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Gut Microbiota Maintains Homeostasis of CD11b$^+$ Macrophages Proliferation and Inflammatory Responses Defense against Enteropathogenic Infection

Yakun Sun[1,2#], Deyan Luo[1,2#], Nianzhi Ning[2], Jiajia Li[2], Tao Li[2], Zhili He[2], Fanghong Chen[2], Zhan Li[2] and Hui Wang[1,2*]

[1]Anhui Medical University, Hefei, 230000, China

[2]State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, 10071, China

[#]These authors contributed equally to this article.

*Address for Correspondence: Hui Wang, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, 20 Dongda Street, Fengtai District, Beijing, 10071, China, Tel: +86 10 66948532; Fax: +86 10 66948532; E-mail: wanghui_dyx@hotmail.com

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Abstract

Aims: The normal microbiota provides a balance microenvironment as a barrier to enteropathogenic infections, such as with enterohemorrhagic *Escherichia coli*. However, the interactions between pathogens and the microbiota are largely unknown.

Methods: High-throughput 16S rRNA gene amplicon analysis showed the disruption of intestinal commensal flora of pharmacologic destruction of gut microenvironment in mice. Nuclear magnetic resonance metabonomics analysis showed a strong metabolic disruption which was correlated with gut microbiota diversity. Pharmacologic destruction of gut microenvironment abrogated immune defense in mice challenged with enterohemorrhagic *Escherichia coli* by lavage.

Results: This report demonstrates for the first time that gut microbiota is an essential part of CD11b$^+$macrophages and pro-inflammatory cytokines mediated defense against enterohemorrhagic *Escherichia coli* infection. We found streptomycin-treated mice displayed reduced survival, increased bacterial burden, increased inflammatory responses, and exacerbated intestinal pathology. At the site of infection, the number of CD11b$^+$ macrophage decreased, and exuberant expression of pro-inflammatory cytokines could result in tissue damage.

Conclusions: The data suggested that gut microbiota played an important role to combat enteropathogenic infection, which was probably by maintaining CD11b$^+$ macrophages proliferation to effectively clear bacteria, and reducing tissue damage by controlling pro-inflammatory responses.

Keywords: Microbiota, Macrophage, Enteropathogen

Introduction

The human intestinal tract harbors trillions of microorganisms referred to as the gut microbiota, which plays an important role in nutrition, physiology, development, and immunity, with disruption of the structure of this community leading to dysbiosis [1]. The microbiota is largely regarded as a barrier to enteropathogenic infections, such as enterohemorrhagic *Escherichia Coli* (EHEC). However, the interactions between enteropathogen and the microbiome are largely unknown [1]. The first appreciation of pathogen-microbiota interactions about bacterium pathogenesis came from the observation that microbiota employs quorum sensing signaling to regulate pathogen's virulence [2,3]. The second appreciation of pathogen-microbiota interactions was nutrient signaling in pathogen's virulence regulation. Enteropathogens generally compete directly against commensals for nutrients and colonization sites within the intestine [4-6]. Since the intestinal microbiota is thought to be an essential contributor to enteropathogenic infections, that impacting the functioning of the immune system should be the third appreciation of pathogen-microbiota interactions [7,8].

Some extrinsic factors, such as with antibiotic or virus, alter the microbiota by reducing diversity and shifting community composition [9,10]. These community shifts cause changes in the metabolic profiles of the intestine including amino acids, fatty acids and glucose, suggesting disruption of a fundamental feature of commensal flora [11]. Several mechanisms by which gut microorganisms can modulate the development of metabolic diseases have been reported [12,13]. Interaction between the host via metabolic capacities of the gut microbiota with immunity is particular interest for infections [14].

EHEC is one of the enteropathogenic bacterium that can be used in our study. It is a gram-negative facultative extracellular bacterium that causes hemorrhagic colitis which can progress to hemolytic uremic syndrome (HUS), a severe kidney disease with immune involvement [15]. It is classified as attaching and effacing pathogens based on their ability to destroy the intestinal barrier [16]. Whereas, the lethality of EHEC is caused by the presence of the potent shiga toxin carried on plasmids. Although much is known about the virulence of EHEC infections, the induction of the host immunity particularly as it relates to intestinal commensal flora is less well understood. Early studies suggested that this interplay between commensal flora and EHEC might be adhering to intestinal epithelium and destroying the epithelial barrier to develop disease [16]. However, some prior reports describing roles for commensal flora during host defense against EHEC and other infections [17] have used naïve mice and focused on innate immune-defense mechanisms. Obrien's group found it was difficult to set up the sensitive mouse model for EHEC infection research only when mice were given drinking water containing streptomycin [18]. We believe streptomycin can reduce the normal facultative intestinal flora of the mice which maybe related with immune defense against EHEC infection. Thus, the streptomycin-treated mouse is a good research animal model to address interactions between microbiota and immunity during enteropathogenic bacterium infection.

Materials and Methods

Mice

All animal studies were conducted in accordance with Beijing Institute of Microbiology and Epidemiology Animal Care and Use Committee (2012-06-21-02) guidelines. BALB/c wild type mice (5-week-old, weighing 14-16g) were obtained from our institute Laboratory Animal Center, Beijing, China. All experimental mice were bred in a specific pathogen-free facility at our institute.

Bacterial infections, fecal transplantations and antibody treatment

Wild-type strain ATCC O157:H7 EDL933 of EHEC was purchased from ATCC center (American Type Culture Collection). For infections, strain O157:H7 EDL933 was incubated in brain heart infusion broth (BHI; Beijing China) under aerobic condition at 37°C for 48 hours. The bacteria were then diluted to an OD of 0.1 at 620 nm, regrown in the same media for 3 to 4 h at 37°C, quantified by measuring the OD, and resuspended in saline at the desired concentration. Where indicated, 20 BALB/c mice were treated pharmacologically by supplementing drinking water with 5 mg/ml streptomycin (Sigma-Aldrich, USA) beginning 3d prior to infection with replenishment every 48h. Twenty BALB/c mice were treated none as controls. Infections were performed by applying 1×10^9 CFU by lavage. Half mice were used for survival analysis and half for harvest. Where indicated, 7 BALB/c mice were treated pharmacologically by supplementing

drinking water with 5 mg/ml streptomycin and 7 BALB/c wild type mice were treated none as controls. Mice were harvested on day 3 to collect sera and fecal pellets.

The intestinal commensal flora was depleted by treating mice with streptomycin in drinking water. Fresh fecal pellets from three donor mice were collected and placed in 1ml transfer buffer (pre-reduced sterile phosphate buffered saline containing 0.05% cysteine HCl) on ice. The supernatant of fecal pellets was collected and diluted (1:3) in transfer buffer. One hundred microliters of diluted fecal supernatant was introduced into recipient mice by oral gavage six times each 48 h.

When indicated, macrophages of 5 BALB/c mice were depleted in vivo by i.p. injection of 0.2 mg CD11b-specificmAb (clone M1/70; BioXCell) on days 1, 3, and 5; Five BALB/c wild type control mice received injections of isotype-matched rat IgG2a mAb (BioXCell).

Measurements of survival and bacterial burden

Mice were monitored at least once daily for 15 days after initiating infection. Unresponsive or recumbent animals were considered moribund and euthanized. To measure bacterial burden, tissues were collected from mice that were euthanized by carbon dioxide. The number of viable bacteria in colon was measured by homogenizing tissues in saline, plating serial dilutions on Columbia agar supplemented with 0.05 mg/ml Nalidixic acid (Sigma-Aldrich), and counting CFU after 24 h growth at 37°C.

16S rDNA amplicon sequencing

Total genome DNA from fecal pellet was extracted using CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. 16S rRNA/ITS genes of distinct regions (16SV4/16SV3/16SV3-V4/16SV4-V5, ITS1/ITS2, Arc V4) were amplified used specific primer (e.g. 16S V4: 515F-806R, *et. al*) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Samples with bright main strip between 400-450bp were chosen for further experiments. Then, mixture PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated.

Nuclear magnetic resonance (NMR) metabonomics analysis

NMR metabonomics analysis was done by Wuhan Anachro Company. In brief, Sera were vortexed, aqueous layer was transferred to 0.5 ml 3KDa ultrafiltration filter (Millipore, USA). Fifty microliters H_2O and 50μl DSS standard solution (Anachro, Canada) was added. Samples were mixed well before transfer to 5mm NMR tube (Norell, USA). Spectra were collected using a Bruker AV III 600 MHz spectrometer equipped with an inverse cryoprobe. The first increment of a 2D-1H, 1H-NOESY pulse sequence was utilized for the acquisition of 1H-NMR data and for suppressing the solvent signal. Experiments used a 100ms mixing time along with a 990ms pre-saturation (~80 Hz gammaB1). Spectra were collected at 25°C, with a total of 64 scans over a period of 7 min. The collected Free Induction Decay (FID) signal was automatically zero filled and fourier transform in Processing module in Chenomx NMR Suite 8.1. (Chenomx Inc., Edmonton, Canada). The data was then carefully phased and baseline corrected by experienced technician in Chenomx Processor. All the spectra were referenced to the internal standard, DSS and analyzed by experienced analysts against Chenomx Compound Library. All metabolites' concentration information was exported to excel and normalized by weight across all parallel samples before used in the later on multivariable analysis. Principal Component Analysis (PCA) and Partial Least Squares Discrimination Analysis (PLS-DA)were performed using the pcaMethods bioconductor package [19] and pls package respectively. Plots were made using ggplot2 package [20].

Measurements of immune parameters

Tissue levels of mRNA encoding TNF-α, IL-6, IL-1β, IFN-γ, IL-8, TF and β-actin were measured by real-time PCR (LightCycle 480), normalized to levels of mRNA encoding β-actin, and expressed as fold change relative to levels in uninfected wild-type mice. The primers were as follows: TNF-α, forward CATCTTCTCAAAATTCGAGTGACAA, reverse TGGGAGTAGACAAGGTACAACCC; IL-6, forward GAACAACGATGATGCACTTG, reverse TGAAGGACTCTGGCTTTGTC; IL-1β, forward TGTAATGAAAGACGGCACACC, reverse TTCTTTGGGTATTGCTTGGGA; IFN-γ, forward CATTGAAAGCCTAGAAAGTCTGAATAAC, reverse TGGCTCTG-

Figure 1: Streptomycin-induced alteration of fecal microbiota profiles.

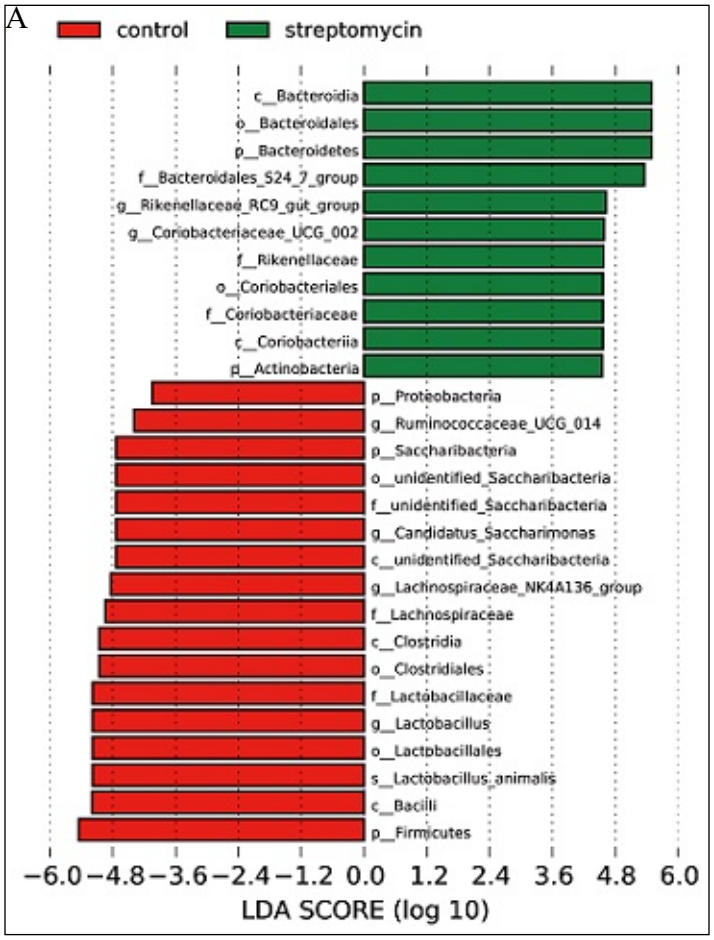

Figure 1A: LEfSe analysis shows differentially abundant genera as biomarkers determined using Kruskal-Wallis test (P<0.001) with LDA score >4.

CAGGATTTTCATG; IL-8, forward TGGCAGCCTTCCTGATTT, reverse AGGTTTGGAGTATGTCTTTATGC; TF, forward CAATGAATTCTCGATTGATGTGG, reverse GGAGGATGATAAAGATGGTGGC;β-actin, forward GGAGGGGGTTGAGGTGTT, reverse GTGTGCACTTTTATTGGTCTCAAG.

Flow cytometry

Preparation of intestinal lymphocytes was described as below. In brief, cells isolated from intestinal lymph nodes were ground with glass rod and were incubated with Fc Block (clone 2.4G2) for 15 min at 4°C, washed 3 times. For enumeration of CD45 cells, macrophages, and neutrophils, intestinal lymphocytes were stained on ice with anti-CD45-EF450 (cloneRM-5), anti-CD11b-APC (clone M1/70), and anti-Ly-6G-FITC (clone 1A8). Data were gated for forward scatter/side scatter and collected on a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Histology

Large intestinal tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The pathological foci in each section were evaluated. (i.e. areas with large numbers of inflammatory cell infiltration accompanied by evidence of edema of submucosa). Representative photomicrographs depict ×200 magnification.

Statistical analysis

Statistical analyses were performed using the program Prism 5.0 (GraphPad Software, Inc., La Jolla, California, USA). Values are expressed as mean±SD. Data were analyzed by unpaired Student's t-test (normal distribution) or one-way ANOVA followed by Dunnett's multiple comparison test. Survival data were analyzed by log rank tests. Statistical analysis of microbiota data was performed in Rhea [21]. EzTaxon [22] was used for the identification of OTUs showing significant differences ($p<0.05$) in relative abundances between feeding groups. $p<0.05$ was considered to be statistically significant.

Results

Streptomycin altered gut microbiota by reducing diversity and shifting community composition

To investigate the importance of diversity of gut microbiota profiles during infections, the High-throughput 16S rRNA gene amplicon analysis was used. The gut microbiota of mice treated with streptomycin corresponding to control mice were analyzed via MiSeq sequencing of 16S rRNAV4 amplicons. A total of 1,812,116 sequences ranging from 85,873 to 98,965 sequences per sample (mean=90,605) were obtained after quality control analyses and Operational Taxonomic Units (OTUs) filtering. From these data, we identified 72-87 OTUs (mean=75) per sample in streptomycin treated group and 310-328 OTUs (mean=324) per sample in the control group. To further investigate the features more likely to explain the differences between mice treated with streptomycin and normal controls, LDA Effect Size (LEfSe) was performed by coupling standard tests for statistical significance with additional analyses examining biological consistency and effect relevance. Features with a linear discriminant analysis (LDA) score cut-off of 4.0 were identified as being different (Figure 1A). A cladogram for family and genus level abundance was shown in Figure 1B. The results showed that *Bacteroides*, *Coriobacteriales* and *Acti-*

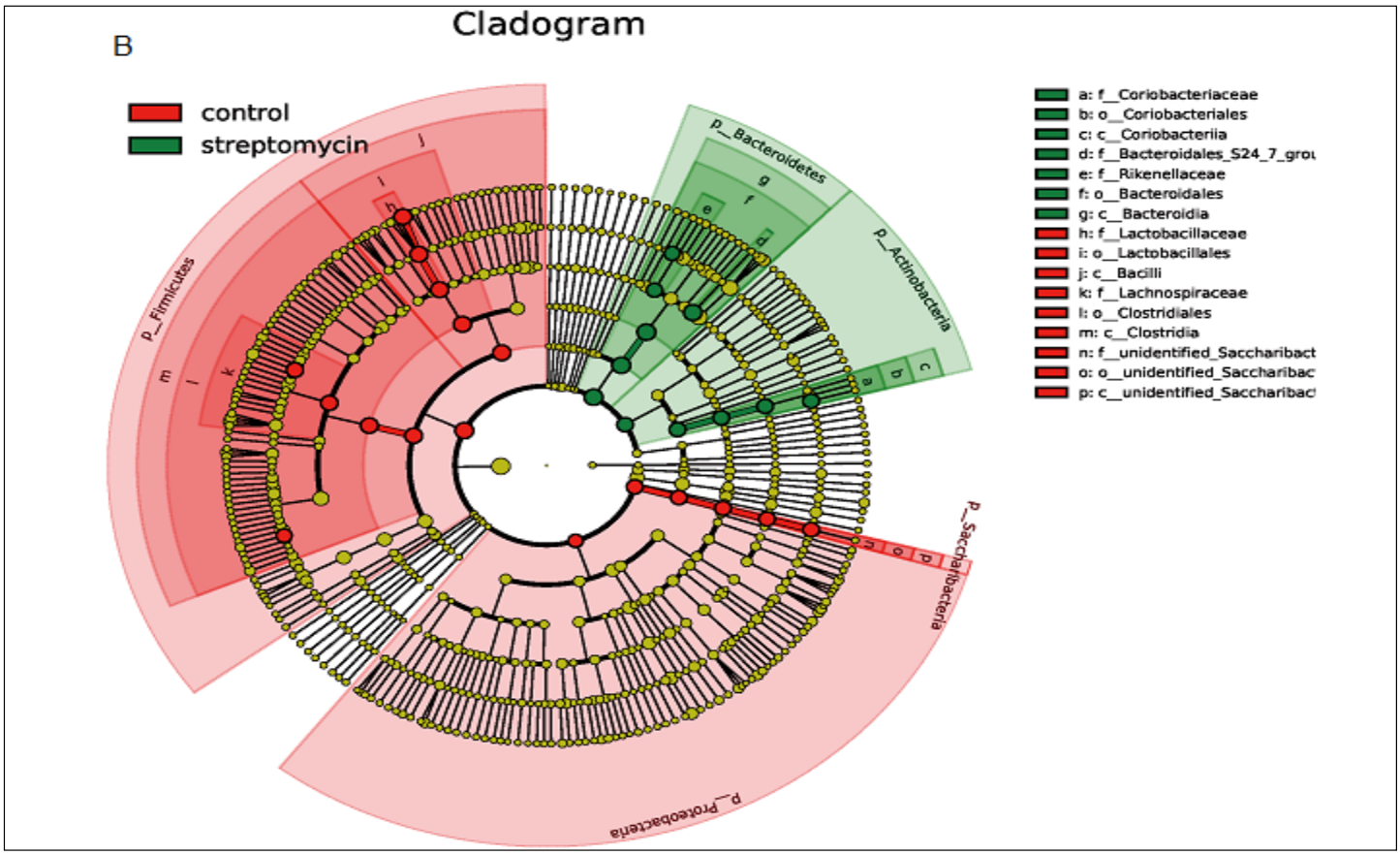

Figure 1B: Cladogram representation of the differentially abundant from phylum to species (only top 50% are plotted here). The root of the cladogram denotes the domain bacteria. The taxonomic levels of phylum, class, order, family, genus and species are labelled, with the colors indicating the test group/control group hosting the greatest abundance. The size of each node represents their relative abundance.

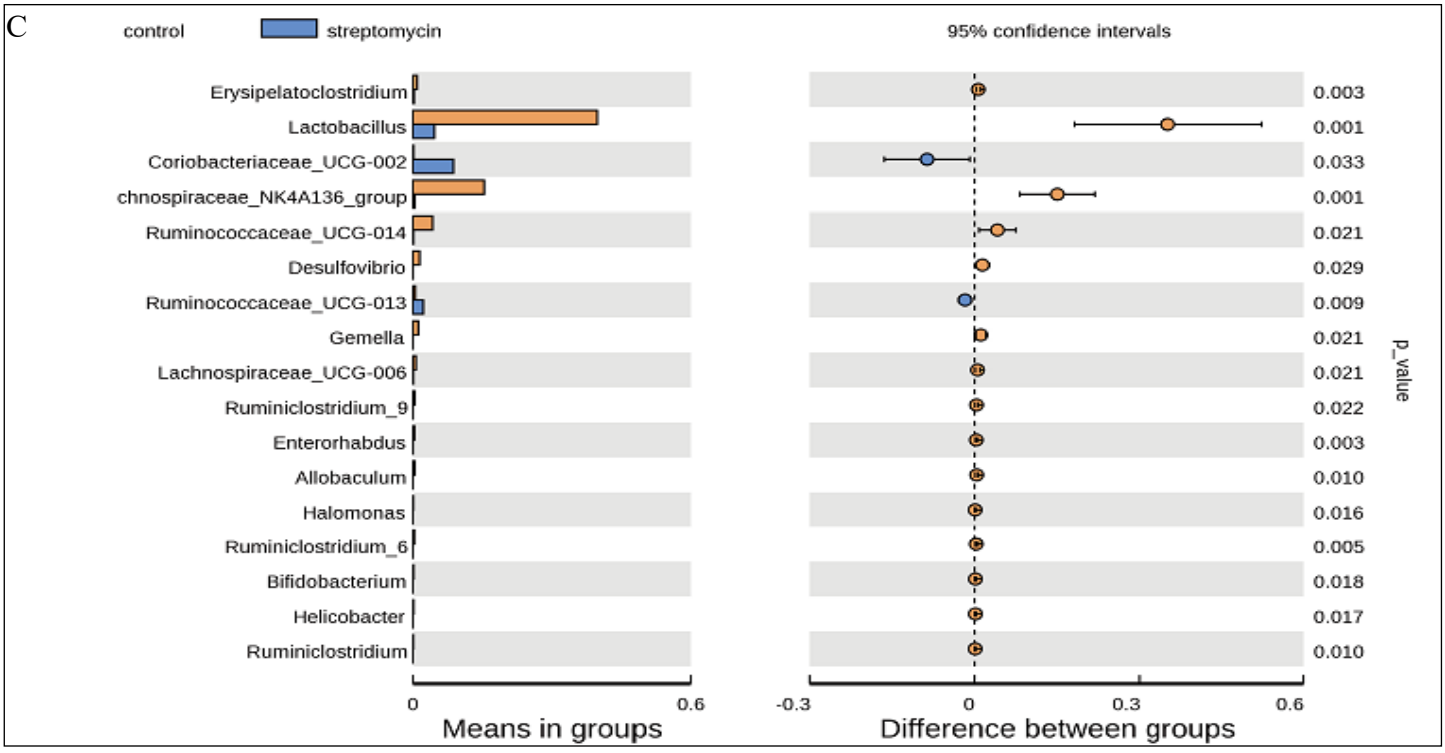

Figure 1C: Extended error bar plot showing the seventeen bacterial genera with a significant difference (T-test; P<0.05) in proportions of at least 1% between samples of control group and samples of streptomycin-treated group. Number of mice: Control, 7; Streptomycin treated group, 7.

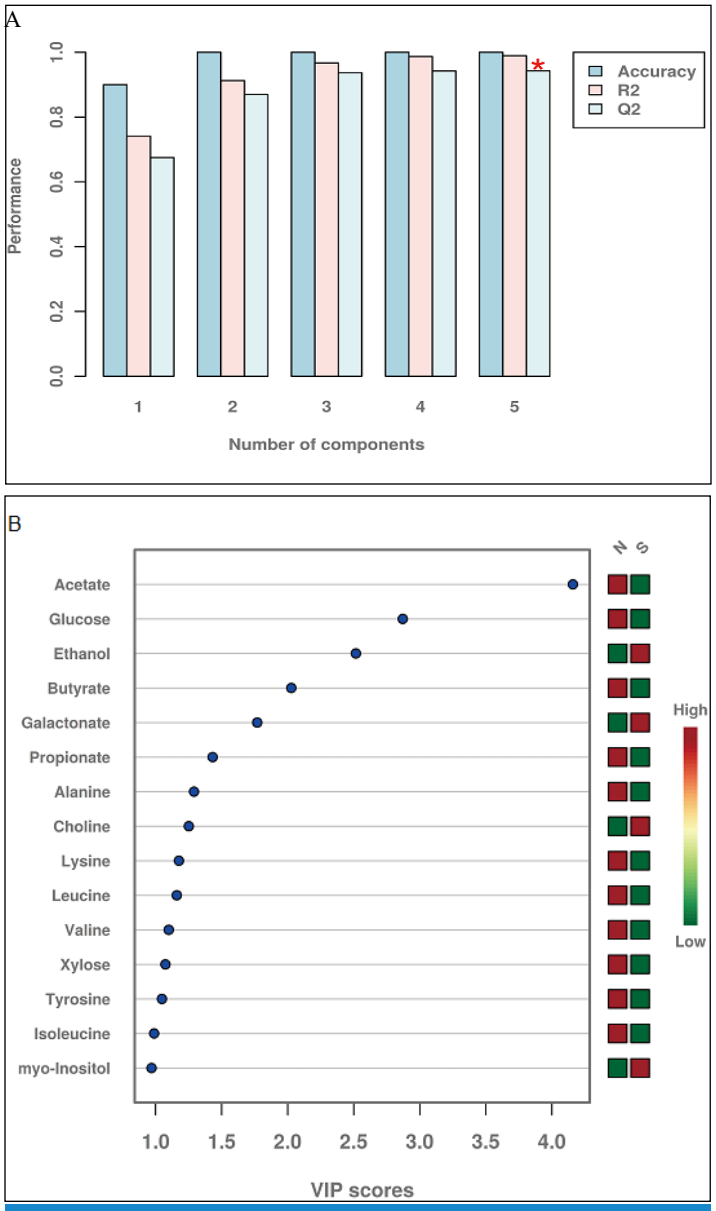

Figure 2: Streptomycin-induced alteration of metabolic characterization. The NMR data sets that included 14 aqueous from different mice. The R2 and Q2 intercept values were 0.99 and 0.83 after 14 permutations (A), The VIP scores (B) of the major metabolites for the separation of samples of mice treated with streptomycin in the PLS-DA derived score plots. Number of mice: Control, 7; Streptomycin treated group, 7.

nobacteria were significantly enriched in guts of mice treated with streptomycin, whereas *Firumicutes, Bacilli, Lactobacillus, Clostridia, Lachnospiraceae, Saccharibacteria, Ruminococcaceae, Proteobacteria* and butyrate-producing bacteria (BPB) *Clostridium* were negatively associated with these mice. Finally, extended error bar plot shows the seventeen bacterial genera with a significant difference in proportions of at least 1% between samples of control group and samples of streptomycin-treated group. Two genera are overabundant within the fecal samples collected from mice treated with streptomycin compared to those collected from control mice, genera abundance (left) and 95% confidence intervals (right), were detected in 7 streptomycin-treated mice (Figure 1C).

Multivariate data analysis and metabolic characterization of samples of mice treated with streptomycin

Next, we need to address the metabolic characterization after gut microbiota diversity was disrupted. The NMR data sets that included 14 aqueous metabolite data from samples of different mice were assessed using PCA and PLS-DA score plotting. Our data show that significant differences were observed among the two groups. Samples of mice treated with streptomycin were clearly separated from samples of control mice (PLS-DA score plots and PCA) (data not shown). PLS component 2 versus PLS component 1 explained separation among samples of mice treated with streptomycin from control group. Combining PLS component 1 and PLS component 2 explained 80.3% of total variance (68.7% and 11.6%, respectively). When cross-validation on PLS-DA was performed, component 5 and parameters, such as R2X = 0.95, R2Y = 0.99, and Q2Y = 0.83, which showed its predictive power and degree of fit to the data, were obtained (Figure 2A). The result shows the PLS-DA-derived Variable importance in the projection (VIP) values of the major compounds contributing to the separation of samples of mice treated with streptomycin in the PLS-DA model. VIP value is a weighted sum of squares of the PLS-DA weight, both with respect to Y as the correlation to all the responses and X as its projection, picking components that play important roles in the separation. It has been indicated that cutting-off for VIP around 1.0 worked well for variable selection. As shown in Figure 2B. We have shown in this work that metabolites implicated in glycometabolism and lipid metabolism: levels of glucose, acetate, butyrate, propionate, alanine, lysine, leucine, valine, tyrosine and isoleucine decreased in fecal samples of mice treated with streptomycin. Going further, we demonstrated that metabolites implicated in protein metabolism: four essential amino acid (lysine, leucine, valine and isoleucine) levels in the guts decreased markedly when the mice were treated with streptomycin.

Gut microbiota plays a protective role during enterohemorrhagic *Escherichia Coli* infection

Since the streptomycin did alter the diversity of gut microbiota and metabolic profile, we need to address the impact on immune

responses of alteration of gut diversity during infections. Here, we used EHEC as model to research the interactions between pathogen and microbiota. To investigate the functional importance of gut microbiota during EHEC infection, we evaluated wild-type mice treated with streptomycin in guts. Specifically, we compared mice treated with streptomycin in drinking water which disrupted the balance of commensal flora and mice treated none as control. Upon lavage inoculation with the sublethal dose (1×10^9 CFU) of EHEC, mice treated with streptomycin began to succumb on day 5, and only 20% survived until day 15 (Figure 3A). In contrast, the control mice showed significantly improved survival. Parallel evaluations of body weight changes over the course of infection suggested that the control mice experienced less severe disease than did mice treated with streptomycin (Figure 3B). On day 5 after the inoculations, mice were euthanized, and the intestinal EHEC bacterial burden (Figure 3C) and shiga toxin levels were measured (Figure 3D). Levels of bacterial burden and shiga toxin increased in streptomycin treated mice. The shiga level of blood was correlated with CFU after sublethal inoculation. Fecal microbiota transplantation was seen as an advanced method for dysbacteriosis treatment. Fecal transplantation 3 days and 6 days after the initiation of infection did suffice to improve the survival of mice treated with streptomycin (Figure 3E). Parallel evaluations of body weight changes over the course of infection suggested that the mice transplanted with fecal commensal flora experienced less severe disease than did control mice, even though these body weight differences did not achieve statistical significance (Figure 3F). On day 5 after the inoculations, mice were euthanized, and intestinal EHEC bacterial burden (Figure 3G) and shiga toxin levels were measured (Figure 3H). Levels of bacterial burden and shiga toxin decreased in fecal transplanted mice. Shiga levels also correlated with CFU after sublethal inoculation. However, there were no significance between 3 days recovery group and 6 days recovery group. In the large intestinal tissues of streptomycin treated mice, the cellular infiltrates tended to be medium to large and were frequently associated with edema (Figure 3L, see arrow), whereas the foci in the tissues of control mice (Figure 3J) and fecal microbiota transplanted mice (Figure 3L) tended to be small to medium. We conclude that gut microbiota performs a critical protective function during sublethal dose EHEC infection. We believe that the diversity of microbiota and followed metabolic profile are essentials for innate immune response against EHEC in streptomycin-treated animal models.

Gut microbiota contributes to anti-inflammation and CD-11b$^+$macrophage maintaining against enterohemorrhagic Escherichia Coli

Next, we need to investigate the mechanisms of protection conferred by gut microbiota. We measured inflammation markers and immune cell accumulation in streptomycin treated mice and control mice. Consistent with our previous discoveries of CFU (Figure 3C), Similar trends were observed for markers of inflammation, including intestinal levels of mRNA encoding TNF-α (Figure 4A), IL-6 (Figure 4B), IL-1β (Figure 4C), IFN-γ (Figure 4D), IL-8 (Figure 4E), and TF (Figure 4F). The differences between mice treated with streptomycin and control mice were highly significant. To investigate the nature of the cellular response during gut microbiota defense against EHEC, immune cells were collected from intestinal lymph nodes and stained on ice with anti-CD4-EF450, anti-CD11b-APC, and anti-Ly6G-FITC. The multi-color flow cytometric studies were performed. In comparison with control mice, the numbers of intestinal CD11b$^+$ macrophage decreased in streptomycin-treated mice (Figure 4G, 4I). Whereas, the streptomycin-treated mice and control mice harbored similar numbers of total pulmonary CD4$^+$ T cells and neutrophils (data not shown). To assess whether a decrease in macrophage numbers played an important role during infection, macrophages were depleted at the time of EHEC challenge. Specifically, mice were treated with CD11b-specific mAb or isotype-matched control mAb on day 1, 3, and 5 relative to challenge with EHEC. Mice treated with CD11b-specific mAb displayed significantly reduced survival in comparison with mice treated with control mAb (Figure 4H).

Discussion

Results of High-throughput 16S rRNA gene amplicon analysis showed the disruption of intestinal commensal flora of streptomycin-treated mice. Reducing diversity and community composition of intestinal commensal flora in mice also changed metabolic characterization in gut. Gut tract harbors trillions of indigenous bacteria whose coexistence relies on the ability of each member to use a few limiting resources. Invading pathogens need to compete with the microbiota to establish their colonization [23]. Although, EHEC is not a stranger to gut track for nutrients. The interplay between the nutrient requirements of normal flora and EHEC is still important to determine its

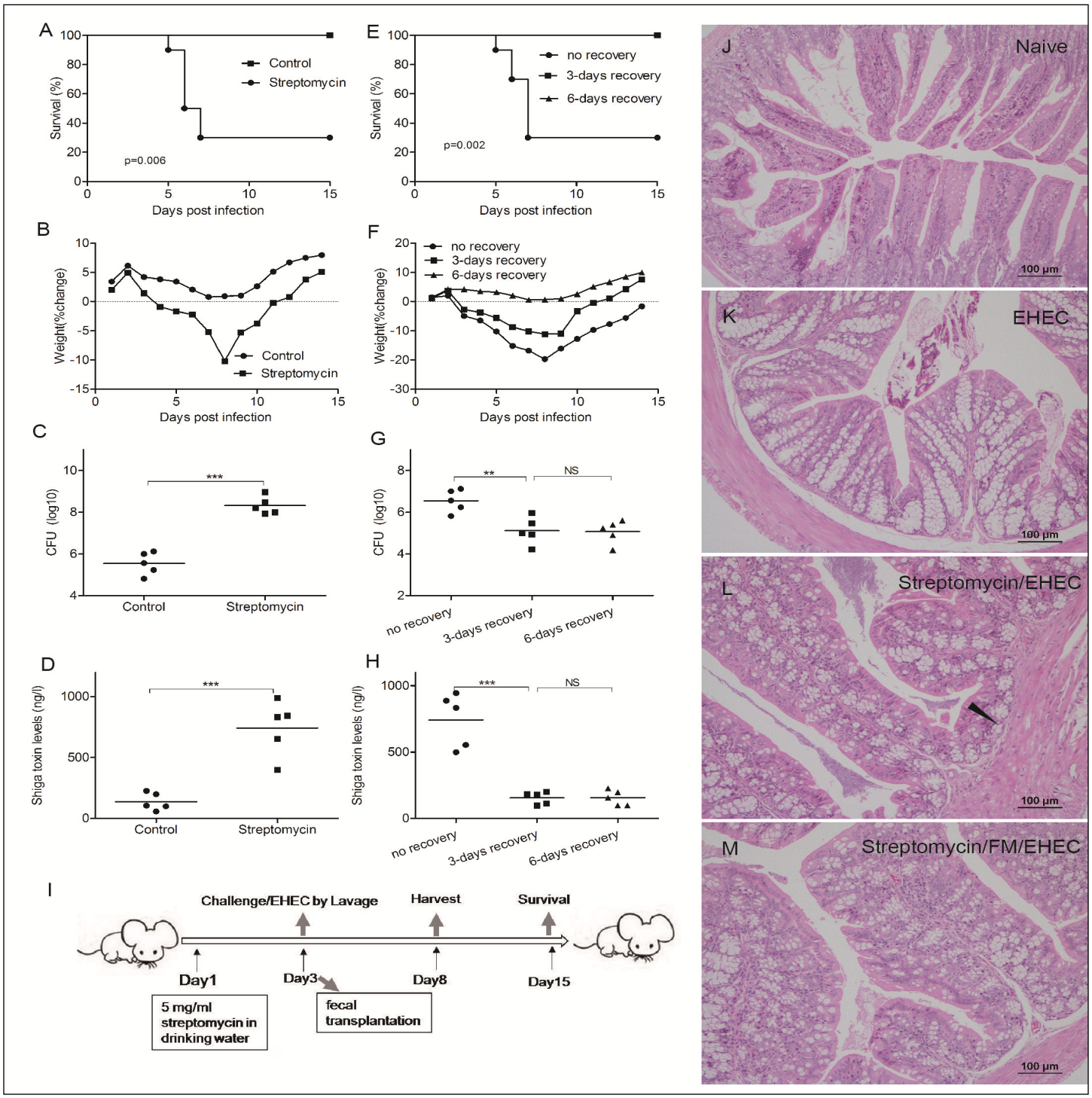

Figure 3: Gut microbiota is dispensable for survival after inoculation with a sublethal dose of EHEC. Streptomycin treated mice and control mice were inoculated with a sublethal dose (1-2×10^9 CFU) of EHEC, (A) survival, (B) percent body weight change, (C) intestinal bacterial burden and (D) shiga toxin levels. All control mice survived (n=10 per group), whereas 80% streptomycin treated mice succumbed (n=10 per group). The streptomycin treated mice showed more weight loss and higher level of shiga toxin. The administration of fecal flora 3 days and 6 days after the initiation of infection did suffice to improve the survival of mice treated with antibiotic, (E) survival, (F) percent body weight change, (G) intestinal bacterial burden and (H) shiga toxin levels. All the recovered mice survived (n=10 per group), whereas 80% control mice succumbed (n=10 per group). The control mice showed more weight loss and high level of shiga toxin. (I) Mice treatment plan. Gut microbiota also helped to reduce pathogen during infection, (J) naïve, (K) lesions observed in hematoxylin-and-eosin-stained intestinal section from mice treated none and then infected with EHEC, and Lesions observed in intestinal section from (L) mice treated with streptomycin and (M) mice transplanted with fecal microbiota, then infected with EHEC. **p<0.01, ***p<0.001. NS, not significant.

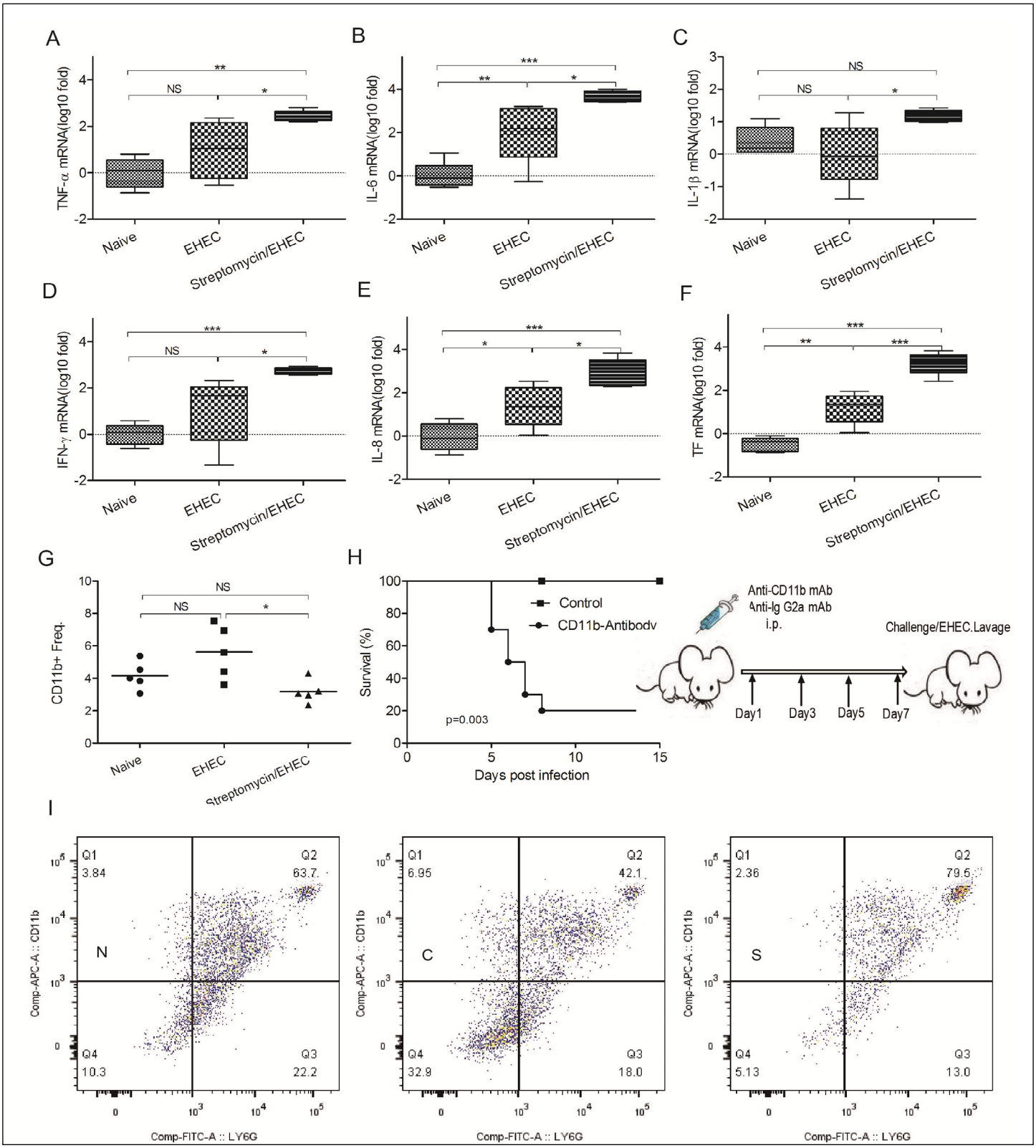

Figure 4: Gut microbiota is essential to reduce inflammation and maintain CD11b$^+$ macrophages proliferation defense against EHEC. Streptomycin treated mice and control mice were inoculated with a sublethal dose (1-2×10^9 CFU) of EHEC. On day 5 after the inoculations, mice were euthanized, and intestinal levels of mRNA encoding TNF-α (A), IL-6 (B), IL-1β (C), IFN-γ (D), IL-8 (E), and TF (F) were measured (n=8-10 per group). The numbers of intestinal CD11b$^+$macrophage decreased in streptomycin-treated mice (G, I: N, naïve; C, control; S, streptomycin). Wild-type mice were injected with CD11b-specific mAb, most mice died with EHEC challenge (n=5 per group) (H). *<0.05, **p<0.01, ***p<0.001. NS, not significant.

virulence [1]. Glucose is an ideal carbon source for EHEC, but these molecules are scarce in the lower GI tract. Njoroge, et al. [24] discovered the importance of glucose availability in regulating T3SS by EHEC. Stemming from the observation that high-glucose growth media suppressed type III secretion while low-glucose conditions induced LEE expression. We detected lower level of glucose in guts of mice treated with streptomycin which may induce LEE expression to improve virulence of EHEC. Another important metabolite is short-chain fatty acids (SCFAs) which provide a preferred energy source to enterocytes, regulating cell differentiation and intestinal immunity [25]. Fermentation of starches within the colon is primarily mediated by *Firmicutes* to produce SCFAs. Butyrate is a SCFA with the strongest effect on cell cycle and plays an anti-inflammatory role in the gut. In our study, we detected lower level of butyrate which was correlated with lower level of butyrate-producing bacteria *Clostridium* (Figure 1) in streptomycin-treated mice. Our results displayed that depletion of butyrate through antibiotic treatment has critical implications in intestinal immunity. Low concentration of butyrate was shown to up regulate EHEC virulence genes involved in motility and formation of attaching and effacing lesions [26]. Mice fed with acetylated starch have increased bacterial acetate levels in their feces, leading the protection against an initial EHEC colonization [27]. In our mouse model, we detected lower level of acetate in mice treated with streptomycin. This is maybe another reason that mouse treated with streptomycin could not survival EHEC infection. Besides *Firmicutes* and *Clostridia*, we also detected lower level of *Lactobacillus*. Whereas, *Lactobacillus* could effectively alleviate diarrhea in mice via modulation of intestinal microflora and improve the function of immune system [28].

Inflammatory responses are initiated in response to pathogen infection, contributing to protective host immunity. However, failure to control exuberant expression of pro-inflammatory cytokines can result in tissue damage. In our study, we detected high levels of pro-inflammatory cytokines expression and inflammatory foci with various degrees of intestinal damage in mice treated with streptomycin. We believe that gut microbiota is very important to control the inflammatory cytokines expression. Du H and his team believed that Peroxisome proliferator–activated receptor-(PPAR) in myeloid-lineage cells played a key role in inhibiting pro-inflammatory cytokine synthesis and myeloid-derived suppressor cells (MDSCs) expansion, and free cholesterol served as the ligand for PPAR [29,30]. In our study, we assessed high levels of inflammatory cytokines and MDSCs proliferation (Figure 4H, 4J: CD-11b^{+}Ly-6G^{+}) in mice treated with streptomycin. We also found increased choline and myo-Inositol levels in same group, which were usually negative related with cholesterol levels. We deduced that metabolites in fecal influence pro-inflammatory cytokine synthesis and MDSCs proliferation through PPAR impacting by cholesterol level. Monocytes are a critical effector component during EHEC infections by regulating innate and adaptive immune responses. Monocytes from patients infected with EHEC exhibited reduced circulatory expression of function-related proteins such as CD11b, CD64, CD62L, and CX3CR, possibly due to monocyte infiltration of tissue lesions [8,31,32]. In our study, we detected reduced expression of CD11b in intestinal tissue, which means the cell count and function of macrophage was influenced by gut commensal flora. Because reducing diversity and community composition of intestinal commensal flora in mice also changed metabolic characterization in gut. We assumed that metabolites of intestinal commensal flora may be the most important contributor to CD11b^{+} macrophages mediated defense against EHEC infection.

The lethality of EHEC is caused by the presence of the potent shiga toxin, which can induce multiple thromboembolism to cause HUS [33,34]. Tissue factor (TF) plays a prominent role in the initiation of vascular procoagulant pathways. TF is expressed primarily by extravascular cells, whereas the proteases that generate thrombin circulate in plasma as inactive precursors. This physical segregation usually ensures that this "extrinsic" coagulation pathway is only activated in response to breaches of vascular integrity [35]. Since TF is very important for disease development, we detected TF level in gut. We found the TF level significantly increased in mice treated with streptomycin, that the key impact factor to extrinsic coagulation pathway by microbiota still need to investigate in the following step.

Conclusion

This report revealed dramatic impairments in macrophage and inflammation mediated defense in mice altered the microbiota by reducing diversity and shifting community composition. This impairment displayed by the mouse used in this study strongly suggests that microbiota is an important contributor to homeostasis of innate immune response defense against enteropathogenic infection. Studies over the past a few years have revealed remarkable interplay between microbiota, immunity, and infec-

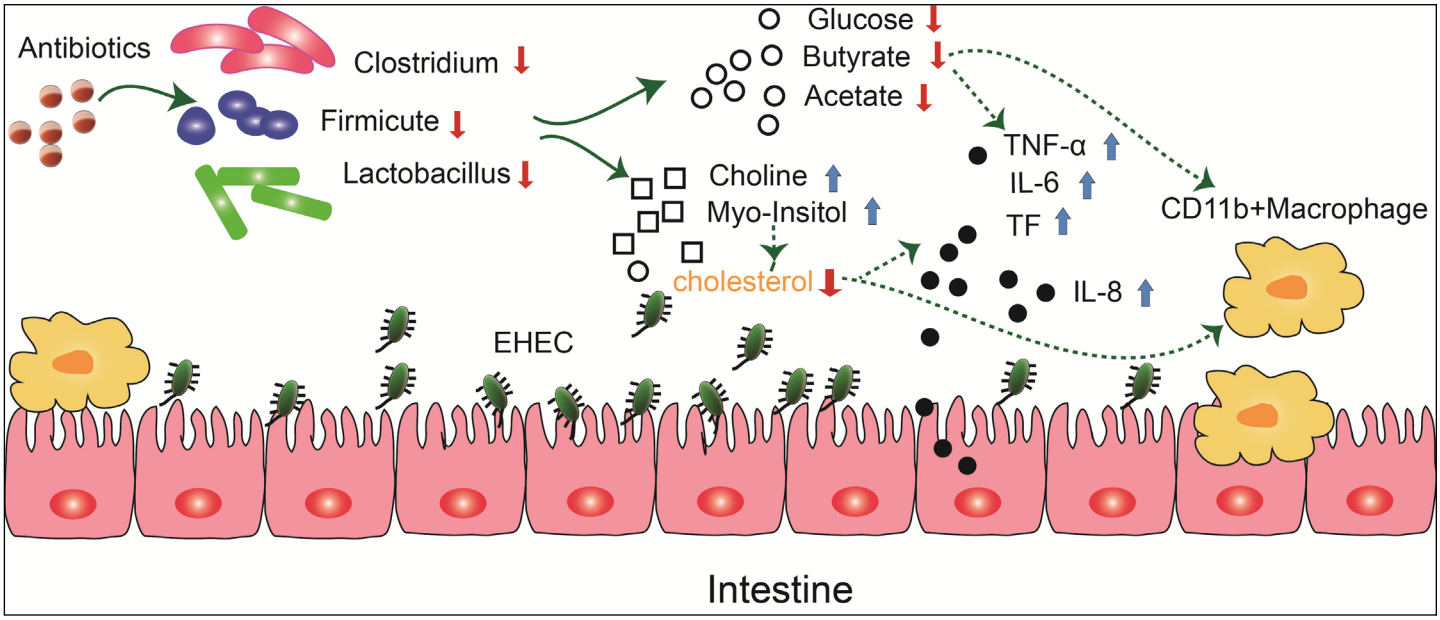

Figure 5: The mechanism described in our work.

Antibiotics changed diversity of gut microbiota and metabolic profiles. The changed metabolic profiles also altered the innate immune homeostasis. Gut microbiota played an important role to combat enteropathogenic infection, which was probably by maintaining $CD11b^{+}$macrophages proliferation to effectively clear bacteria, and reducing tissue damage by controlling the pro-inflammatory responses.↑increase, ↓decrease.

tion [36]. This report extends those connections by demonstrating that gut microflora leading to changing metabolic characterization can critically influence the homeostasis of innate immune response against EHEC (Figure 5). The accumulating evidence for essential protective roles for microbiota during a variety of infections could help to explain the details of mechanism and suggests that treatments for serious infection should consider the homeostasis of gut microbiota

Conflicts of Interest

The authors declare that they do not have a commercial or other association that might pose a conflict of interest.

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Availability of Data and Materials

The datasets generated during the current study are available in the [figshare] repository, [http://doi.org/10.6084/m9.figshare.11938602.v1]

Authors' Contributions

All authors discussed the results and implications of the manuscript. H.W and D.L. conceived the study, supervised the project, analyzed data, and wrote the paper. Y.S., N.N., J.L., T.L., Z.H., L.Z., F.C., Z.L., and L.X. performed experiments and analyzed data. D.L. advised on statistical evaluations. D.L. and Y.S. contributed equally to this work.

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