

Original Article

Different immunization methods lead to altered gut flora and varied responses to *Mycobacterium tuberculosis* infection in mice

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Abstract

Introduction: Vaccination is an essential means for prevention of tuberculosis infection, but the effects of various vaccines on the intestinal flora of mice and their response to *Mycobacterium tuberculosis* (*Mtb*) infection remain poorly understood.

Methodology: In this study, two different vaccinations – ESAT6 and ESAT6 + TLR8 agonists - were administered to mice transgenic for human TLR8 to investigate gut microbiota characteristics following vaccination. Gut microbiota was investigated by next generation sequencing in the MiSeq Sequencing System. Adonis analysis was used to evaluate the effect of variables on gut bacterial community structure. Chao1, Shannon index, and phylogenetic diversity index were used to explore the gut bacterial diversity.

Results: The results showed that different vaccines have significant influence on mice intestinal bacteria (adonis analysis, $p < 0.01$), with gut bacterial diversity within the ESAT6 + TLR8 agonists group being significantly decreased compared to the ESAT6 treatment group ($p < 0.01$). Following infection with *Mtb* via tail vein injection, the bacterial community structure within the control versus vaccinated groups altered significantly (adonis analysis, $p < 0.01$), and the altered changed genera were markedly different between the groups. Following infection, *Bifidobacteria* differed between the groups, indicated that they play a vital role in the response to infection.

Conclusions: Our results indicated that different vaccines might have distinct influences on intestinal flora, and their role should not be ignored.

Key words: Gut microbiota; vaccine; TLR8; *Mycobacterium tuberculosis*.

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Introduction

Tuberculosis (TB) remains a significant threat to human health with an increasing number of cases infected with *Mycobacterium tuberculosis* (*Mtb*) worldwide. During the invasion of *Mtb* in the human body, many host-pathogen-recognition receptors can recognize *Mtb* surface molecules, such as toll-like receptors (TLRs). It has been reported that several TLRs work to recognize *Mtb* and activate the innate immune responses accompanied by mycobacterial infection [1]. Also, gut microbiota play an important role in the function of host immune system [2]. This microbiome is vital for the development of host immune processes responding to stimuli, which is essential for maintenance of normal homeostasis [3-7]. Thus, the roles of human microbiota within TB infection should not be overlooked. Since the characteristic of sputum microbiota during TB infection

was studied by next generation sequencing in 2013 [8], dramatic changes were observed in gut microbiota of tuberculosis patients [9]. The gut microbiota of TB patients was primarily characterized by significantly decreased bacteria related by short-chain fatty acids production [9]. Much evidence indicated the close relationship between the changes of gut and lung microbiota and the *Mtb* infection [10]. It was also proved in mice [11]. The gut microbiota is also crucial for the response to *Mtb* in the lung [12]. Gut microbiota rapidly modified following *Mtb* infection and formed a gut bacterial community structure that was significantly different from uninfected samples [11]. Vaccination is an essential means for prevention of TB. Work is ongoing to improve the vaccine effectiveness in various ways. Evidence indicates that dysbiosis of gut microbiota could affect the function of DCs and the response of T cell against *Mtb* [12]. Whether different

immunization methods could affect gut microbiota as well as how they affect vaccine effectiveness remains poorly understood. Our previous study evaluated the impact of ESAT6 (Abcam)-aluminum hydroxide gel (group C) and ESAT6-aluminum hydroxide gel-TL8-506 (group D) on *Mtb* vaccine effectiveness. It was demonstrated that group D gained better protection against *Mtb* infection compared to group C as supported by CFU determination in the lung, bacterial load in lung and liver, lung histology, and percentage of granuloma area within lung lobes (Figure S1) [13]. In the present study we investigated the fecal bacterial community from our previous research and compared the bacterial community structure of group B and group D within the gut microbiota responses to *Mtb* infection, which could lead to a better understanding of the shift of gut species within the context of infection.

Methodology

Experimental process

In brief, 12 six-week-old female human TLR8 transgenic mice were used for experiments. Mice were divided into three equal groups, which were intramuscularly injected with either PBS (group B), ESAT6 (Abcam)-aluminum hydroxide gel (group C), or ESAT6-aluminum hydroxide gel-TL8-506 (group D). A second immunization with the same agent was performed four weeks later. Feces were collected four weeks following the second vaccination. Then, mice were challenged with 10^5 CFU of *Mtb* H37Rv in 200 μ L of PBS, and feces were subsequently collected on days 18 and 90 post-*Mtb* infection (Figure 1). Fecal samples were immediately subjected to DNA extraction. All the animal experiments were permitted by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Sciences, CAMS &

PUMC. Detailed information could refer to Tang's paper [13].

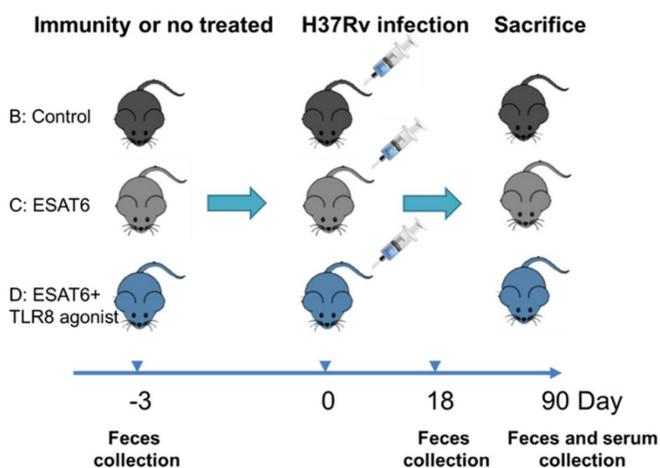
Next-generation sequencing

Feces DNA was extracted according to the manufacturer's recommendations using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Firstly, the DNA was diluted to 1ng/uL and stored at -20°C for further process. Secondly, PCR of V3-V4 variable regions was performed using Nossa's (2010) primers [14] with the diluted template. The same amplification system was employed using sterile deionized water as the template for the negative control. Thirdly, Amplicon quality was detected using gel electrophoresis, after which amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), amplified with another round of PCR, and purified again using AMPure XP beads. The batch containing no visible band at 400 bp for the negative control was treated using the following steps. Equal amounts of the purified amplicons were pooled for subsequent sequencing using the MiSeq Sequencing System (Illumina, Inc., San Diego, CA, USA).

Sequence processing

Raw sequencing data were processed according to the protocol followed by Chen Lin [15] to remove the low quality sequences. Further denoising of the sequences involved first abandoning reads containing ambiguous, homologous sequences or that were < 200 bp, retaining reads with 75% of bases above Q20, and finally detecting and removing any reads containing chimeras using the QIIME software (version 1.8.0) [16]. Clean reads were subjected to primer sequence removal and clustering to generate operational taxonomic units (OTUs) using the UPARSE software with a 97% similarity cutoff [17]. Representative reads for each OTU were then selected using the QIIME package. All representative reads were annotated and blasted against the Silva database Version 123 (16s rDNA) using the Ribosomal Database Project Classifier (confidence threshold = 70%) [18]. Each sample was rarefied to 16,973 sequences. The Chao1 richness estimator, Shannon index, and phylogenetic diversity (PD) [19] index were calculated using the mother software. We used the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to predict the KEGG category and functional enzyme [20].

Figure 1. Experimental flow chart.



Statistical analysis

The α -diversity [21] of the bacterial community was analyzed on the basis of Chao1, Shannon–Wiener, and Faith’s phylogenetic diversity (PD) [22] index using the OTU matrix file. Phylogenetic tree was built using approximately maximum likelihood based on the evolutionary distance calculated by Jukes and Cantor model with QIIME. Phylogenetic tree was used for PD index. Principal coordinates analysis (PcoA) and a permutational multivariate analysis of variance were carried out using distance matrices (adonis) with Bray-Curtis distance in the package “vegan” in the R environment. All other statistical analyses were performed in the package “state” in the R environment. *p* values below 0.05 indicated statistically significant differences.

Results

Effects of different immunization modes on intestinal flora within mice

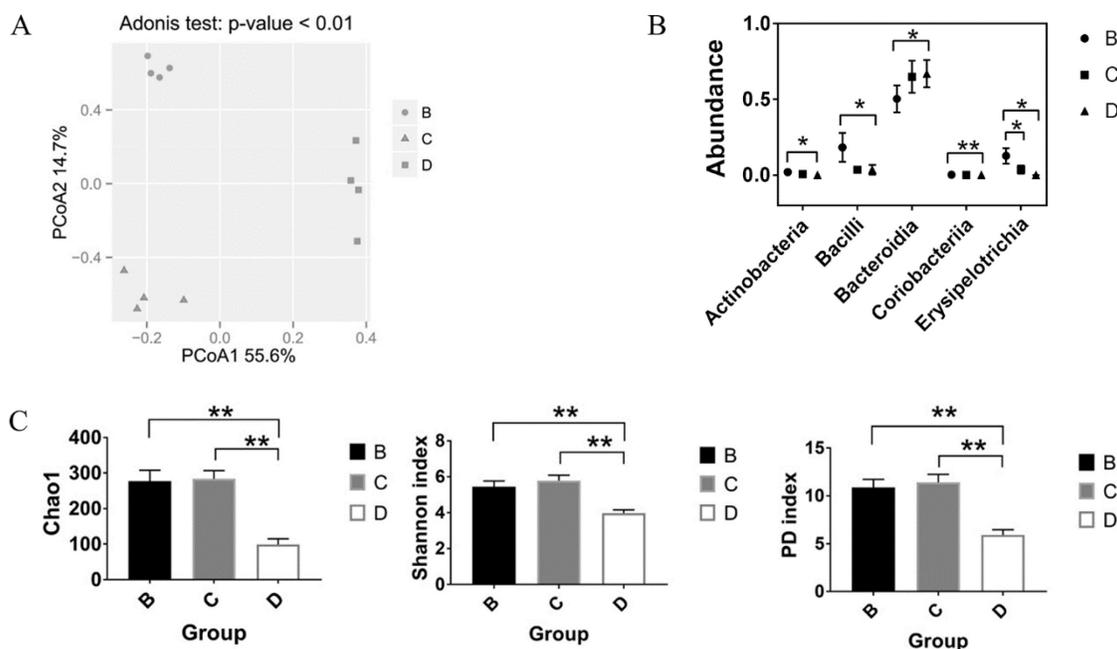
PcoA based on the Bray-Curtis distance showed that the samples were clustered into three groups consistently within the method of immunization, and the gut microbiota structure differed significantly among the groups (Figure 2A). Furthermore, it was found the prevalence of classes such as Actinobacteria, Bacilli, Bacteroidia, Coriobacteriia, Erysipelotrichia were significantly different among the groups (Figure

2B). Of note, the abundances of Bacilli, Bacteroidia, and Erysipelotrichia displayed considerable changes (Figure 2B). Analysis of bacterial diversity showed that the ESAT6-aluminum hydroxide gel-TL8-506 had a highly significant effect on Chao1, Shannon diversity, and PD diversity (Figure 2C). However, the ESAT6 (Abcam)-aluminum hydroxide gel inoculation had no effect on bacterial diversity (Figure 2C).

Changes of intestinal flora within immunized mice following Mtb challenge

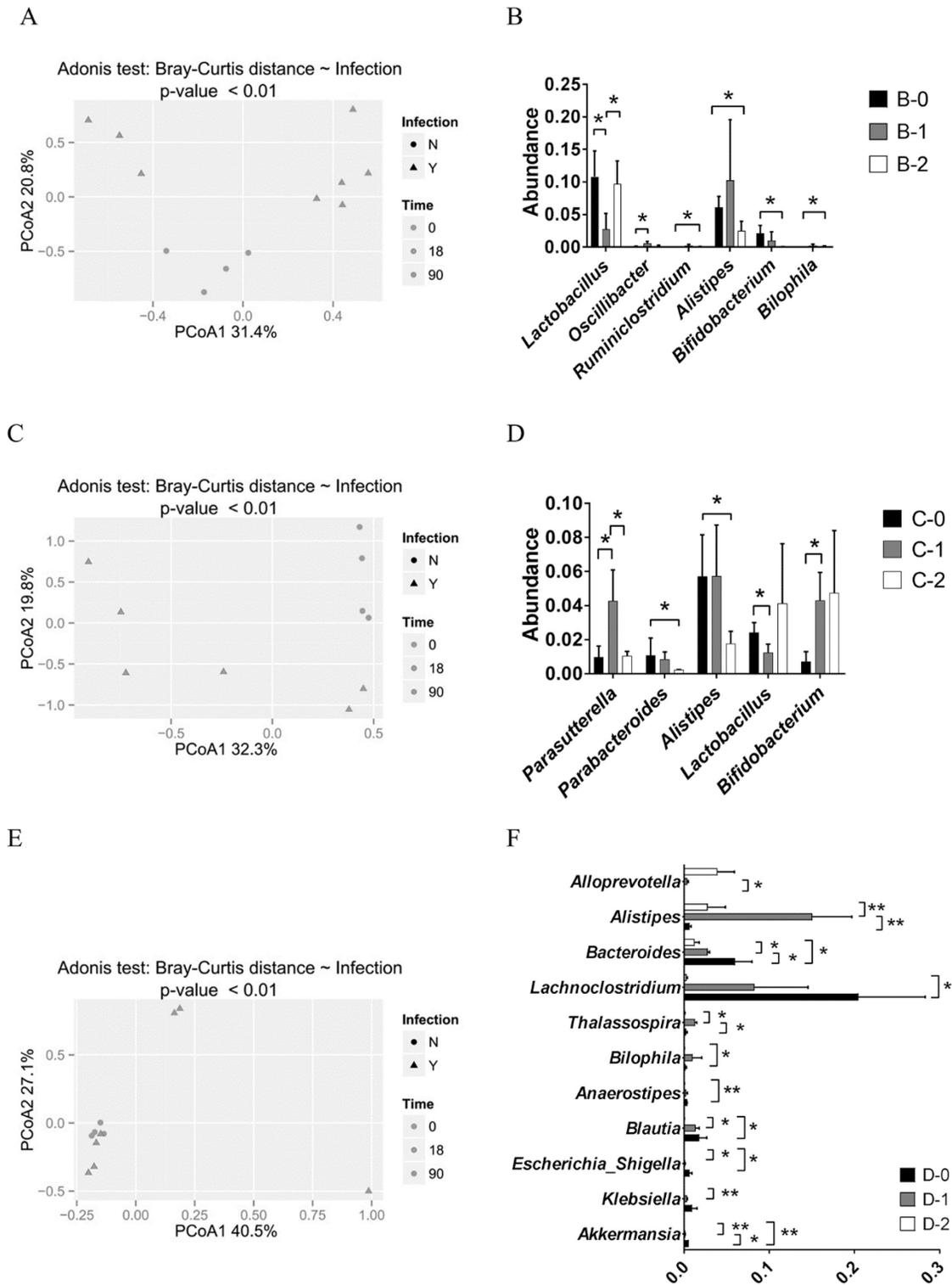
Bacterial community structures significantly changed following *Mtb* infection (Figure 3A). Taxa at the genus level with abundances greater than 0.001 were analyzed to compare the differences among groups. The communities of *Lactobacillus*, *Oscillibacter*, *Ruminiclostridium*, *Alistipes*, *Bifidobacterium*, and *Bilophila* were significantly changed (Figure 3B), with greatest variation occurring within the *Lactobacillus*, *Alistipes*, and *Bifidobacterium*. *Lactobacillus* were suppressed on day 18 following *Mtb* infection and recovered to original levels by day 90; *Alistipes* and *Bifidobacterium* remained unchanged on day 18 but were suppressed on day 90 (Figure 3B). Bacterial community structures within groups C and D also significantly changed following *Mtb* infection (Figure 3C and 3E).

Figure 2. Characteristics of gut microbiota affected by different immunization methods.



(A) Principal coordinates analysis (PCoA) of samples based on Bray-Curtis distances following immunization. (B) Abundances of significantly different classes within different groups. (C) Chao1, Shannon index, and PD index in groups. Adonis and t-tests were performed in the R environment. * $P < 0.05$; ** $P < 0.01$.

Figure 3. Response of gut microbiota within different groups to *Mtb* infection.



(A) (C) (E) PCoA of samples based on Bray-Curtis distances in group B, C, and D. (B) (D) (F) Abundances of significantly different genera following *Mtb* infection in groups B, C, and D. Adonis and t-tests were performed in the R environment. * $P < 0.05$; ** $P < 0.01$.

However, the significantly changed genera within these groups were quite different from those of group B. *Parasutterella*, *Parabacteroides*, *Alistipes*, *Lactobacillus*, *Bifidobacterium* were the most abundance genera present within mice in group C; *Akkermansia*, *Klebsiella*, *Escherichia*, *Shigella*, *Blautia*, *Anaerostipes*, *Bilophila*, *Thalassospira*, *Lachnoclostridium*, *Bacteroides*, *Alistipes*, *Alloprevotella* were the most abundant within group D mice (Figure 3D and 3F). *Alistipes* and *Lactobacillus* showed similar behavior to group B, while *Bifidobacterium* was stimulated following *Mtb* infection on day 18 post-infection within group C (Figure 3B and 3D). In group D, *Alistipes* prevalence was increased on day 18 and recovered to original levels on day 90, while the abundances of *Lactobacillus* and *Bifidobacterium* were unaffected by *Mtb* infection (Figure 3F). A marked increase in *Bacteroides* and *Lachnoclostridium* levels were observed following *Mtb* infection (Figure 3F).

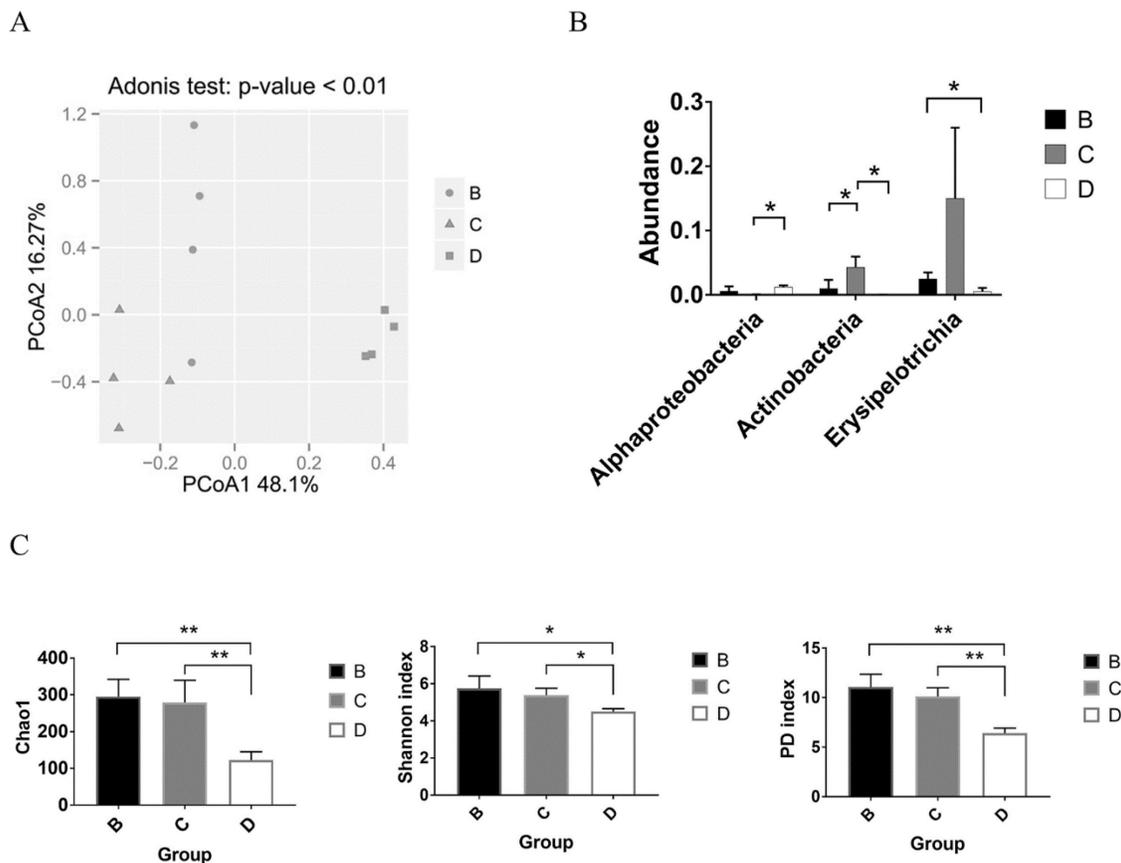
Differences in intestinal flora within immunized mice following Mtb infection

The bacterial community structure was separated according to group (Figure 4A). Alphaproteobacteria, Actinobacteria, Erysipelotrichia differed among groups (Figure 4B). Bacterial diversity within infected samples was similar result to that of samples collected prior to *Mtb* infection (Figure 4C). On day 90 post-infection, the bacterial community still significantly differed between the groups (Figure 5A). Betaproteobacteria was the only class that differed significantly between groups B and D (Figure 5B). *Chao1*, Shannon and PD indexes showed no differences among groups on day 90 (Figure 5C).

Discussion

The gut microbiota affect mammalian both during homeostasis and disease states [23]. The relationship between humans and gut microbiota is vital for the maintenance of health. Vaccines provide active acquired immunity to particular pathogens, and their effectiveness is critical for disease prevention.

Figure 4. Differences in gut microbiota between different groups on day 18 post-infection.



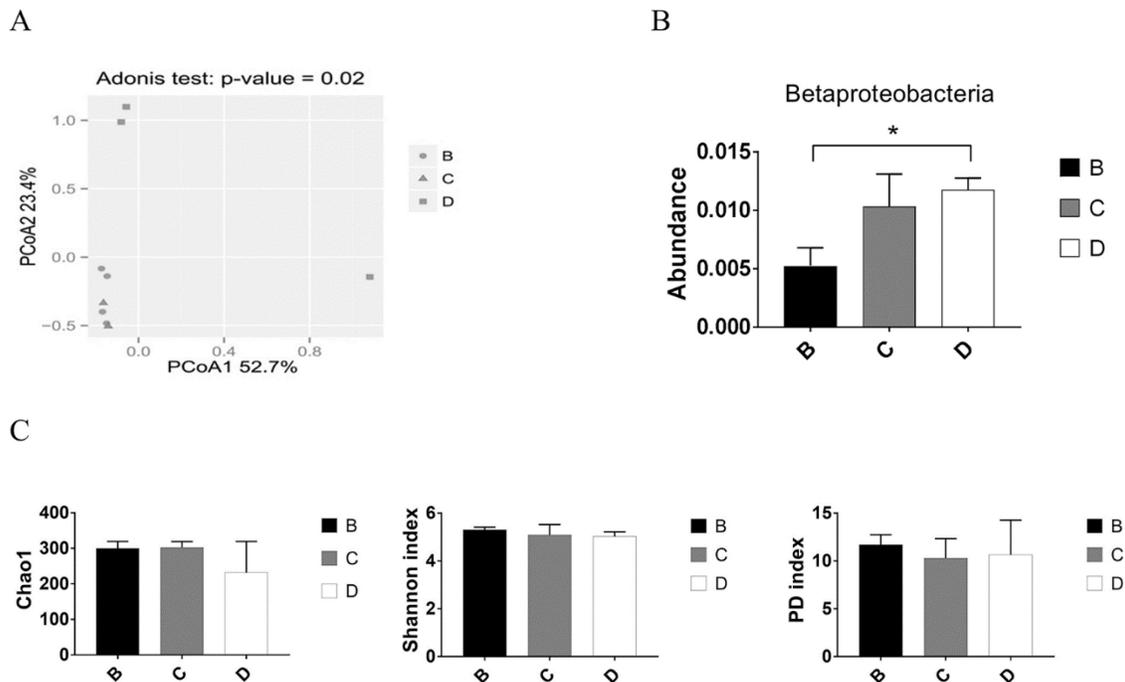
(A) PCoA of samples based on Bray-Curtis distances collected on day 18. (B) Abundances of significantly different classes between groups B, C, and D. (C) Chao1, Shannon, and PD indexes within different groups. Adonis and t-tests were performed in the R environment. * $P < 0.05$; ** $P < 0.01$.

Therefore, gut microbiota composition may affect how individuals respond to vaccinations. Evidence indicates that many factors related to gut microbiota could affect the host response to vaccines. Kim *et al.* (2017) showed that malnutrition and poor sanitation, which affects the composition of gut microbiota, can impair the efficacy of vaccines. It was shown that antibiotic-driven intestinal dysbiosis could lead to decreased responses to adjuvant and live vaccines, and that restoration of the gut microbiota could recover these responses [24]. Evidence from human and mouse models both suggested that the gut microbiome could affect immune responses to vaccines [25]. However, limited studies have investigated the influence of the intestinal microbiome on vaccine responses [26]. Tuberculosis, commonly known as consumption for many years, results from infection by *Mtb*. The vaccine currently available for TB prevention is only BCG [27]. However, it was reported that the BCG vaccine has several limitations such as a low rate of protection [28, 29]. Therefore, the development of novel vaccines that are more effective and safer should be considered as a priority. In this study, we selected two vaccination variations: ESAT6 and ESAT6 + TLR8 agonists. The effect of immunization on intestinal flora may affect its response to *Mtb* infection. We found that vaccination with ESAT6 + TLR8 agonists had a great effect on gut

bacterial community and bacterial diversity compared to ESAT6 alone (Figure 2 and Figure S2). Toll-like receptor 8 is a protein in the TLR family in humans [30]. Agonists of the TLR family have been proven to enhance the protective effect of the *Mtb* vaccine [31-33]. Our previous study demonstrated that vaccination with ESAT6 + TLR8 agonists could strengthen the protective efficacy of *Mtb* immunization [13]. A diverse bacterial community could contribute to the improvement of protective efficiency. It has been shown that members of Bacteroidetes, which contain a great number of beneficial commensal microorganisms, decreased in patients experience repeated TB infections [34]. We found that the abundance of Bacteroidia increased following vaccination with ESAT6 + TLR8 agonists (Figure 2B), indicating that Bacteroidia played an important role in vaccination efficacy. *Mtb* infection could also affect human sputum microbiota [35]. Murine gut microbiota significantly changes following *Mtb* infection [11]. Our results also confirmed that *Mtb* infection could alter the gut microbiota following challenge with *Mtb* infection (Figure 3A), as *Lactobacillus* and *Bifidobacterium* were both affected by *Mtb* infection (Figure 3B).

Reduction of probiotics could indicate a decreased benefit of the presence of gut microbiota. Conversely, the abundance of *Bifidobacterium* in mice vaccinated

Figure 5. Differences between gut microbiota among different groups on day 90 post-infection.



(A) PCoA of samples based on Bray-Curtis distances collected on day 90. (B) Abundances of significantly different classes between groups B, C, and D. (C) Chao1, Shannon, and PD indexes within different groups. Adonis and t-tests were performed in the R environment. * $P < 0.05$; ** $P < 0.01$.

with ESAT6 increased after *Mtb* infection (Figure 3D) and no change occurred within the group immunized with ESAT6 + TLR8 agonists, possibly indicating no apparent negative influence of *Mtb* infection. However, the gut microbial community of vaccinated mice within both groups changed after *Mtb* infection (Figure 3C and 3E). Probiotics could benefit host by several mechanisms such as restoration of disordered microbiota [36]. It was found that probiotics benefit the immune response to oral vaccinations which could improve the vaccine efficacy [26]. Thus, application of probiotics accompanied by vaccination may increase the efficiency of *Mtb* infection prevention. Differences in gut microbiota between groups B, C, and D remained following *Mtb* inoculation on day 18 (Figure 4A), with the trend of bacterial diversity remaining unchanged (Figure 4C). Significantly different taxa at the class level decreased over time (Figure 4B, 5B, and Figure S3), and the bacterial diversity indices of the groups became indistinguishable (Figure 5C). We also found that several genera were significantly correlated with inflammation factors, including *Alloprevotella*, *Clostridium sensu stricto* 1, *Romboutsia*, *Ruminococcaceae* UCG_013, *Ruminococcus* 1, *Thalassospira*, and *Turicibacter* (Table S1). These genera could possibly be considered as enhancers of immunity. The host-microbiota could initially protect lung from *Mtb* colonization [37]. Gut commensal bacteria could affect the outcome of BCG vaccination [38], and treatment of TB significantly alters the composition of the gut microbiota [39]. It was demonstrated that the addition of certain bacteria was an effective strategy for the process of prevention and treatment of tuberculosis.

Conclusions

In sum, vaccination is an effective method for prevention of tuberculosis. Our study found that different methods of vaccination distinctly influenced mice intestinal bacteria. TLR8 agonists vaccinated in combination with ESAT6 significantly affected gut microbiota and bacterial diversity. Behaviors of probiotics within the gut exhibited a significantly different response following *Mtb* challenge. The gut microbiota communities between the vaccinated groups also showed different patterns throughout the course of infection. All these data confirmed that vaccination strategy can affect gut microbiota structure and result in a shift of gut microbial communities following *Mtb* infection, and probiotics may be considered as a functional treatment to recover gut microbiota function.

These results could ultimately facilitate advances in TB treatment.

Data availability

All bacterial 16S rRNA gene sequences generated for the present study were deposited in the National Centre for Biotechnology Information Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra>) under the accession number PRJNA588481.

Acknowledgements

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Conflict of interests: No conflict of interests is declared.

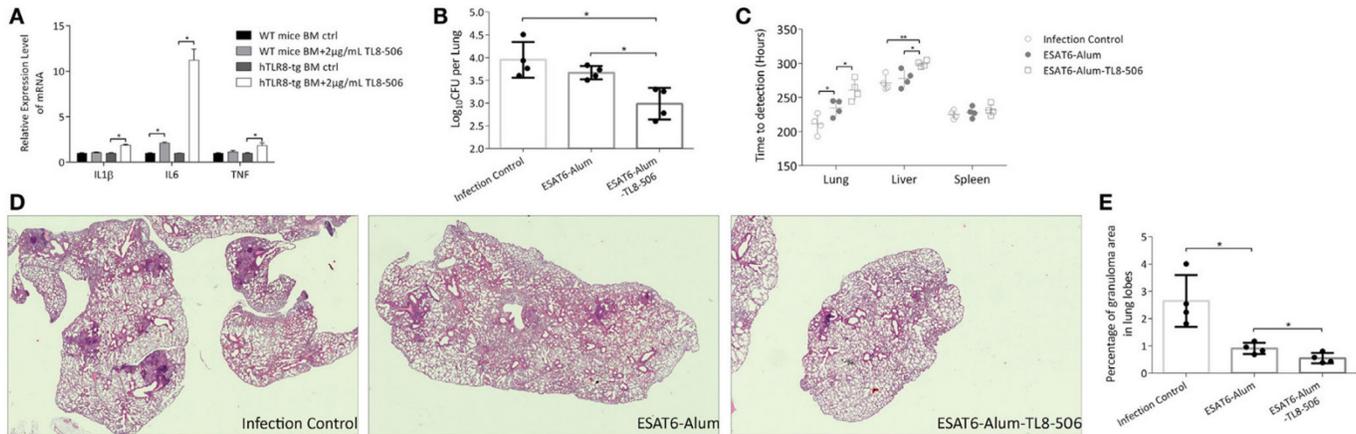
Annex – Supplementary Items

Supplementary Table 1. The Pearson correlation analysis between gut genera (abundance > 0.001) and inflammatory factor in serum.

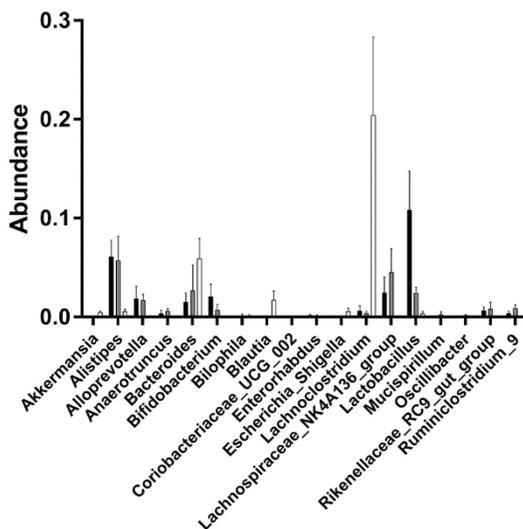
	IFN γ	IL-12p70	IL-4	IL-5	IL-6	TNF α	IL-10	IL-2	IL-17A
<i>Aggregatibacter</i>	-0.194	-0.157	-0.062	-0.204	-0.159	-0.196	-0.021	-0.368	-0.187
<i>Akkermansia</i>	0.265	-0.141	-0.152	-0.161	-0.143	-0.13	-0.136	-0.239	-0.091
<i>Alistipes</i>	0.256	0.458	0.447	-0.054	0.288	0.578	0.436	0.421	0.09
<i>Allobaculum</i>	-0.318	-0.262	-0.17	0.228	-0.275	-0.474	-0.39	-0.111	0.319
<u><i>Alloprevotella</i></u>	0.054	0.856**	-0.43	-0.305	0.114	0.07	0.614	0.314	0.118
<i>Anaeroplasma</i>	-0.549	-0.27	0.081	-0.022	-0.297	-0.411	-0.574	-0.364	-0.268
<i>Anaerotruncus</i>	0.07	-0.147	0.452	0.202	0.052	-0.047	-0.393	-0.069	0.307
<i>Bacteroides</i>	-0.267	0.149	-0.506	-0.028	-0.224	-0.293	-0.358	-0.365	-0.103
<i>Bifidobacterium</i>	-0.338	-0.165	-0.038	0.35	-0.13	-0.367	-0.302	0.061	0.454
<i>Bilophila</i>	-0.097	-0.144	0.572	0.038	-0.017	0.021	-0.29	-0.106	0.1
<i>Blautia</i>	-0.021	0.262	-0.206	-0.231	-0.153	0.219	0.231	-0.197	-0.032
<i>Campylobacter</i>	-0.188	-0.162	-0.056	-0.205	-0.159	-0.19	-0.02	-0.369	-0.191
<u><i>Clostridium sensu stricto 1</i></u>	0.744*	-0.119	0.54	0.911**	0.925**	0.322	-0.357	0.52	0.618
<i>Coprococcus 1</i>	0.123	-0.324	0.324	-0.044	0.037	-0.133	-0.305	-0.358	-0.069
<i>Coriobacteriaceae_UCG_002</i>	-0.298	0.426	-0.36	0.173	-0.036	-0.517	-0.075	0.253	0.628
<i>Desulfovibrio</i>	-0.003	-0.313	0.013	-0.15	-0.143	-0.199	0.059	-0.13	-0.063
<i>Enterorhabdus</i>	0.208	0.388	-0.251	-0.034	0.147	0.155	0.567	0.612	0.229
<i>Erysipelatoclostridium</i>	0.414	-0.287	-0.074	0.193	0.044	0.025	-0.294	-0.15	0.032
<i>Family XIII_UCG_001</i>	-0.31	-0.371	-0.09	-0.155	-0.371	-0.427	-0.231	-0.484	-0.031
<i>Filifactor</i>	-0.194	-0.157	-0.06	-0.205	-0.159	-0.194	-0.019	-0.367	-0.189
<i>Fusobacterium</i>	-0.194	-0.158	-0.06	-0.203	-0.159	-0.195	-0.022	-0.368	-0.187
<i>Haemophilus</i>	-0.188	-0.178	-0.047	-0.197	-0.163	-0.186	-0.04	-0.392	-0.189
<i>Intestinimonas</i>	-0.039	-0.178	0.444	-0.132	-0.13	-0.11	-0.346	-0.268	-0.059
<i>Lachnoclostridium</i>	-0.026	-0.105	-0.168	0.064	-0.168	-0.15	0.164	0.313	0.273
<i>Lachnospiraceae_NK4A136_group</i>	-0.064	0.022	0.607	0.043	0.112	0.05	-0.276	-0.034	0.108
<i>Lachnospiraceae_UCG_001</i>	0.2	-0.24	0	0.636*	0.389	-0.144	-0.638*	0.035	0.238
<i>Lachnospiraceae_UCG_006</i>	-0.351	-0.311	0.002	0.309	-0.207	-0.471	-0.47	-0.051	0.36
<i>Lactobacillus</i>	0.201	0.488	-0.148	0.008	0.128	0.304	0.476	0.41	0.35
<i>Leptotrichia</i>	-0.195	-0.158	-0.061	-0.203	-0.16	-0.195	-0.021	-0.368	-0.187
<i>Marvinbryantia</i>	-0.444	-0.246	-0.186	0.006	-0.265	-0.437	-0.565	-0.386	-0.289
<i>Mucispirillum</i>	-0.038	-0.322	0.032	-0.179	-0.318	-0.246	-0.509	-0.637*	-0.197
<i>Neisseria</i>	-0.202	-0.161	-0.04	-0.214	-0.165	-0.189	-0.023	-0.377	-0.198
<i>Oscillibacter</i>	0.158	-0.278	0.611	-0.062	-0.005	0.21	-0.214	-0.154	-0.153
<i>Parabacteroides</i>	0.071	0.601	-0.193	-0.217	0.066	0.327	0.626	0.451	-0.062
<i>Parasutterella</i>	-0.054	0.225	-0.221	0.045	0.068	-0.047	-0.127	0.069	-0.143
<i>Parvimonas</i>	-0.199	-0.164	-0.038	-0.215	-0.165	-0.189	-0.023	-0.377	-0.201
<i>Peptococcus</i>	-0.201	-0.163	-0.061	-0.204	-0.167	-0.203	-0.027	-0.373	-0.184
<i>Porphyromonas</i>	-0.194	-0.158	-0.06	-0.204	-0.16	-0.195	-0.021	-0.368	-0.188
<i>Prevotella</i>	-0.194	-0.158	-0.061	-0.204	-0.159	-0.195	-0.022	-0.369	-0.188
<i>Prevotella_7</i>	-0.195	-0.161	-0.054	-0.206	-0.161	-0.193	-0.022	-0.37	-0.192
<i>Prevotellaceae_NK3B31_group</i>	-0.149	-0.039	-0.092	-0.243	-0.226	0.069	0.21	-0.074	-0.163
<i>Prevotellaceae_UCG_001</i>	-0.314	0.276	-0.366	-0.234	-0.24	-0.103	0.046	-0.058	-0.296
<i>Rikenellaceae_RC9_gut_group</i>	-0.513	-0.421	-0.3	0.092	-0.342	-0.611	-0.596	-0.553	-0.073
<u><i>Romboutsia</i></u>	0.733*	-0.101	0.539	0.911**	0.93**	0.321	-0.348	0.531	0.625
<i>Roseburia</i>	0.278	-0.065	0.022	-0.167	-0.102	0.059	-0.108	-0.258	-0.059
<i>Ruminiclostridium</i>	0.245	0.073	0.099	-0.234	-0.05	0.009	-0.026	-0.156	-0.012
<i>Ruminiclostridium_5</i>	0.279	0.287	0.387	-0.048	0.204	0.601	0.553	0.54	0.009
<i>Ruminiclostridium_6</i>	-0.017	-0.246	0.294	0.685*	0.252	-0.179	-0.435	0.262	0.666*
<i>Ruminiclostridium_9</i>	-0.059	-0.301	0.624	0.34	0.124	-0.076	-0.557	-0.031	0.242
<i>Ruminococcaceae_NK4A214_group</i>	-0.289	-0.455	0.216	0.216	-0.089	-0.358	-0.456	0.041	0.013
<i>Ruminococcaceae_UCG_004</i>	0.366	-0.243	0.551	0.313	0.329	0.244	-0.12	0.289	0.235
<i>Ruminococcaceae_UCG_005</i>	-0.258	-0.305	0.333	0.106	-0.208	-0.327	-0.584	-0.302	0.148
<i>Ruminococcaceae_UCG_010</i>	-0.032	0.374	0.028	0.586	0.381	-0.185	-0.293	0.364	0.781**
<u><i>Ruminococcaceae_UCG_013</i></u>	0.505	-0.258	0.556	0.848**	0.758*	0.134	-0.599	0.321	0.452
<i>Ruminococcaceae_UCG_014</i>	-0.251	-0.373	0.008	0.458	-0.065	-0.485	-0.662*	-0.08	0.337
<u><i>Ruminococcus 1</i></u>	0.669*	-0.272	0.369	0.858**	0.689*	0.086	-0.536	0.314	0.629
<i>Streptococcus</i>	-0.194	-0.143	-0.068	-0.241	-0.171	-0.167	0.046	-0.335	-0.219
<i>Subdoligranulum</i>	-0.404	-0.189	0.512	-0.309	-0.284	-0.038	-0.117	-0.189	-0.326
<i>Thalassospira</i>	-0.24	0.818**	-0.214	-0.285	0.069	-0.288	0.162	0.219	0.102
<i>Treponema 2</i>	-0.194	-0.158	-0.061	-0.203	-0.159	-0.195	-0.021	-0.368	-0.188
<i>Turicibacter</i>	0.252	0.852**	-0.338	-0.071	0.317	-0.129	0.294	0.429	0.418

Pearson Correlation analysis were carried out in the R environment. Pearson Correlation Coefficient (r) were showed in the table. * $p < 0.05$; ** $p < 0.01$. Strong correlated genera ($r > 0.8$) were marked with underline.

Supplementary Figure 1. TLR8 transgenic mice immunized with TLR8 agonist carry lower bacilli load and display ameliorated pathological lesions in tissues following a challenge. (A) Bone marrow cells were cultured and treated ex vivo as indicated for 12 h before the mRNA level of IL1 β , IL6, and TNF was determined. Three mice per group. * $p < 0.05$, by Student’s t-test. (B) CFU (log10-transformed) of lungs in differently immunized TLR8transgenic mice 10 weeks post Mycobacterium tuberculosis challenge. (C) Time-to-detection (transformed into hours) by the MGIT 960 system in lung, spleen, and liver. (D) Lung sections stained with H&E. (E) Percentage of granuloma area in lung lobes determined by NanoZoomer S60 (Hamamatsu) and software, 3–5 sections per mice. * $p < 0.05$, ** $p < 0.01$, by one-way ANOVA with a Bonferroni posttest. Data are representative of two independent experiments, each with four mice per group (Tang, *et al.* 2017: 1972).



Supplementary Figure 2. The significantly different genera among group B, C, and D before Mtb infection. The genera (the mean abundance > 0.001) which were significantly different among groups were showed in bar chart.



Supplementary Figure 3. The significantly different genera among groups before Mtb infection and on day 18 and day 90 challenged with Mtb. Red box indicated the abundance of the corresponding genus was significantly greater in former group; blue box indicated the abundance of the corresponding genus was significantly lower in former group; white box indicated no difference.

