb* expression profile vis-à-vis that of the microbiota was not assessed.

Hyper-expression of virulence factors such as ESAT-6, alteration in mode of iron acquisition, potential evasion of immune surveillance, suppression of dormancy, and induction of cell-wall remodelling were observed in transcripts of *M. tb* growing in both HIV^- and HIV^+ blood, albeit the expression levels were higher in HIV^+ blood than the former. Hence, *M. tb* grew quickly in blood with repression of *mprAB* persistence and DevR (DosR) dormancy genes, and upregulation of PDIM and PGL virulence genes etc., suggesting that the *M. tb* cell can distinguish between environments and easily coordinate its responses to colonise the host [81]. These findings indicate that *M. tb* in sputum hides its virulence factors and conserves energy in a near-dormant state to evade the hosts' immune factors. Therefore, genes that were hyper-expressed in these sputum *M. tb* strains have been suggested as potential biomarkers for the design of novel nucleic acid diagnostics [83].

It would also be of great interest to determine the expression profile of *M. tb* in the midst of several bacterial commensals instead of in macrophages. *M. tb* mutants lacking the above-mentioned essential virulence, transport, stress etc. genes can also be exploited by growing them in alveolar or macrophage cell lines to identify novel drug targets and potential biomarkers for vaccine and diagnostics design. Bukka et al. (2012) for instance, showed that transcripts of the ESAT-6 subfamily genes *esxKL* and *esxJI*, were differentially expressed under different growth conditions, with Δ*esxKL*

mutants failing to grow or growing relatively slowly in macrophages [78]. Upon the addition of a wild-type *M. tb* cell to the macrophage cell culture, the Δ*esxKL* recovered their normal growth rates as measured in bacterial colony forming units (CFU)/mL. Thus, the potential of *M. tb* to secrete and transport growth and virulence factors from one macrophage to another as a means of communication and coordination of infection in the granuloma was suggested. This study shows the importance of undertaking mutational analysis of hyper-expressed or repressed genes to characterize *M. tb* pathogenesis, persistence and resistance. More importantly however, would be the analysis of *M. tb* expression profiles in connection with surrounding microbiota in cavitaceous and non-cavitaceous granulomas.

4. Conclusion

Available evidence suggests that *M. tb* has little impact on airway and gut microbiota. Although studies on TB microbiome are relatively few due to challenges in obtaining lung samples, available bioinformatic tools also hamper efficient resolution of microbiome data. Nevertheless, important successes have been chalked so far, with most studies agreeing to the substantial and long-term impact of TB antimicrobials on the gut microbial ecology and immunity, which could predispose a cured patient to another TB reinfection. Notwithstanding, antimicrobials activated autophagy and improved phagocytosis in macrophages, induced ROS and NO production, and prevented *M. tb* phagosome escape in macrophages. The ability of INH and PZA to pre-dispose mice to TB is concerning as it means patients placed on anti-TB prophylaxis and those on antitubercular drugs are being disadvantaged as their alveolar macrophages are unable to efficiently clear *M. tb.* The dysbiotic effect of antimicrobials and their subsequent immune dysregulation also put persons on antibiotics at high risk of getting TB.

While the absence of autophagy caused dysbiosis and hyper-inflammation, dysbiosis in turn induced autophagy. Autophagy is also hampered by nicotine in cigarette smoke, SCFAs produced by anaerobes in the microbiota, glucocorticoids and PGE2, while it was induced by commensals' LPS, vitamin D, and atRA. AMP and gut metabolites such as IPA were found to be more anti-mycobacterial, giving promise to the potential availability of anti-mycobacterial molecules in the

microbiome. Hybrid sequencing of TB patients' microbiome plus meta-transcriptomics using RNA-seq and Cappable-seq holds much promise for unravelling the seeming conundrum surrounding *M. tb*-microbiota-immunity–antimicrobials interactions and discover potential drug targets, diagnostic biomarkers and vaccine antigens.

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Figure 1. Factors affecting the gut microbiome and their indirect effect on *M. tuberculosis* pathogenesis.

Figure. 2. The Lung-Gut microbiome cross-talk. Microbiota disturbances in the gut affects immune function in the lungs, increasing susceptibility to M. tuberculosis. Restoration of gut eubiosis however, increases host resistance to *M. tuberculosis.*

Figure 3. A diagrammatic representation of *Mycobacterium tuberculosis*-microbiota-antimicrobials-immunity (autophagy) cross-talk. Coloured lines have been used to show the interconnections or cross-talk between antimicrobials, autophagy (immunity), the microbiota (in a dysbiotic state), and *M. tuberculosis,* with each colour chosen to represent each determinant. Intermediary inhibitors and inducers have also been shown around these four major players in this complex interaction. The cross sign (**X**) represents inhibition, killing, or attenuation of *M. tuberculosis,* or a physiological process or condition. The tick sign (✓) represents the inducement, activation, increment, production or enhancement of a process or physiological condition. ROS is reaction oxygen species, NO is nitric oxide, AMPs is antimicrobial peptides, PEG2 is prostaglandin E2, atRA is all trans retinoic acid, SCFA is short chain fatty acids, IPA is indole propionic acid, TNF is tumour necrosis factor, IL-10 is interleukin 10, IFN-γ is interferon gamma