

# A murine model of tuberculosis/type 2 diabetes comorbidity for investigating the microbiome, metabolome and associated immune parameters

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## Abstract

Tuberculosis (TB) is one of the deadliest infectious diseases in the world. The metabolic disease type 2 diabetes (T2D) significantly increases the risk of developing active TB. Effective new TB vaccine candidates and novel therapeutic interventions are required to meet the challenges of global TB eradication. Recent evidence suggests that the microbiota plays a significant role in how the host responds to infection, injury and neoplastic changes. Animal models that closely reflect human physiology are crucial in assessing new treatments and to decipher the underlying immunological defects responsible for increased TB susceptibility in comorbid patients. In this study, using a diet-induced murine T2D model that reflects the etiopathogenesis of clinical T2D and increased TB susceptibility, we investigated how the intestinal microbiota may impact the development of T2D, and how the gut microbial composition changes following a very low-dose aerosol infection with *Mycobacterium tuberculosis* (*Mtb*). Our data revealed a substantial intestinal microbiota dysbiosis in T2D mice compared to non-diabetic animals. The observed differences were comparable to previous clinical reports in TB patients, in which it was shown that *Mtb* infection causes rapid loss of microbial diversity. Furthermore, diversity index and principle component analyses demonstrated distinct clustering of *Mtb*-infected non-diabetic mice vs. *Mtb*-infected T2D mice. Our findings support a broad applicability of T2D mice as a tractable small animal model for studying distinct immune parameters, microbiota and the immune-metabolome of TB/T2D comorbidity. This model may also enable answers to be found to critical outstanding questions about targeted interventions of the gut microbiota and the gut-lung axis.

## KEYWORDS

gut microbiota, host microbe interaction, infectious diseases, tuberculosis, type 2 diabetes

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## 1 | INTRODUCTION

Tuberculosis (TB) is a serious global public health concern. Despite combined international efforts to halt the TB epidemic by 2030, nearly 1.5 million people still lose their lives to *Mycobacterium tuberculosis* (*Mtb*) infections every year, making TB the world's leading infectious killer.<sup>1</sup> In addition, one-quarter of the global population has latent TB infection (LTBI).<sup>1</sup> The absence of an effective life-long vaccine, the emergence of drug-resistant *Mtb* strains and comorbid immunosuppressive conditions pose a challenge to the current global TB eradication commitments.<sup>2</sup> Recent studies reported that people living with the metabolic disease type 2 diabetes (T2D) have a 3- to 4-fold increased risk of developing active TB<sup>3</sup> and that its impact on the global TB burden is much higher than human immunodeficiency virus (HIV) coinfection, the most potent known risk factor for TB.<sup>4</sup> The link between TB and T2D is disquieting, as the global incidence of diabetes is rapidly increasing and predicted to reach 629 million by 2045, representing a large repository of potential active TB cases and LTBI reactivators.<sup>5</sup> Whilst we and others have found a number of immune defects associated with elevated TB risk using multiple animal models of diabetes,<sup>6,7</sup> the exact underlying mechanisms are not yet fully understood.

A growing body of evidence suggests a role for microbiota, especially gut or intestinal microbiota, in the development and progression of a number of chronic disease conditions, including T2D.<sup>8</sup> Significantly, it has become increasingly clear that the microorganisms living on host mucosal surfaces play a substantial role in shaping the anti-TB immune response, thus influencing the disease susceptibility and outcome.<sup>9</sup> In a more recent study, we evaluated whether an altered lung microbiota in T2D is associated with an increased susceptibility to aerosolized *Mtb*.<sup>6</sup> The microbial composition in murine T2D lungs was largely comparable to non-diabetic (ND) mice, suggesting that lung microbiota alone may not directly facilitate the increased TB susceptibility.<sup>6</sup> Using a tractable model of diet-induced murine T2D which encompasses key metabolic, immunological and pathophysiological features of human T2D,<sup>10</sup> in this study we investigated how the intestinal microbiota changes during the development of T2D and how it impacts on the development of TB. Our findings indicate that in line with observations obtained from humans with T2D,<sup>11</sup> diabetic mice have a significantly altered gut microbiota and that the microbial diversity in T2D is further reduced following aerosol *Mtb* infection. Collectively, these findings further support the utility of our T2D mice as a tractable small animal model for studying immune parameters, microbiota and the immunometabolome in TB/T2D comorbidity.

## 2 | METHODS

### 2.1 | Ethics

All the animal experimental procedures were approved by the animal ethics committee (A2400) of James Cook University, Australia

and conducted in accordance with the National Health & Medical Research Council (NHMRC) animal care guidelines.

### 2.2 | Diet-induced murine T2D model

This murine T2D model was developed and extensively characterized using male C57BL/6 mice.<sup>10</sup> Estrogen protects female mice from developing high-fat diet induced adipocyte hypertrophy and liver steatosis and from becoming insulin resistant<sup>12</sup> and thus female mice were not included. Four- to six-week-old male mice were randomly divided into two groups and fed either an energy dense diet (EDD, SF03-030, Specialty Feeds, Western Australia; high glycemic index semi-pure rodent diet containing 23% fat, 19.4% protein, 50.5% dextrose, and 7.5% fiber) or an isometric quantity of standard rodent diet (SD, SF08-020, Standard AIN93M rodent diet containing 4% fat, 13.6% protein, 64.3% carbohydrate, and 9.4% fiber) for 30 weeks (Figure 1A). Animals were then assessed for body weight, fasting blood glucose (FBG), plasma HbA1c and glucose tolerance to confirm the status of diabetes.<sup>10</sup> To measure FBG levels, mice were fasted for 6 hours prior to the test. Similarly, for the glucose tolerance test (GTT), the baseline blood glucose levels were measured on 6-hour fasted mice. Glucose solution (100  $\mu$ L of 2 g/kg) was administered IP using a 27G needle and blood glucose levels were measured at 15-, 30-, 60- and 120-minute post administration intervals. Areas under the curve (AUC) from GTT readings were calculated in Graphpad Prism using the trapezoid rule;  $AUC = \Delta X * ((Y1 + Y2) / 2) - \text{Baseline}$ .

### 2.3 | *Mtb* culture and aerosol infection

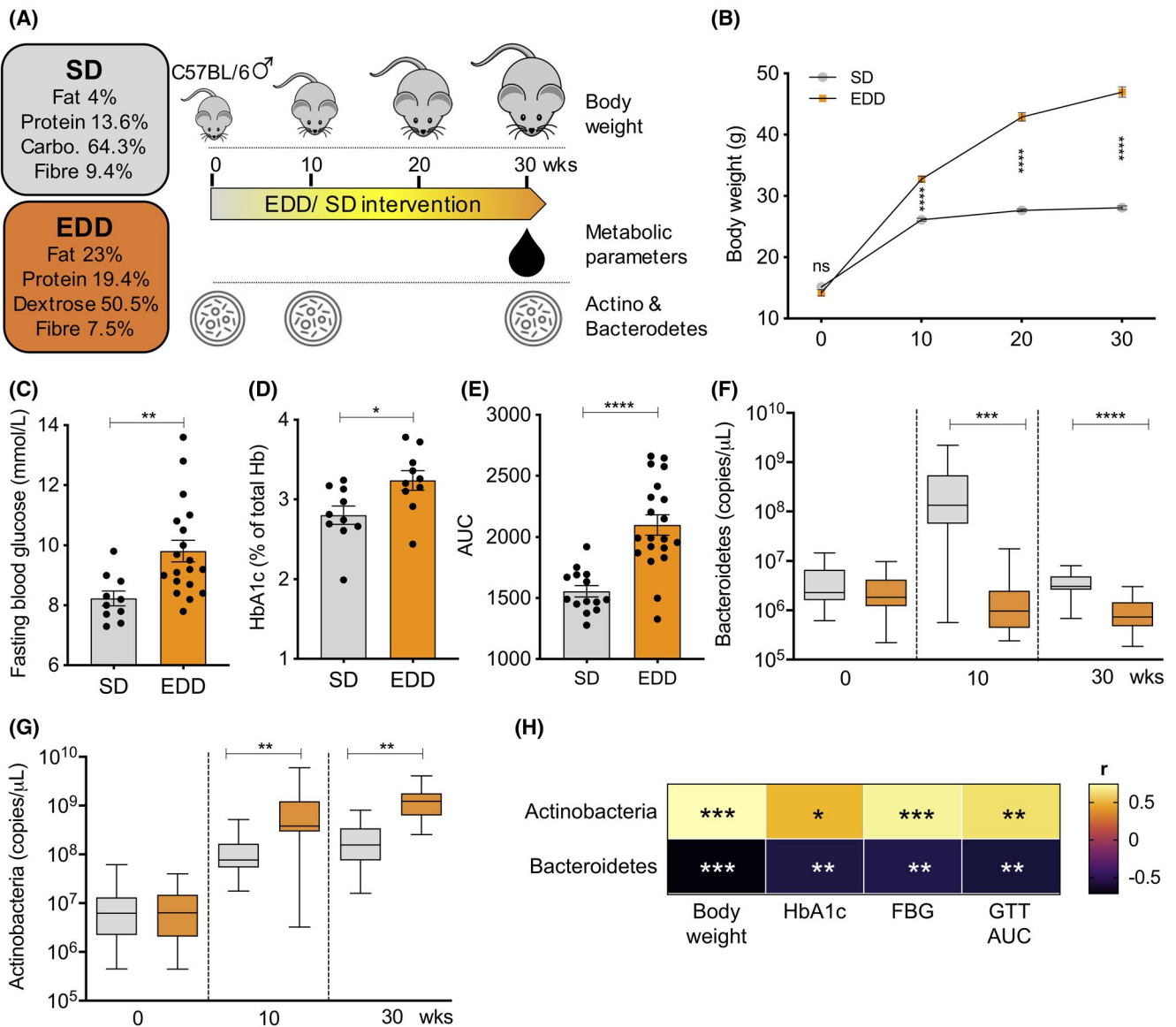
*Mtb* H37Rv were cultured in 10% ADC enriched Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.2% glycerol and 0.05% Tween 80 until mid-logarithmic phase of growth. Animals were subjected to a very low-dose (10-20 CFUs) of aerosol *Mtb* infection using a Glas-Col inhalation exposure system.

### 2.4 | CFU enumeration

At multiple time points post infection (p.i.), serial dilutions of lung homogenates in sampling bags containing 1 mL of sterile PBS/0.05% Tween 80 were plated on 10% OADC enriched 7H11 agar plates supplemented with 10  $\mu$ g/ $\mu$ L cycloheximide and 20  $\mu$ g/mL ampicillin and incubated for 3-4 weeks at 37°C to enumerate organ bacterial loads.

### 2.5 | DNA extraction, qPCR analysis and 16S rRNA microbiome sequencing

Fecal samples from both SD- and EDD-fed mice were collected at designated time points during the diet intervention and DNA was extracted using the PowerSoil DNA extraction kit (MoBio Laboratories)



following manufacturer's instructions for qPCR analysis. The number of Actinobacteria and Bacteroidetes copies within each DNA sample was quantified using sequence specific probes and PCR primers (Table S1; Macrogen Inc, South Korea) in ImmoMix reaction mix (Bioline, Australia). In addition, fecal samples collected prior (naïve) and 45 days p.i. (*Mtb*) were subjected to comprehensive microbial profiling using 16S rRNA microbiome sequencing. Bacterial DNA was extracted with a commercially available Microbiome DNA purification kit (Invitrogen), purity assessed using NanoDrop (Thermo Scientific),

quantified with a Qubit 4.0 Fluorometer (Invitrogen) and the final DNA concentration was adjusted to 5 ng/ $\mu$ L. The 16S rRNA gene encompassing the V5-V8 regions was targeted using the 803F and 1392wR primers<sup>13</sup> modified to contain an Illumina specific adapter sequence. Preparation of the 16S library was performed as per the workflow outlined by Illumina. Indexed amplicons were pooled together in equimolar concentrations and sequenced on a MiSeq Sequencing System (Illumina) using paired end sequencing with V3 300 bp chemistry at the Australian Centre for Ecogenomics (ACE)

according to established protocols. Sequence data was analyzed as previously described<sup>6</sup> using a modified version of MetaDEGalaxy.<sup>14</sup>

## 2.6 | Statistics

Student's *t* test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test in GraphPad Prism version 8 were primarily used for group comparisons. Pearson's correlation coefficient was used to assess the association between fecal bacterial concentrations and metabolic variables.  $P < .05$  was considered significant.

## 3 | RESULTS

To induce murine T2D, C57BL/6 male mice were subjected to an extended period of diet intervention (Figure 1A). Mice that received ad libitum access to EDD gained weight more rapidly than SD-fed mice (Figure 1B). After 30 weeks, EDD-fed mice had a significantly higher body mass compared to SD-fed mice (Figure 1B, SD,  $28.08 \pm 0.2889$  g vs EDD,  $46.96 \pm 0.8504$  g; mean  $\pm$  SEM,  $P < .0001$ ). Significantly increased levels of fasting blood glucose (Figure 1C, SD,  $8.230 \pm 0.2463$  mmol/L vs EDD,  $9.811 \pm 0.3614$  mmol/L; mean  $\pm$  SEM,  $P = .0062$ ) and glycosylated hemoglobin (HbA1c) (Figure 1D, SD,  $2.801 \pm 0.1153\%$  vs EDD,  $3.238 \pm 0.1231\%$ ; mean  $\pm$  SEM,  $P = .0184$ ) are indications of poorly controlled diabetes in EDD-fed mice. Moreover, these mice had impaired glucose tolerance as reflected by significantly higher AUCs from the glucose tolerance test (GTT) compared to SD-fed mice (Figure 1E, SD,  $1556 \pm 46.66$  vs EDD,  $2098 \pm 82.53$ ; mean  $\pm$  SEM,  $P < .0001$ ), a key metabolic feature associated with the development of clinical T2D.

We next assessed the impact of diet on two of the five phyla of bacteria present in the gut, namely Actinobacteria and Bacteroidetes, during the 30-week diet intervention period (Figure 1F,G). There was no significant difference in Bacteroidetes abundance between the EDD- and the SD-fed mice at baseline (0 weeks;  $P = .1381$ ). After 10 weeks of diet intervention a significant difference in Bacteroidetes levels had developed, with mice consuming the SD having more Bacteroidetes present in the feces than mice on the EDD ( $P = .0009$ ). After 30 weeks, the Bacteroidetes levels remained significantly higher in SD-fed mice than in EDD-fed mice ( $P < .0001$ ) (Figure 1F). In contrast to Bacteroidetes, significantly more copies of Actinobacteria were present in the feces of EDD-fed mice compared to SD-fed mice after

10 and 30 weeks of diet intervention (Figure 1G). Pearson's correlation coefficient test revealed a negative correlation between Bacteroidetes abundance and (a) body weight ( $r = -.7157$ ,  $P = .0004$ ), (b) fasting blood glucose ( $r = -.5738$ ,  $P = .0082$ ), (c) HbA1c ( $r = -.5859$ ,  $P = .0066$ ), and (d) GTT AUC ( $r = -.6108$ ,  $P = .0042$ ) (Figure 1H & Figure S1). However, a positive relationship was observed between Actinobacteria abundance and (a) body weight ( $r = .7394$ ,  $P = .0002$ ), (b) fasting blood glucose ( $r = .6912$ ,  $P = .0007$ ), (c) HbA1c ( $r = -.4692$ ,  $P = .0369$ ), and (d) GTT AUC ( $r = .6179$ ,  $P = .0037$ ) (Figure 1H & Figure S1).

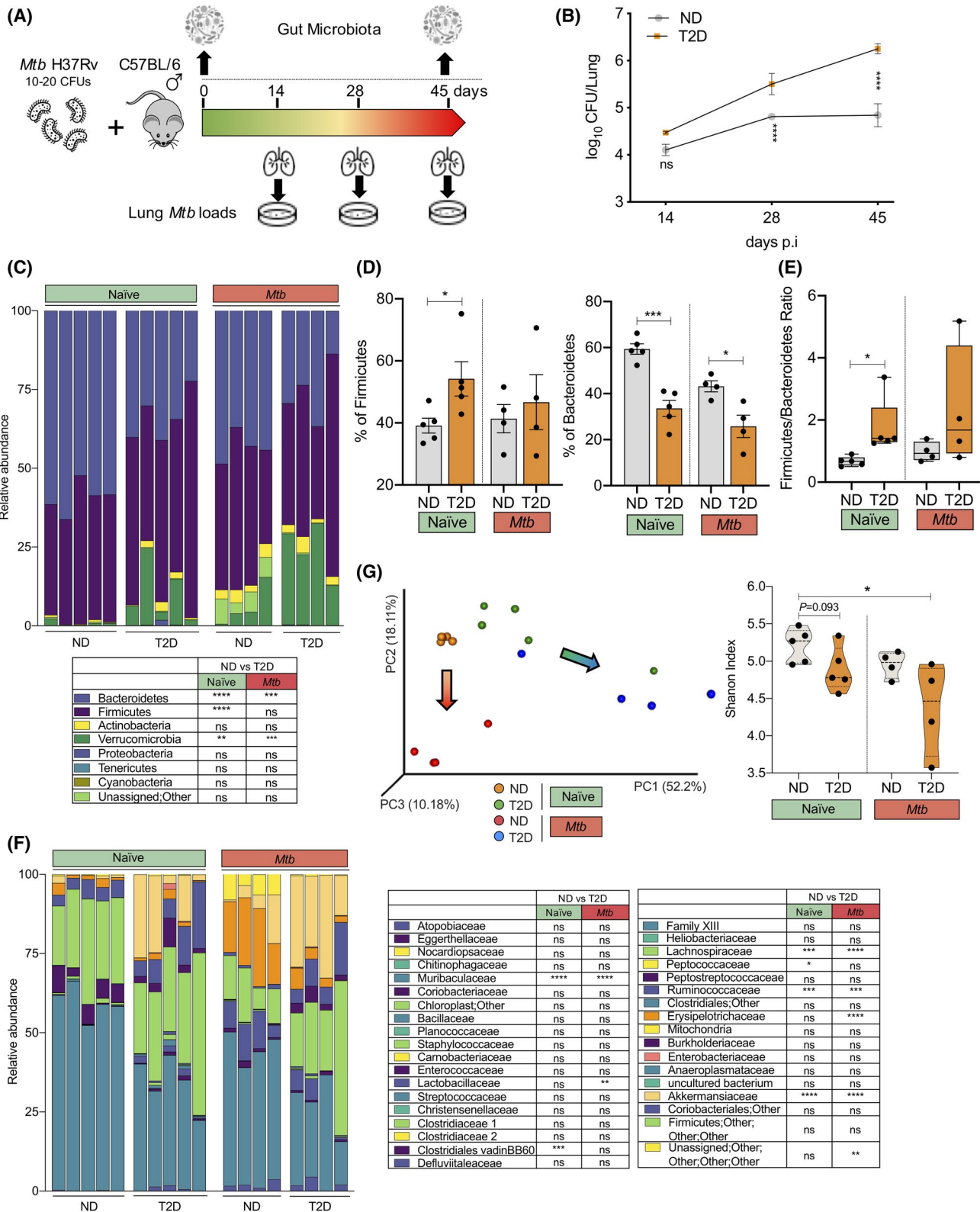
To further dissect the role of the intestinal microbiota in increased TB susceptibility in T2D hosts, following 30 weeks of diet intervention, confirmed T2D and ND control mice were infected with aerosolized *Mtb* (Figure 2A). Whilst the lung *Mtb* CFU levels in both ND and T2D mice were mostly comparable at 14 days p.i., *Mtb*-infected T2D lungs had a significantly higher *Mtb* burden at 28 and 45 days p.i. (Figure 2B; Day 28 = ND,  $\log_{10} 4.813 \pm 0.0481$  vs T2D,  $\log_{10} 5.502 \pm 0.2255$ ; mean  $\pm$  SEM,  $P = .0174$ ; Day 45 = ND,  $\log_{10} 4.842 \pm 0.2436$  vs T2D,  $\log_{10} 6.252 \pm 0.1062$ ; mean  $\pm$  SEM,  $P = .0007$ ), indicating increased TB susceptibility in T2D mice. In addition to lungs, we have previously shown that T2D mice also had substantially higher bacterial burdens in both spleen and liver compared to ND mice.<sup>6,15,16</sup> Increased bacterial burdens were also reflected in significantly higher lung and liver immunopathology in T2D hosts.

We next investigated how aerosol *Mtb* infection alters the intestinal microbiota in both ND and T2D mice. Comprehensive 16S rRNA-based microbiome profiling revealed marked alternations in three main phyla of intestinal microbiota in uninfected naïve T2D mice compared to ND mice (Figure 2C). Naïve T2D mice harbored a significantly higher percentage of Firmicutes ( $P < .0001$ ) and a lower level of Bacteroidetes ( $P < .0001$ ) (Figure 2D) leading to an elevated Firmicutes to Bacteroidetes (F/B) ratio (Figure 2E) compared to ND mice. Moreover, 6 differentially abundant families between ND and T2D were detected prior to *Mtb* infection (Figure 2F). Among which, 4 families – Akkermansiaceae, Ruminococcaceae, Lachnospiraceae and Peptostreptococcaceae – were highly abundant in T2D guts compared to ND mice. However, the reverse was true for the Muribaculaceae and Clostridiales vadinBB60 families, which were significantly more abundant in ND mice. Principal component analysis (PCA) of the operational taxonomy units (OTU; species found in fecal samples) also revealed a distinct clustering of naïve-ND (orange) and naïve-T2D (green) samples (Figure 2G). Although not significant, naïve T2D mice had a substantially lower overall gut microbial diversity compared to ND mice, as reflected in the Shannon diversity index (Figure 2G).

**FIGURE 2** Type 2 diabetes and aerosol *Mtb* infection markedly alter the intestinal microbial composition in mice. A, T2D and ND mice were infected with a very low dose of aerosol *Mtb* (10-20 CFUs) and lung bacterial loads and gut microbiota changes were assessed. B, *Mtb* numbers in the lung at 14, 28 and 45 d p.i. in T2D and ND mice. C, Gut microbiota composition at the phylum level was characterized. D, Abundance of Firmicutes and Bacteroidetes in naïve and *Mtb*-infected T2D and ND mice at 45 d p.i. E, Firmicutes to Bacteroidetes (F/B) ratio in naïve and *Mtb*-infected T2D and ND mice at 45 d p.i. F, Gut microbiota composition at the family level was also characterized. G, PCA plot and Shannon diversity index in naïve and *Mtb*-infected ND and T2D mice at 45 d p.i. The arrows indicate the shift of the ND and T2D clusters following *Mtb* infection. Results are presented as pooled data means  $\pm$  SEM (B), relative proportions (C, F) and individual data points (D, E, G) from 4-5 mice per group (B-G). \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ ; \*\*\*\* $P < .0001$ ; ns: not significant by unpaired two-tailed Student's *t* test (B-F) and one-way ANOVA followed by Tukey's test (G). Data are mean  $\pm$  SEM (B, D, E), and quartiles in violin plots (G). ND, non-diabetic; T2D, type 2 diabetes

Interestingly, 45 days following the low-dose aerosol *Mtb* infection, *Mtb*-infected ND mice still harbored a significantly higher percentage of Bacteroidetes compared to *Mtb*-infected T2D mice (Figure 2D), despite the overall reduction of Bacteroidetes in comparison to their naïve counterparts (Figure 2C). The F/B ratios in *Mtb*-ND and *Mtb*-T2D were comparable (Figure 2E). Similar to the

phylum level, compared to naïve mice, *Mtb* infection led to an overall reduction in the bacterial families Muribaculaceae, Akkermansiaceae, Ruminococcaceae and Lachnospiraceae (Figure 2F). In addition, a significantly higher abundance of Lactobacillaceae and Erysipelotrichaceae families was observed in *Mtb*-ND compared to *Mtb*-T2D (Figure 2F). These families were not differentially abundant



prior to the infection (Figure 2F). Collectively, *Mtb* infection significantly altered the gut microbial composition in both ND and T2D groups and caused a greater diversity loss in T2D mice indicated by a lower Shannon diversity index (Figure 2G). In addition, discrete clustering of *Mtb*-ND (red) and *Mtb*-T2D (blue) in the PCA matrix is suggestive of the characteristic microbial composition in each disease phenotype (Figure 2G).

## 4 | DISCUSSION

The human gut constitutes a substantial microbial habitat and the gut microflora has even been considered to be an essential organ.<sup>17</sup> Compelling evidence suggests microbiome involvement in basic biological processes such as metabolism, immune modulation, mucosal barrier maintenance and major disease conditions. These include metabolic diseases, respiratory diseases, mental and physiological diseases, autoimmune diseases and infectious diseases, including pulmonary TB.<sup>18</sup> Interestingly, the microbiota has been shown to exert effects at distal sites across the body via the so-called gut-lung, gut-brain and gut-liver axes.<sup>19</sup> Factors such as antibiotic consumption, nutrition or infections can cause dysbiosis, thus influencing host defense and immunity, which in turn contribute to disease susceptibility and exacerbation.<sup>20</sup> Moreover, a majority of TB risk factors, such as excessive alcohol consumption, smoking and diabetes have been shown to affect the host microbiota.<sup>9</sup> In this study, we investigated how the intestinal microbiota changes during the development of T2D and whether it influences the elevated risk of TB susceptibility using a robust mouse model of T2D that mimics many cardinal features of human T2D.<sup>10</sup>

T2D is a progressive metabolic disease.<sup>8</sup> Our data suggest that extended feeding of EDD to mice caused dysbiosis in the intestine and is reflected in increased levels of Firmicutes (Gram-positive) and a reduced abundance of Gram-negative Bacteroidetes compared to SD-fed ND mice. A majority of human gut-resident bacterial species (~90%) also belongs to Bacteroidetes and Firmicutes.<sup>21</sup> In accordance with our findings, several other studies have previously shown similar patterns of Firmicutes and Bacteroidetes abundance in other animal models of diabetes,<sup>22</sup> as well as in T2D patients.<sup>11</sup> In addition, diet-induced obese animals<sup>23</sup> and obese individuals with insulin resistance<sup>24</sup> presented with an elevated F/B ratio. However, contradictory findings with opposite tendencies have also been reported.<sup>25</sup> Patients with T2D have a characteristic increase in gut permeability,<sup>26</sup> resulting in metabolic endotoxemia which leads to chronic inflammation and insulin resistance in T2D.<sup>27</sup> However, prebiotic feeding can significantly improve this phenotype in obese individuals.<sup>28</sup> In a recent study, the increased abundance of Firmicutes and Verrucomicrobia (similar to our observations in T2D mice) was found to be associated with deleterious metabolic measures that hindered cellular functions.<sup>29</sup> The findings of this study suggest that some but not all of the phyla of bacteria change over time as T2D develops.

*Mtb* infection significantly alters the composition of the gut microbiota in humans and animals.<sup>9</sup> Potential anti-tubercular activity of certain bacterial infections and bacterial metabolites such as

*Helicobacter pylori* infections in non-human primates<sup>30</sup> and Indolepropionic acid from gut *Clostridia* spp.,<sup>31</sup> respectively, have been previously reported. On the other hand, antibiotic-mediated induction of dysbiosis in gut microbiota provokes early susceptibility to *Mtb* infection and facilitates bacterial dissemination in mice.<sup>32</sup> This appears to be associated with the suppression of Th1 immunity and reduced levels of IFN- $\gamma$  and TNF- $\alpha$ .<sup>32</sup> Intriguingly, these phenotypes could be reversed by microbial reconstitution via fecal transplantation, a possible method for ameliorating metabolite imbalance, signifying the importance of gut microbiota in conferring resistance to *Mtb*.<sup>32</sup> Similarly, a strong correlation has been established between reduced gut microbial diversity and increased risk of allergic asthma in young age<sup>33</sup> and respiratory infections such as influenza A virus.<sup>34</sup> The latter study points towards an association between depletion of gut microbiota and impaired function of dendritic cells (DCs) and alveolar macrophages (AMs), two important players in anti-TB innate immunity.<sup>35</sup> Antibiotic-treated mice show lower levels of NLRP3-dependent IL-1 $\beta$  and IL-18 secretion, resulting in failure of DCs to migrate from lung tissues to mediastinal lymph nodes and diminished antigen presenting capacity following influenza A infection.<sup>34</sup> DCs from gut microbiota-depleted mice were associated with lower expression levels of co-stimulatory CD80, CD86 and MHCII molecules.<sup>34</sup> A more recent study demonstrated that gut microbiota dysbiosis impairs the phenotype and cytokine response of Mincle<sup>+</sup> lung DCs followed by defective Th1 and Th17 immune responses, thus promoting *Mtb* survival.<sup>36</sup> In addition, AMs derived from gut-microbiota depleted mice had a diminished phagocytic capacity and low TNF- $\alpha$  levels upon *Streptococcus pneumoniae* ex vivo infection.<sup>37</sup>

How an altered intestinal microbiota in T2D influences anti-TB immunity is not yet fully understood. However, it has been suggested that translocation of immune cells, bacteria and bacterial metabolites such as short chain fatty acids (SCFAs) from intestine to lung via the blood circulation could shape the immune responses via the gut-lung axis.<sup>38</sup> SCFAs are known to induce anti-inflammatory IL-10, tolerogenic T cell profiles in humans,<sup>39</sup> particularly butyrate which modulates mucosal immune responses acting mainly on innate immune cells, thereby suppressing the activation, differentiation and recruitment of neutrophils, macrophages and DCs.<sup>40</sup> Our T2D mice also had an increased abundance of butyrate-producing Firmicutes. Human PBMCs treated with butyrate demonstrate a significantly reduced production of *Mtb*-induced pro-inflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-17A and increased IL-10,<sup>41</sup> further supporting the correlation between increased butyrate levels and dampened anti-TB immune responses. In a recent study, Nastasi and colleagues have shown that butyrate inhibited the activation and expansion of antigen-specific TNF- $\alpha$  and IFN- $\gamma$  producing CD8<sup>+</sup> T cells by affecting the antigen-presenting cells.<sup>42</sup> In contrast, beneficial roles of butyrate, such as promoting memory potential of activated CD8<sup>+</sup> T cells have also been reported.<sup>43</sup> Furthermore, butyrate treatment strongly reduced the secretion of IL-12p70, accompanied by reduced expression of co-stimulatory molecules CD40, CD80, CD83, CD86, MHC-I and MHC-II.<sup>42</sup> Similarly, butyrate suppressed LPS-induced activation of human DCs and promoted IL-10-secreting T regulatory cells.<sup>44</sup> We

have recently demonstrated defective co-stimulatory and innate pathogen recognition receptor expression in TB/T2D comorbid lungs.<sup>6</sup> Moreover, a recent animal study implicated a role of SCFAs in priming myeloid cells in the bone marrow, potentially establishing a gut-bone marrow-lung axis.<sup>45</sup> Taken together, this evidence supports the notion that altered microbiota, along with other factors associated with T2D, renders innate cells including antigen-presenting cells ineffective, thus leading to increased susceptibility to TB.

More importantly, based on the evidence presented above, it is tempting to speculate that the microbiota alone or specific cocktails of microbiota-derived metabolome could potentially be developed as therapeutic agents in the context of TB/T2D. However, further studies are warranted and the T2D/TB comorbidity model described above is an ideal model for such research endeavors.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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### SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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