Alteration in the gut microbiota provokes susceptibility to tuberculosis

Nargis Khan¹, Aurobind Vidyarthi¹, Sajid Nadeem¹, Shikha Negi¹, Girish Nair¹, Javed N. Agrewala¹*

¹Institute of Microbial Technology, India

Submitted to Journal:
Frontiers in Immunology

Specialty Section:
Mucosal Immunity

ISSN:
1664-3224

Article type:
Original Research Article

Received on:
14 Aug 2016

Accepted on:
10 Nov 2016

Provisional PDF published on:
10 Nov 2016

Frontiers website link:
www.frontiersin.org

Citation:
doi:10.3389/fimmu.2016.00529

Copyright statement:
© 2016 Khan, Vidyarthi, Nadeem, Negi, Nair and Agrewala. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution and reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.
Alteration in the gut microbiota provokes susceptibility to tuberculosis

Nargis Khan¹, Aurobind Vidyarthi¹, Sajid Nadeem¹, Shikha Negi¹, Girish Nair¹ and Javed N Agrewala¹*

¹Immunology Division, CSIR-Institute of Microbial Technology Chandigarh, India

*Address correspondence: Javed N. Agrewala, CSIR-Institute of Microbial Technology, Chandigarh-160036, India. E-mail: javed@imtech.res.in
Abstract
The microbiota that resides in the gastrointestinal tract provides essential health benefits to the host. In particular, they regulate immune homeostasis. Recently, several evidences indicate that alteration in the gut microbial community can cause infectious and non-infectious diseases. Tuberculosis (TB) is the most devastating disease, inflicting mortality and morbidity. It remains unexplored, whether changes in the gut microbiota can provoke or prevent TB. In the current study, we have demonstrated the antibiotics driven changes in the gut microbial composition and their impact on the survival of Mtb in the lungs, liver and spleen of infected mice, compared to those with intact microbiota. Interestingly, dysbiosis of microbes showed significant increase in the bacterial burden in lungs and dissemination of Mtb to spleen and liver. Further, elevation in the number of Tregs and decline in the pool of IFN-γ and TNF-α releasing CD4 T cells was noticed. Interestingly, fecal transplantation in the gut microbiota disrupted animals exhibited improved Th1 immunity and lesser Tregs population. Importantly, these animals displayed reduced severity to Mtb infection. This study for the first time demonstrated the novel role of gut microbes in the susceptibility to TB and its prevention by microbial implants. In future, microbial therapies may help in treating patients suffering from TB.

Keywords: Antibiotics, gut microbiota, tuberculosis, Mycobacterium tuberculosis, fecal transplant
Introduction

Approximately one-third of the world population is infected with Mycobacterium tuberculosis (Mtb), but only 5%–10% contract active tuberculosis (TB), whereas the remaining 90%–95% develop effective immunity (Druszczyńska et al., 2012). An intriguing possibility is that there exists an intricate balance between host and pathogen; where the host develops remarkably powerful immunity, which does not allow the pathogen to replicate and inflict disease. However, any disturbance in this finely-tuned balance may lead to the development of TB.

The gut microflora is an immense health asset for human beings (Flint et al., 2012). The mammalian gut harbours trillions of commensals. These microbes not only influence local but also systemic immunity. Recently, various reports signify that the gut microbes can modulate, tune and tame the host immune response (Maslowski and Mackay, 2011). Importantly, an ever-growing number of disorders have been linked with resident microbiota and gastrointestinal diseases, such as intestinal bowel disease (IBD) (Hansen, 2015). More importantly, imbalance in the gut microbiome has been shown to be associated with extra-intestinal ailments such as cancer, cardiovascular diseases, obesity and non-alcoholic fatty liver disease (Arthur et al., 2012; Howitt and Garrett, 2012; Ray, 2012; Moreno-Indias et al., 2014). Consequently, it advocates the significance of the microbial composition that can influence our health. The microbiota provides a fine equilibrium to the host by regulating immune homeostasis (Wu and Wu, 2012).

Antibiotics are often used in the clinics to treat bacterial infections but they are also major factor in disturbing the gut microbial composition (Modi et al., 2014). Antibiotics driven changes in gut microbiota provokes host susceptibility to enteric infection. However, impact of antibiotics induced changes of gut microbiota on the TB progression has not yet been studied. Therefore, we designed our study taking into consideration two models pre and post-antibiotics treatment models of experimental TB. In pre-antibiotics model, animals were treated with broad spectrum antibiotics prior to Mtb infection, mimicking a condition wherein the individuals undergo treatment with various antibiotics before being exposed to Mtb and may have some impact on the progression of TB. In post-antibiotics model, animals were treated with antibiotics after Mtb infection. Post-antibiotics treatment study was designed to consider those individuals who are exposed to Mtb and take broad spectrum antibiotics for some other infections. Interestingly, we observed that animals with disruption in gut microbiota by both pre- and post-antibiotics treatment showed higher susceptibility towards Mtb and promoted its dissemination. Intriguingly, faecal transplantation (FT) from normal mice reconstituted the gut microbiota of the animals, which subsequently decreased the Mtb load in the lungs and prevented the dissemination of the disease. In essence, this finding signifies that the alteration in the gut microbiome may facilitate the development of TB.
**Materials and methods.**

*Animals.* C57BL/6 mice, 6-8 weeks were procured from the CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India.

*Ethics statement.* All the experiments were approved by the Institutional Animal Ethics Committee of the IMTECH and performed according to the National Regulatory Guideline issued by Committee for the Purpose of Supervision of Experiments on Animals (No. 55/1999/CPCSEA), Ministry of Environment and forest, Govt. of India.

*Cultivable microbes.* Fecal samples (200-300mg) were collected aseptically in 1 ml of 1X PBS. Samples were homogenized and supernatant were collected after centrifuging samples at 2000 rpm for 2 min to pellet down debris. Later, serial dilutions were made and supernatants (100 μl) were plated on different media to culture both aerobic and anaerobic microbes. To cultivate anaerobic microbes, plates were kept in vacuum tight container in the presence of anaerobic gas pack (Himedia, Mumbai, India) for overnight.

*Bacterial diversity:* To assess the bacterial diversity; supernatant from fecal samples was plated on different media to culture both aerobic and anaerobic microbes for overnight as described above. The identification of colony morphotypes were carried out using five parameters: colony size, form, colour, texture. A phenotypic variant was considered when it differed in at least one of the referred morphological parameters. Diversity was calculated by counting the total number of different colonies of bacteria grown on different media plates. Total different colonies from the faeces of healthy animals were considered as a reference number. Decrease in the bacterial diversity was calculated as [(reference number - total different colonies calculated from infected or pre-antibiotics or post-antibiotics) X 100]/reference number.

*Pre-antibiotics treated Mtb model (pre-antibiotics).* Mice were pre-treated with vancomycin (100 mg/L), polymixin B (60 mg/L), carbenicillin (50 mg/L), trimethoprim (20 mg/L) and amphotericin B (50 mg/L) *ad libitum* in drinking water for 21d. The water containing antibiotics were replaced on every 3d. Control mice were fed antibiotics free water. After 21d, mice were challenged with *Mtb* (H37Rv) with deposition of 100 CFU in the lungs. The animals were administered antibiotics in the water for additional 21d. Later, the mice were sacrificed and tissues were harvested aseptically for immunological, microbiological and histopathological studies. Pictorial representation of methodology is embedded in the Supplementary data.

*Post-antibiotics treated Mtb model (post-antibiotics).* Mice were challenged with *Mtb* with the deposition of 100 CFU in the lungs. After 21d, mice were treated with antibiotics for subsequent 21d with replacement of water every 3d. Later, mice were sacrificed and tissues were harvested aseptically for immunological, microbiological and histopathological studies. Pictorial representation of methodology is embedded in the Supplementary data.

*Reconstitution of gut microbiota.* Antibiotics treated mice underwent fecal transplant (FT) to reconstitute the gut composition. Fecal samples (200-300mg) from 5 healthy mice were collected aseptically in 5 separate tubes in 1 ml of 1X PBS. Samples were homogenized and supernatant
were collected after centrifuging samples at 2000 rpm for 2 min to pellet down debris. Supernatant slurry was collected and pooled together and 100 µl was gavaged into mice within 15-20 minutes of excretion. Five doses with a gap of 3d interval were orally administered to mice prior to 15d of sacrificing animals. Pictorial representation of methodology is embedded in Supplementary as Methodology 2.

Expression of IFN-γ, TNF-α and Foxp3 by flowcytometry. Spleen were harvested and single cell suspension was prepared. Briefly, lymphocytes from spleen were prepared by lysing RBCs with ACK lysis buffer (NH₄Cl 0.15M, KHCO₃ 10mM, EDTA 88mM), washed 3X with PBS and resuspended in RPMI-1640-FBS-10%. Viability of the cells was assessed by trypan blue dye-exclusion method. The experiments were performed to detect intracellular cytokines and FoxP3 expression on T cells. To detect cytokines, splenocytes were restimulated in vitro with purified protein derivative (PPD). Splenocytes stimulated with PPD (20 µg/ml) were cultured for 48h at 37º/CO₂ (5%). During last 4h, cells were incubated with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) and ionomycin (1 µM) plus brefeldin A (5 µg/ml) for 2h. The cells were stained with anti-CD4 Abs. After surface staining, cells were washed and resuspended in permeabilization-fixation solution (BD Cytofix/Cytoperm kit; BD Pharmingen, San Diego, CA), and intracellular cytokine staining was performed with fluorescence-labeled Abs to TNF-α, IFN-γ, according to manufacturer's protocol. FoxP3 staining was performed (Foxp3/Transcription Factor Staining Buffer Set, Ebioscience) according to manufacturer’s instructions. Data were collected using FACS Aria and analyzed with BD DIVA software.

Statistical analysis. Statistical analysis was done using unpaired ‘Student t’ test and one way ANOVA for group analysis with Graph pad prism software 6.

Results

Antibiotics treatment modulates the gut microbiota composition. Recent studies have shed new light on an impact of antibiotics on the gut microbes, which eventually may affect the severity of disease. Hence, we were curious to explore the influence of altered gut microbiota on the progression of TB. To test this hypothesis, mice were fed with broad spectrum antibiotics, prior to Mtb infection (pre-antibiotics) (for detailed methodology, please see supplementary data). Broad spectrum antibiotics were selected for the study. These antibiotics exhibited no impact on Mtb recovery in a Middlebrook 7H11 agar formulation (Chang et al., 2002). Animals treated with antibiotics for initial 5d showed significant (p<0.001) decrease in the CFU of the gut microbiota (Fig. 1A). In contrast, when the antibiotics treatment was prolonged for 42d, significant (p<0.001) elevation in the number of microbes was observed (Fig. 1A). Similar results (p<0.01) were observed in the case of mice prior challenged with Mtb and then treated with antibiotics (post-antibiotics) (Fig. 1B). Results suggest that initially antibiotics sensitive bacteria were eliminated, which resulted in the decline of CFUs (Fig. 1A). However, later antibiotics resistant microbes predominated by over proliferation. However, we observed significant (p<0.01) decrease in the diversity of the microbial taxa (Fig. 1C), since the majority of the colonies were of sister clones, as identified through their morphology. Further, we noticed enlargement in the size of caecum (Fig. 1D). This change in the intestine was due to the decreased ability of antibiotics treated mice to digest the food; as it was apparent by the presence
of indigested food in the intestine. Similar results were observed in post-antibiotics treated \textit{Mtb} animals (Fig. 1D).

\textbf{Animal treated with antibiotics showed higher \textit{Mtb} burden in the lungs and its dissemination.}
The aim of the study was to assess the consequence of antibiotics driven alteration in the gut microbiota on \textit{Mtb} survival in \textit{Mtb} challenged mice. Interestingly, we observed that disruption of microbiota in pre-antibiotics (p<0.05) and post-antibiotics (p<0.01) treatment, significantly enhanced the growth of \textit{Mtb} in the lungs of the infected animals (Fig. 2A, B). This information was further corroborated with histopathological analysis of the lungs (Fig. 2C). \textit{Mtb} infected mice on pre-antibiotics treatment showed larger and greater number of granulomas in their lungs, compared to control (\textit{Mtb} infected but not treated with antibiotics) (Fig. 2C; upper panel). In addition, we observed significant increase in the granulomatous or tuberculous region (p<0.01) in lungs of pre-antibiotics \textit{Mtb} infected animals (Fig. 2D). Similar trend was observed in the post-antibiotics treated animals as shown in histopathological images (Fig. 2C; lower panel).

Dissemination of \textit{Mtb} from lungs to other parts of the body is a probable factor responsible for extra-pulmonary infection. Interestingly, significant increase in the bacterial load of \textit{Mtb} was observed in the spleen (p<0.01) and liver (p<0.05) of pre-antibiotics \textit{Mtb} infected model (Fig. 3A). Similarly, post-antibiotics infected mice showed greater \textit{Mtb} burden in the spleen (p<0.01) and liver (p<0.01), as compared to controls (Fig. 3B). These data suggests that gut the microbiota play a crucial role in restricting the proliferation and dissemination of \textit{Mtb}.

\textbf{Fecal transplantation in antibiotics treated mice reconstitutes the gut microbiota.}
Before sacrificing animals, both pre-antibiotics and post-antibiotics treated groups were fecal transplanted (FT) orally. Interestingly, we observed significant (p<0.001) decline in the number of gut microbes in pre-antibiotics model after fecal transplantation and their number was comparable with normal mice (Fig. 4A) Further, there was significant (p<0.001) but partial restoration in the microbial diversity, as noticed through morphology of microbes in FT group (Fig. S1). The variation was not resilient i.e., did not return to its original percentage. It suggests that lowering of microbial diversity by antibiotics treatment could not be fully restored to its original frequency after FT for 15d. It is reported that antibiotics treatment decreases the number of beneficial microbes (Noverr and Huffnagle, 2004). Therefore, we have confirmed the alteration in gut microbiota due to antibiotics treatment, by identifying microbes in the fecal samples by RT-qPCR. We observed significant increase in the number of Enterococcus (p<0.05) but decline in the level of Bifidobacterium (p<0.01), Lactobacillus (p<0.05), Camphylobacter (p<0.05) and \textit{Bacteroides} (p<0.05) (Fig. 4B). These results further support the partial reconstitution of gut microbial composition through FT. This information was further corroborated by PCR (Fig. S2). As compared control animals (2.3 cm), antibiotics treated group showed increase in the size of caecum (3.2 cm). Interestingly, FT restored the size of caecum to near normal (2.6) (Fig. 4C). Furthermore, as compared to controls, the histopathological studies conducted on the ileum of pre-antibiotics treated animals exhibited distorted structure of microvilli. The microvilli structure of ileum was reinstated to normal in the FT animals (Fig. 4D). Similar results were observed in post-antibiotics model (Fig. S3). It was interesting observation that FT recovers antibiotics associated abnormities.
Fecal transplantation restricts the growth as well as dissemination of Mtb. Since, we observed significant augmentation in the growth of Mtb in the lungs of pre-antibiotics (p<0.05) and post-antibiotics (p<0.01) groups (Fig. 2, 3), therefore we thought to study the influence of FT on pre-antibiotics and post-antibiotics animals infected with Mtb. Interestingly, FT significantly reduced the bacterial load in the lungs (p<0.001) and spleen (p<0.01) of both pre- and post-antibiotics mice exposed to Mtb (Fig. 5A, B).

We also examined the progression of disease by studying the histopathological changes in the lungs. As compared to control group, larger size and number of granulomas in the lungs of pre-as well as post-antibiotics group were noted (Fig. 5C, D). It was observed that antibiotics treated mice after FT, exhibited granulomas with smaller size and number, lesser infiltration of lymphocytes and consolidated lung architecture, compared to antibiotics group (Fig. 5C, D). These results suggest that gut microbiome can contribute in controlling Mtb growth and its dissemination.

Antibiotics treatment augments Tregs but suppresses Th1 cells. The role of Th1 cells is established in protection, whereas Tregs promote susceptibility to TB. Consequently, it was imperative to analyze the impact of antibiotics treatment on Tregs and Th1 cells and correlation in the modulation in their frequency with predisposition to TB. We observed substantial decline in the expression of IFN-γ and TNF-α upon treatment with antibiotics (Fig. 6A, C). It was of interest to note the restoration of the production of IFN-γ and TNF-α in the mice with FT (Fig.6A, C). Expression of IFN-γ in the spleen was further confirmed by RT-qPCR (Fig. 6B). In contrast, antibiotics treatment augmented the frequency of Tregs, whereas FT downregulated their number as evidenced by the expression of FoxP3 (Fig. 6D). This observation suggests that the antibiotics driven fluctuation in the gut microbiota can modulate the frequency of Tregs and Th1 cells, which may be responsible for proneness to TB.

Discussion
TB is one of the world’s leading killer diseases with approximately two million deaths and eight million new cases annually. It is public health and economic burden on the country (Singh et al., 2014). However, majority of Mtb exposed individuals remain asymptomatic but exhibit varying level of immunity against Mtb infection. Several host factors have been identified in both mice and humans that contribute to susceptibility towards Mtb infection. The nramp1/SLC11A1 confers resistance in the murine model of TB, typhoid and leishmaniasis (Blackwell et al., 2001). The role of diet also considerably contribute in prompting disease symptoms (Dore and Blottiere, 2015).

Recently, research related to gut microbiota has gain considerable impetus, following the observation of its correlation with many immune disorders (Ochoa-Reparaz et al., 2009;Kamada et al., 2013). Shifts in the composition of the microbiota, whether induced by dietary changes, antibiotics treatment or invasive pathogens, can disturb the balance of gut microbes and dysregulate the function of local as well systemic immune system (De Filippo et al., 2010;Salzman, 2011;Wu and Wu, 2012). Perturbation in the gastrointestinal microbiota composition is also strongly associated with allergies and asthma (Noverr and Huffnagle, 2004;Penders et al., 2007).
Current study revealed the role of gut microbiota in controlling the pathogenesis of TB. Key findings emerged from the study suggest that disruption of gut microbiota with antibiotics of *Mtb* infected animals revealed (i) significant alteration in the gut microbiota; (ii) higher *Mtb* burden in the lungs; (iii) dissemination of *Mtb* to spleen and liver; (iv) fecal implants reconstituted the gut microbiota and recuperated TB by declining the *Mtb* burden.

We selected antibiotics that were effective against both Gram positive and negative bacteria. Further, dose of antibiotics was carefully chosen that showed no effect on *Mtb* viability, even when administrated for 42d. Importantly, the selected dose significantly induced dysbiosis in the gut. Importantly, alteration in gut microbiota promotes the survival of *Mtb* in the lungs. This finding emphasizes that composition and function of the gut community are important factors in conferring host resistance to invading pathogens. Our study revealed very interesting findings that antibiotics mediated disruption of gut microbiota not only increases the growth of *Mtb* in the lungs but also promotes its dissemination to other organs. Impact of antibiotics driven changes of gut microbiota on *Mtb* survival was further supported by fecal transplantation. Interestingly, fecal transplantation of antibiotics treated mice restores the gut microbiota and decreases the *Mtb* burden in their lungs and prevents dissemination to spleen.

Microbiota plays an active role in the development and function of both pro- and anti-inflammatory T-cell pathways (Round and Mazmanian, 2009; Kamada and Nunez, 2013). Frequency of the gut microbiota should be well-tuned to mount host response against pathogens. Imbalance in the number of Tregs changes the microbial composition and vice-versa (Round and Mazmanian, 2010). CD4 T cells are the major players in imparting immunity against *Mtb*. It is important to mention that the gut microbiota contributes substantially in the development of CD4 T cells, both within and outside the intestine (Noverr and Huffnagle, 2004; Garidou et al., 2015). We also observed that mice treated with antibiotics showed suppression of Th1 immunity but increase in the frequency of Tregs. Fascinatingly, fecal transplantation of antibiotics treated mice restored the gut microbiota and reinvigorated immunity by augmenting the pool of IFN-γ and TNF-α releasing Th1 cells. In contrast, inhibition in the population of Tregs was noted. Currently, it is difficult to explain how this phenomenon is operating. However, this observation is quite interesting and may open a new line of investigation.

Antibiotics have been a cornerstone of innovation in the field of public health but their negative effect on immune system and health cannot be ignored. Antibiotics driven compositional changes in the intestinal microbiota lead to severe dysregulation in the physiological and immunological intestinal homeostasis, creating serious and adverse consequences for the host (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011). To overcome such effect, new treatment strategies should be developed and designed to counteract the negative effect of antibiotics. One strategy could be to provide probiotics to supplement antibiotics-induced deficits in the microbiota. Another, yet better approach could be to use immunomodulators to enhance the efficacy of immune system to combat infectious agents. With better understanding of the correlation between gut microorganisms and *Mtb*, one hope is that their manipulation/supplementation might prove to be a future targeted therapy for treating diseases. Advances in understanding how gut microbiota regulate the pathogenesis of TB, may pave a novel way toward new therapeutic intervention.
Acknowledgments. We are grateful to Dr. BN Dutta, former Professor of the Postgraduate Institute of Medical Education and Research, Chandigarh, India for histopathological analysis.

Funding. This study is supported by the Council of Scientific and Industrial Research (CSIR), New Delhi, India. NK, SN, SKN are recipient of fellowship of DBT; AV and GN of CSIR, India.

Conflict of Interest: Authors declare no conflict of interest

Contribution of Authors. Concept or design of the work (JNA and NK); experiments performed (NK, AV, SN, SKN, GN); analysis, or interpretation of data for the work (NK and JNA)

References


**Figure Legends**

Fig. 1. *Antibiotics altered the gut microbial composition.* (A-C) Mice were pre-treated with broad spectrum of antibiotics for 42d. In between, on day 21 animals were challenged with *Mtb*. (A) Cultivable microbes on 5d and 42d; (C) microbial diversity on 42d were enumerated in fecal samples of treated mice. (B) In post-antibiotics model, after 21d of *Mtb* challenge; mice were treated with antibiotics daily for subsequent 21d. Later, cultivable microbes were enumerated in the fecal samples. (D) Increment in the size of caecum in both pre-antibiotics and post-antibiotics animals was measured; bar graph represents the length (cm) of caecum. Data shown as mean±SEM are representative of 3 independent experiments (n=4-5 animals/group). *p<0.05, **p<0.01, ***p<0.001.

Fig. 2. *Disturbance in gut microbiota by antibiotics increased the survival of Mtb in the lungs and its dissemination to other organs.* Mice treated with antibiotics prior and post exposure to *Mtb* infection. Later, animals were sacrificed and lungs were isolated. Bacterial burden in (A) pre-antibiotics; (B) post-antibiotics treated group was estimated by plating serial dilutions of lung homogenate on 7H11 agar plates. Colonies were enumerated on 21d of plating. Bar graph depicts the bacterial burden in lungs. Data shown as mean±SEM are representative of 3 independent experiments (n=4-5 mice/group). (C) Histopathology sections of lungs of pre-antibiotics and post-antibiotics group were H and E stained and imaged at a magnification 40X. (D) Bar graphs depict the percentage of tuberculous region of pre-antibiotics-*Mtb* group. Data are representative of 3 independent experiments (n=4-5 animals/group). *p<0.05, **p<0.01.

Fig. 3. *Disrupted gut microbiota promotes the dissemination of Mtb.* Mice were treated with (A) pre-antibiotics; (B) post-antibiotics to *Mtb* infection. After 42d, mice were sacrificed and *Mtb* load was estimated in the spleen and liver by enumerating CFUs. Bar graph depicts the *Mtb* load. Data shown as mean±SEM are representative of 3 independent experiments (n=4-5 animals/group). (*p<0.05, **p<0.01.

Fig. 4. *Faecal transplantation reconstitutes the gut microbiota.* (A) pre-antibiotics mice were administered 5 doses of FT, 15d prior to sacrificing animals. Later, (A) enumeration of cultivable microbes was assessed in the fecal samples. (B) Alteration in the number of microbes *Enterococcus; Bifidobacterium; Lactobacillus; Campylobacter; Bacteroides* was studied in the fecal samples by RT-qPCR. (C) Increment in the size of caecum was measured. (D) Histopathology sections of ileum were H and E stained and photomicrographs are shown at 100X magnification. Data shown as mean±SEM are representative of 2 independent experiments (n=4-5 animals/group). *p<0.05, **p<0.01, ***p<0.001.

Fig. 5. *Restoration of gut microbiota restrains the growth of Mtb in the lungs and prevents its dissemination.* Mice with (A,C) pre-antibiotics; (B,D) post-antibiotics treatment were provided 5 doses of FT, 15 d prior to sacrificing. Later, *Mtb* load was estimated in lungs and spleen. Bar graph depicts the *Mtb* load. Histopathology sections of lungs of (C) pre-antibiotics; (D) post-antibiotics treated group were H and E stained and imaged at 40X magnification. Data shown as mean±SEM are representative of 2 independent experiment (n=5 animals/group).*p<0.05, **p<0.01, ***p<0.001.

11
Fig.6. Dysbiosis of gut microbiota imbalanced the frequency of Th1 and Tregs cells. Mice with pre-antibiotics treatment were provided 5 doses of FT, 15d prior to sacrificing. Later, intracellular expression of (A) IFN-γ; (C) TNF-α; (D) FoxP3 was monitored in CD4 gated T cells by flowcytometry. (B) IFN-γ expression was detected at mRNA level by RT-qPCR. Data shown are representative of 2 independent experiments (n=4-5 mice/group).