

Short- and Long-Term Effects of Different Antibiotics on the Gut Microbiota and Cytokines Level in Mice

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Background: Antibiotics are the first line of treatment for infectious diseases. However, their overuse can increase the spread of drug-resistant bacteria. The present study analyzed the impact of different types of antibiotics on the gut microbiome and cytokines level of mice.

Methods: A total of five groups of 8-week-old male BALB/c mice (n = 35) were treated with piperacillin-tazobactam (TZP), ceftriaxone (CRO), tigecycline (TGC), levofloxacin (LEV) or normal saline (Ctrl), respectively, for up to 4 weeks. Fecal samples were analyzed by bacterial 16S rRNA gene sequencing for bacterial identification. Blood samples were used for the determination of 23 serum cytokines using multiplex immunoassay.

Results: Exposure to antibiotics was shown to affect the normal weight gain of mice. Significant changes in gut composition caused by TZP, CRO and TGC treatment included the decreased abundance of *Bacteroidetes* (p < 0.01), *Muribaculaceae* (p < 0.01) and *Lachnospiraceae* (p < 0.01), and the increased abundance of *Proteobacteria* (p < 0.05), *Enterobacteriaceae* (including *Klebsiella* and *Enterobacter*) (p < 0.01) and *Enterococcaceae* (including *Enterococcus*) (p < 0.01). After 4-week treatment, the TZP, CRO and LEV groups had significantly lower concentrations of several serum cytokines. Correlation analysis of the top 30 bacterial genera and cytokines showed that *Enterococcus* and *Klebsiella* were strongly positively correlated with tumor necrosis factor- α (TNF- α), interleukins (IL) IL-12p70 and IL-1 β . *Desulfovibrio*, *Candidatus Saccharimonas*, *norank_f_norank_o_Clostridia_UCG-014*, *Lactobacillus*, and *Roseburia* were strongly negatively correlated with these cytokines.

Conclusion: This study demonstrates the effects of various antibiotics on the intestinal microflora and immune status of mice. Compared with TZP, CRO and TGC, LEV had minimal impact on the gut microbiota. In addition to TGC, long-term TZP, CRO and LEV intervention can lead to a decrease in serum cytokine levels, which may depend on the intestinal microflora, antibiotic used and the duration of treatment.

Keywords: gut microbiota, piperacillin-tazobactam, ceftriaxone, tigecycline, levofloxacin, cytokine, immunity

Introduction

Since the discovery of penicillin by Alexander Fleming in 1928, antibiotics have been widely used to save millions of lives.¹ However, the overuse and misuse of antibiotics have become a worldwide problem. There is increasing concern that antibiotic exposure may result in short- and/or long-term non-beneficial consequences, including an increased risk of necrotizing enterocolitis, bronchial allergy and asthma, obesity and autoimmune diseases.²⁻⁶ In recent years, disruption

of the intestinal microbiota by antibiotics has received increasing attention. Some studies have demonstrated a direct link between antibiotic use and alterations in the intestinal microbiota.^{7–10} Moreover, it was found that antibiotic inducing changes in the gut microbiota may affect normal immune function or immune status by disrupting metabolic homeostasis.^{11–13}

Among various antibiotics, the side effect of broad-spectrum antibiotics is easy to be depreciated as they are widely used in human and animal disease prevention and treatment. However, long-term high-dose application of broad-spectrum antibiotics can lead to intestinal microecological imbalance, reduce the colonization ability of normal bacteria, increase the number of potential pathogenic bacteria and even cause endogenous infection in severe cases.¹⁴ The amount of disturbance is closely related to the spectrum, dosage, route of administration, pharmacokinetic and pharmacodynamic properties.¹⁵ For example, β -lactams are widely used to treat infectious diseases and disrupt cell wall and peptidoglycan synthesis, leading to cell death. Among them, penicillin/ β -lactamase inhibitor combinations (piperacillin/tazobactam, TZP) and Third-generation cephalosporin (ceftriaxone, CRO) are broad-spectrum antimicrobials commonly used in clinical.¹⁶ They have very similar pharmacokinetic and pharmacodynamic characteristics and spectra of activity against medically important pathogens.¹⁷ Gamage et al found the administration of either of two broad-spectrum antibiotics TZP or CRO could result in significant changes fecal microbiota of ICU patients.¹⁸ Tigecycline is an important glycylcycline antibiotic, which was designed to circumvent common tetracycline resistance mechanisms that is administered intravenously, while Levofloxacin is a type of fluoroquinolone antibiotic that is used to treat a wide range of infections caused by Gram-negative and Gram-positive bacteria with a wide margin of safety and efficacy.^{19,20} However, the specific correlation and mechanism between these broad-spectrum antibiotics with intestinal microbiota composition still remain elusive, which may can ultimately induce the body's immune status imbalance.

A better understanding of this aspect will help in the rational use of broad-spectrum antibiotics in clinical practice. Therefore in our work, we have studied the effects of continuous administration of four types of widely-used broad-spectrum antibiotics on the intestinal microbiota and cytokines level of mice and attempted to establish the correlation between them. The main purpose of the experiment was to compare the differences in intestinal microbiota composition and cytokine levels, which can reflect the body's immune status caused by different antibiotics under short- and long-term intervention. The secondary purpose was to understand the correlation between serum cytokines and intestinal microbiota changes through correlation analysis.

Materials and Methods

Experimental Groups

Six-week-old male BALB/c mice ($n = 35$) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., acclimated for 2 weeks to reduce the impact of environmental stimuli on experimental results and properly fed under specific pathogen-free conditions. The mice were randomly divided into five groups ($n = 7$ per group) according to treatment: piperacillin-tazobactam (TZP), ceftriaxone (CRO), tigecycline (TGC), levofloxacin (LEV) or normal saline (Ctrl). The drugs were administered subcutaneously once a day for 4 weeks at the following doses (mg/20 gm body weight): 40.55 (TZP), 3 (CRO), 0.6 (TGC) and 1.5 (LEV), where the doses are equivalent to those recommended for adult human patients.²¹ The study was approved by the Research Ethics Committee of the First Affiliated Hospital from the School of Medicine of Zhejiang University (2022–1503) and conformed to the 2011 National Institutes of Health Guide for the care and use of laboratory animals. The experimental design scheme is shown in [Figure 1A](#).

Sample Collection and Serum Cytokines Test

We collected fresh stool samples at 3 time points— day 0 (0W), one-week (1W) and four-week (4W) of treatment for 16S rRNA sequencing for bacterial identification. The blood samples were also collected at the 3 time points for the cytokines 23-plex assay.²² The blood samples were immediately frozen and stored at -80 C until analysis. Then before test, the blood samples were centrifuged at 2500 to 3000 revolutions per minute to obtain the serum in the supernatant. The cytokine levels in the serum were analyzed by Bio-Plex™ 200 System (Bio-Rad) with the Bio-Plex murine 23-Plex Panel Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The measured cytokines were as

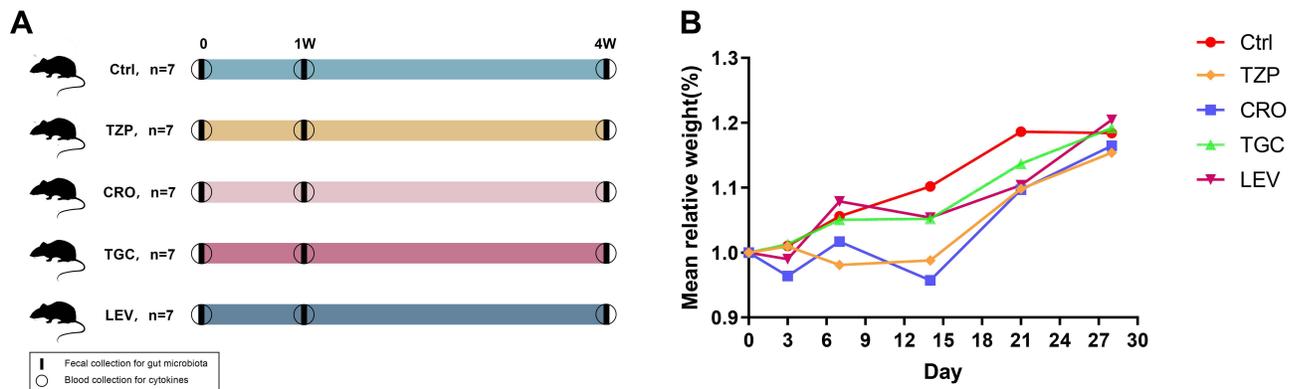


Figure 1 (A) Scheme of the research design. A total of five groups of 8-week-old male BALB/c mice (n = 35) were continuously treated with piperacillin-tazobactam (TZP, n=7), ceftriaxone (CRO, n=7), tigecycline (TGC, n=7), levofloxacin (LEV, n=7) or normal saline (Ctrl, n=7), respectively, for up to 4 weeks. The fecal samples and serum before (0W) and after treatment for 1 and 4 weeks were used for 16S rRNA gene sequencing. Blood was before (0W) and after treatment for 1 and 4 weeks for the determination of serum cytokines. **(B)** Effect of antibiotic treatment on mouse growth performance. Time curve of body weight ratio changes at different time points: 0: before treatment, 3, 7, 14, 21 and 28: 3, 7, 14, 21 and 28 days after treatment.

Abbreviations: TZP, piperacillin/tazobactam; CRO, ceftriaxone; TGC, tigecycline; LEV, levofloxacin; Ctrl, control.

follows: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, IFN- γ , eotaxin, G-CSF, GM-CSF, KC, MCP-1, MIP-1 α , MIP-1 β , RANTES, and TNF- α . Standard curves for each cytokine (in duplicate) were generated using the reference cytokine concentrations supplied in the kit and then used to calculate the cytokine concentrations in the serum.

16S rRNA Gene Sequence Analysis

The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from 200 mg of frozen stool, which was subjected to 16S rRNA gene sequencing.²³ The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR with barcoded primers 338F and 806R. The quality of amplified products was checked on 1% agarose gel and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). The PCR cycling conditions were as follows: 95°C for 1 min; 30 cycles of 98°C for 10s, 50°C for 30s, and 72°C for 30s; and a final extension for 5 min at 72°C. The Illumina MiSeq platform (Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China) was used for sequencing after identification and purification of the amplified products. The raw data were filtered for quality and trimmed of any sequencing adapters using Fastp v0.19.6 software,²⁴ and the sequences were assembled and error-corrected using FLASH 1.2.11 software.²⁵ All sequence data were deposited in the NCBI BioProject: PRJNA890946. Single-end reads were separated by barcode, and barcodes and primers were removed. High-quality reads were obtained using Cutadapt. Chimeric sequences were detected and removed using UCHIME.²⁶ Sequences were analyzed using UPARSE software and the sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic units (OTUs).²⁷ The median OTU count per sample was 30,869. Classified, unfiltered OTU counts were used to calculate the relative abundance of bacterial groups (phylum, family and genus). Main compositional shifts in the microbiome were visualized using bar charts generated in QIIME. Diversity within samples as species richness (a-diversity) was measured by comparison of Shannon and Abundance-based coverage estimate (ACE) diversity indices calculated in Mothur.²⁸ Principal-coordinate analysis (PCoA) was used to assess community similarity among samples (b-Diversity) using Bray-Curtis based on the generated phylogeny tree.

Statistical Analysis

For the test of two groups of data, Student's *T*-test was used for normally distributed data, while the Wilcoxon test was used for non-normally distributed data. For data tests of three or more groups: one-way ANOVA was used for normal distribution and Kruskal–Wallis *H*-test was used for non-normal distribution. Correlations were performed using a non-parametric Spearman's test. Data were analyzed using SPSS version 26.0 (SPSS Inc., Chicago IL, USA) and GraphPad version 9.0.

Results

Changes in Mouse Body Weight During Antibiotic Treatment

After antibiotic treatment, the weight gain of mice in TZP, CRO, TGC and LEV groups was delayed when compared to the Ctrl group (Figure 1B). The body weight of the mice was significantly lower ($p < 0.05$) than the Ctrl group on day 7, 14, and 21 for the TZP group, and only on day 14 and 21 for the mice in the CRO and LEV groups while the same as the group TGC on day 21.

Effect of Long-Term Antibiotic Treatment on Cytokine Levels

Treatment with antibiotics can induce immune responses in mice, which could be dependent on the antibiotic class and the timing of treatment. We compared the levels of all cytokines between 0W, 1W and 4W in the control mice (Figure 2). There were no significant differences in cytokine levels between different groups on 0W and 1W. However, after 4 weeks, the levels of IL-1 α , IL-3, IL-9, IL-12(P40), IL-17, eotaxin, GM-CSF, IFN- γ , MCP-1, MIP-1 α , MIP-1 β , RANTES, TNF- α ,

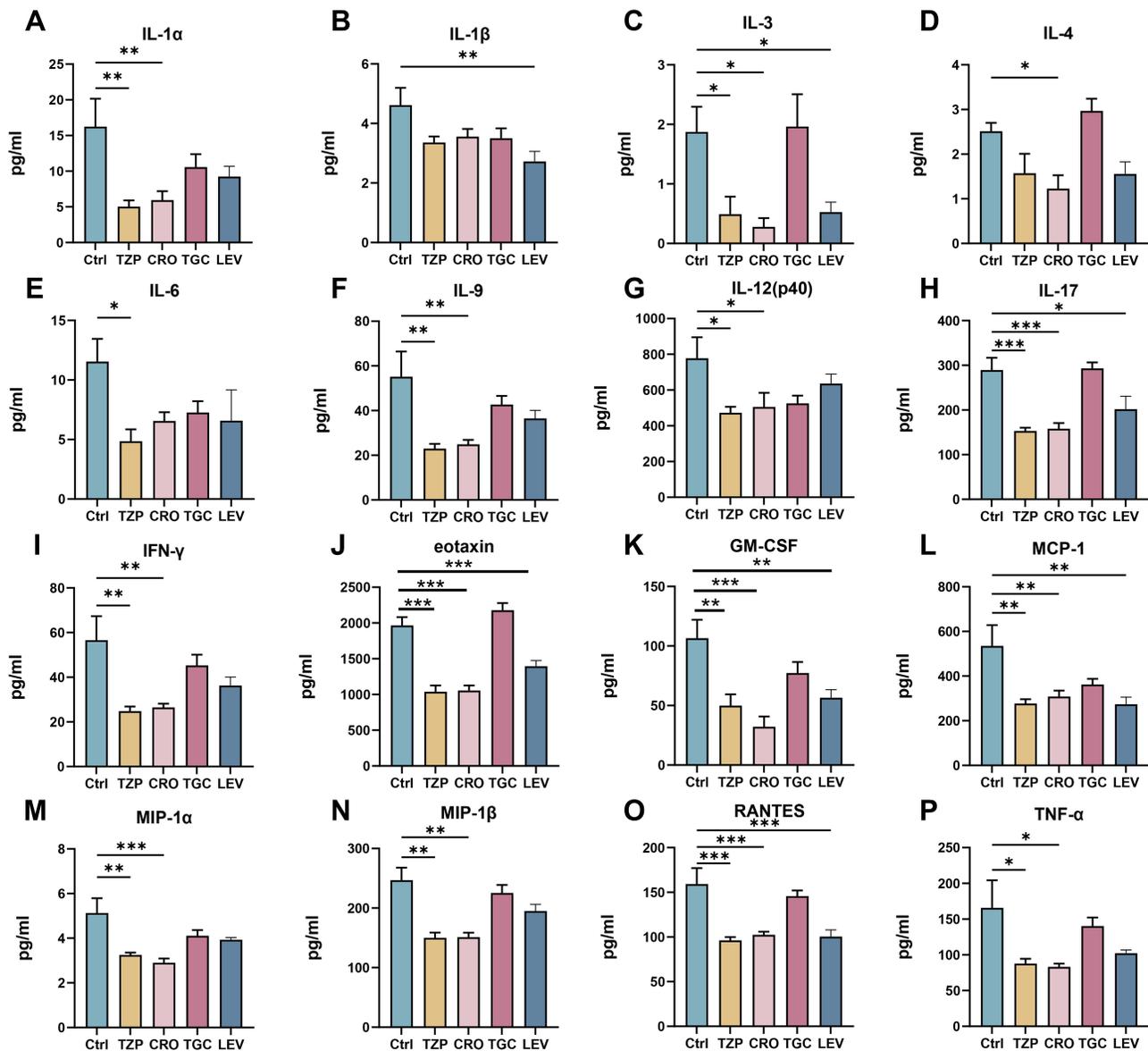


Figure 2 Changes in levels of serum cytokines after antibiotics treatment for 4 weeks. (A) IL-1 α , (B) IL-1 β , (C) IL-3, (D) IL-4, (E) IL-6, (F) IL-9, (G) IL-12 (p40), (H) IL-17, (I) IFN- γ , (J) eotaxin, (K) GM-CSF, (L) MCP-1, (M) MIP-1 α , (N) MIP-1 β , (O) RANTES, (P) TNF- α . Significant differences between the two groups were marked, and *Indicates $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

Abbreviations: TZP, piperacillin/tazobactam; CRO, ceftriaxone; TGC, tigecycline; LEV, levofloxacin; Ctrl, control.

TNF- α in the TZP and CRO groups were significantly lower than Ctrl group ($p < 0.05$). In addition, there was a significant decrease of IL-6 in TZP group ($p < 0.01$) and IL-4 in CRO group ($p < 0.05$). Serum IL-1 β , IL-3, IL-17, eotaxin, GM-CSF, MCP-1 and RANTES cytokine concentrations were significantly decreased in LEV group ($p < 0.05$). There were no significant changes in serum cytokine concentrations in the TGC group. Only long-term treatment with TZP, CRO and LEV was found to have an effect on the levels of serum cytokines. TGC did not cause any drastic changes in the cytokine levels in the short- and long-term.

Effect of Antibiotic Treatment on Gut Microbiome Structure

The diversity and richness of the mouse gut microbiota can be evaluated by calculating alpha diversity (Shannon and Ace indexes) (Figure 3A–D). Our data suggested that α -diversity of the antibiotic groups was dramatically reduced on 1W and 4W when compared to control group ($p < 0.05$). In the fourth week, the α -diversity of LEV was higher when compared to other treatment groups ($p < 0.05$). β -diversity can reflect the degree of similarity or difference in community composition in different samples. Principal coordinate analysis (PCoA) was subsequently performed using distance matrix analysis to compare microbiota structure. The results demonstrated that there were obvious differences among the different groups on 1W and 4W (Figure 3E and F). But it was observed that the mice treated with LEV resembled the control group more closely, compared to the other groups.

The Impact of Different Antibiotics on the Gut Microbial Composition

During the experiment, antibiotic exposure greatly disturbed the microbial composition at the phylum, family and genus levels of distribution (Figure 4A–C). The composition of gut microbiota of mice in the control and antibiotic groups was similar before treatment. *Firmicutes*, *Bacteroidetes*, *Desulfobacterota* and *Patescibacteria* were the dominant bacterial phyla, accounting for more than 85% of the gut microbiota, of which *Firmicutes* and *Bacteroidetes* accounted for more than 75% (Figure 4A).

After short-term (1W) antibiotic exposure, *Proteobacteria* showed significant increase in TZP, CRO and TGC ($p < 0.05$), while *Bacteroidetes*, *Patescibacteria*, and *Desulfobacterota* decreased significantly ($p < 0.05$) (Figure 5A). The dominant bacterial phylum was composed of *Firmicutes* and *Proteobacteria*, accounting for more than 90%. A significant decrease in *Desulfobacterota* was observed in LEV ($p < 0.001$). At the family and genus levels, the concentrations of several beneficial symbionts from the families *Muribaculaceae* and *Lachnospiraceae* ($p < 0.01$) were dramatically decreased in the TZP, CRO and TGC groups. This was largely due to a significant reduction in *norank_f_Muribaculaceae*, *Lachnospiraceae_NK4A136_group*, and *norank_f_Lachnospiraceae* ($p < 0.05$). In addition, *Roseburia* was found to decrease in the CRO and TGC groups ($p < 0.01$), while *Lachnospiraceae_NK4A136_group* also showed a decrease in LEV group ($p < 0.001$). However, a significantly higher relative abundance of opportunistic pathogens in TZP, CRO and TGC groups. The population of *Enterococcaceae* and its genus *Enterococcus* ($p < 0.01$) was higher in TZP and TGC groups. The population of *Enterobacteriaceae* and its genus *unclassified_f_Enterobacteriaceae* ($p < 0.001$) was higher in TGC while the abundance of *Bacillaceae* and its genus *Bacillus* ($p < 0.001$), *Enterobacteriaceae* ($p < 0.01$) and its genus *Enterobacter*, and *Klebsiella* ($p < 0.001$) was higher in CRO (Figure 5B and C).

After long-term (4W) antibiotic intervention, compared to control group, a significant increase of *Proteobacteria* ($p < 0.001$) was observed in the TZP group, accompanied by a decrease in *Firmicutes* and *Bacteroidetes* ($p < 0.001$). Except, *Proteobacteria* ($p < 0.05$), *Firmicutes* ($p < 0.01$) also increased in CRO as well as TGC, and was accompanied by a significant decrease in *Bacteroidetes* ($p < 0.01$). In addition, *Verrucomicrobiota* was significantly increased in LEV ($p < 0.01$) (Figure 5D). At the family and genus levels, families *Muribaculaceae* and *Saccharimonadaceae* ($p < 0.01$) dramatically decreased in the TZP, CRO and TGC groups, which was largely due to a significant reduction in *norank_f_Muribaculaceae* and *Candidatus_Saccharimonas* ($p < 0.01$). *Lachnospiraceae* ($p < 0.001$) decreased only in TZP; however, its genus *Lachnospiraceae_NK4A136_group* ($p < 0.05$), *unclassified_f_Lachnospiraceae* ($p < 0.05$), *norank_f_Lachnospiraceae* ($p < 0.01$) was decreased in TZP, CRO and TGC groups. *Enterococcaceae* (including *Enterococcus*) and *Enterobacteriaceae* ($p < 0.05$) were higher in the TZP, CRO and TGC. *Enterobacter* ($p < 0.01$) and *Blautia* ($p < 0.01$) were higher in the CRO and TGC. *Klebsiella* was tremendous higher in the TZP ($p < 0.0001$). *Akkermansiaceae* and its genus *Akkermansia* ($p < 0.001$) increased in LEV, accompanied with the decrease of *Saccharimonadaceae* and its genus *Candidatus_Saccharimonas* ($p < 0.001$) (Figure 5E and F).

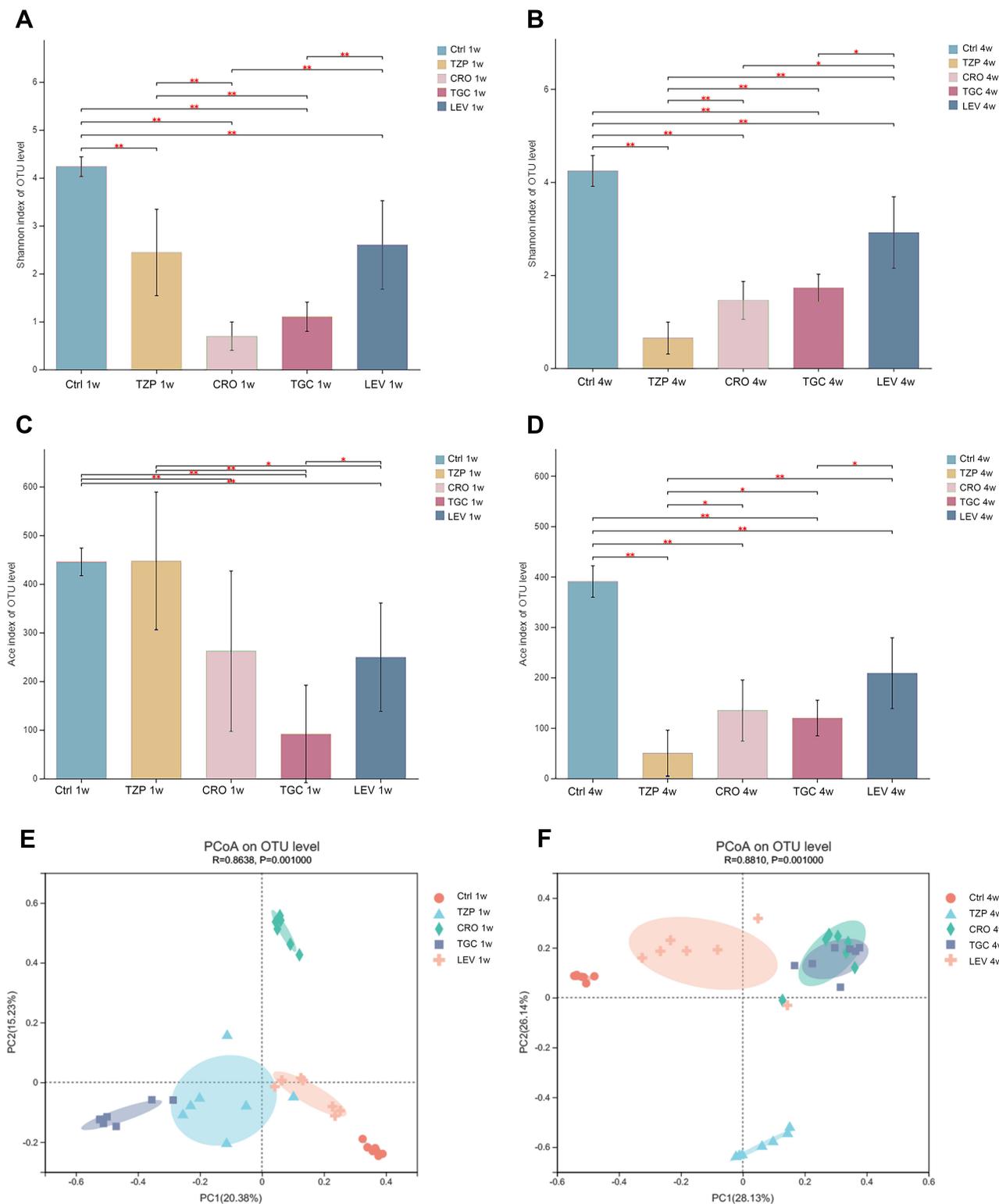


Figure 3 Microbial diversity measured by the Shannon index and Ace index was altered by antibiotic treatment across the treatment groups on 1W (**A** and **B**) and 4W (**C** and **D**). * $P < 0.05$, ** $P < 0.01$ vs the control group. Analysis of microbial communities by principal coordinate analysis (PCoA) at different time points after antibiotic treatment on 1W and 4W (**E** and **F**). 1 W and 4 W: 1 W and 4W after treatment.

Abbreviations: TZP, piperacillin/tazobactam; CRO, ceftriaxone; TGC, tigecycline; LEV, levofloxacin; Ctrl, control.

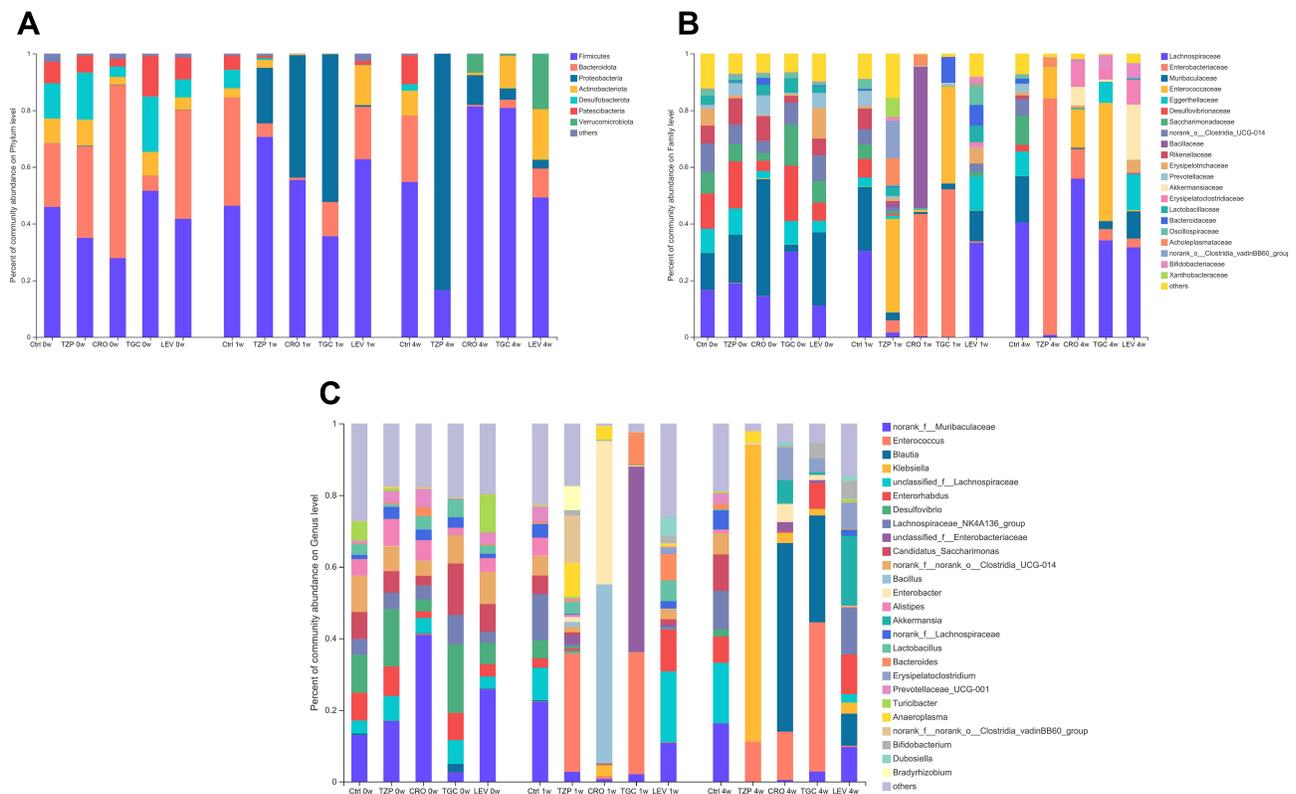


Figure 4 Antibiotic-induced composition changes of gut microbiota. Distribution of the predominant bacteria with antibiotics-treated at (A) phylum level, (B) family level and (C) genus level on 0W, 1W and 4W. Species with less than 0.5% abundance in all samples were merged into the “Others” category. 0W: before treatment. 1W and 4W: 1W and 4W after treatment.

Abbreviations: TZP, piperacillin/tazobactam; CRO, ceftriaxone; TGC, tigecycline; LEV, levofloxacin; Ctrl, control.

Correlation Between Gut Microbiota and Cytokines

To study the interaction between the intestinal microbiota and the host immune system, we calculated the correlations between microbiome compositions (at the genus level) and the 23 serum cytokine levels of host immune markers by Spearman correlation analysis (Figure 6A and B). The significance thresholds were absolute correlation coefficients higher than 0.1 and P values lower than 0.05. *Enterococcus*, *Klebsiella*, *Enterobacter* and *Akkermansia* were found to be positively correlated with most cytokines. *Desulfovibrio*, *Candidatus_Saccharimonas*, *norank_f__norank_o__Clostridia_UCG-014*, *Roseburia*, *Lactobacillus* and *Helicobacter* were negatively correlated with most cytokines. Next, the significance thresholds were defined as absolute correlation coefficients higher than 0.3. *Enterococcus* and *Klebsiella* were strongly positively correlated with IL-1 β , IL-12 (p70) and TNF- α . *Erysipelatoclostridium* was strongly positively correlated with IL-1 α , IL-6, IFN- γ and MIP-1 β . *Desulfovibrio*, *Candidatus_Saccharimonas*, *norank_f__norank_o__Clostridia_UCG-014*, *Roseburia*, *Lactobacillus* and *Helicobacter* were strongly negatively correlated with IL-1 β , IL-12 (p70) and TNF- α .

Discussion

Antibiotics are still indispensable for the treatment of infectious diseases, which makes their rational use even more important.^{29,30} The various factors influencing the choice of the antibiotic include the administration, antibacterial spectrum, drug resistance, and organ functions of the patients. However, a lot of research in recent years has focused on the impact of antibiotics on gut microbiota and host immunity. In spite of the interspecies differences as the human gut microbiome is prone to fluctuations due to various factors including diet and environment, mouse models remain valuable for studying the human microbiome.¹⁷ In our study, four types of antibiotics were continuously administered to mice for 4 weeks. Subsequent studies analyzed the impact of different antibiotics on the intestinal microbiota and host

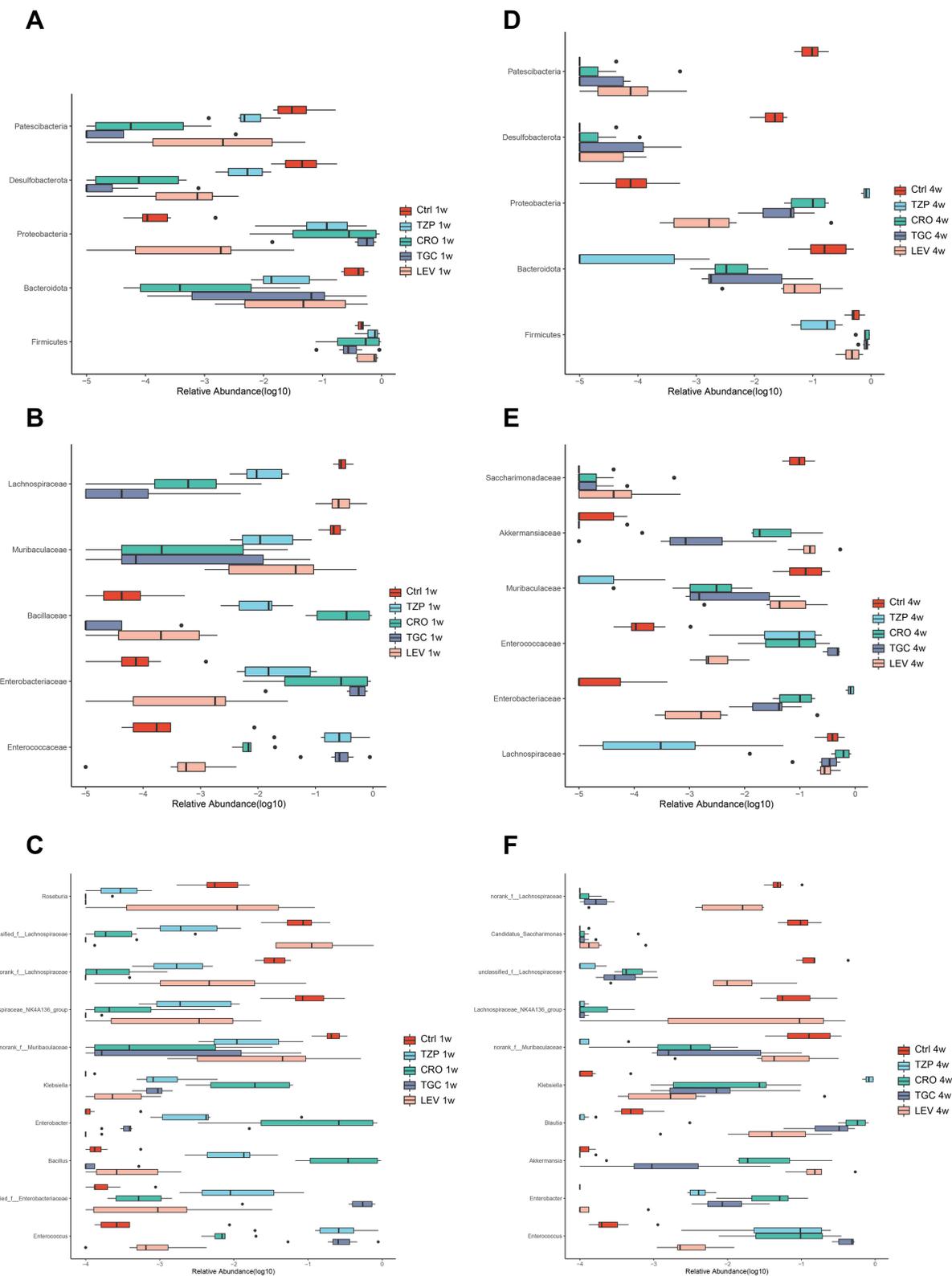


Figure 5 Comparison of dominant bacteria at the (A) Phylum, (B) Family and (C) Genus levels among five groups on 1W. Comparison of dominant bacteria at the (D) Phylum, (E) Family and (F) Genus levels among five groups on 4W. 1 W and 4 W: 1 W and 4W after treatment. **Abbreviations:** TZP, piperacillin/tazobactam; CRO, ceftriaxone; TGC, tigecycline; LEV, levofloxacin; Ctrl, control.

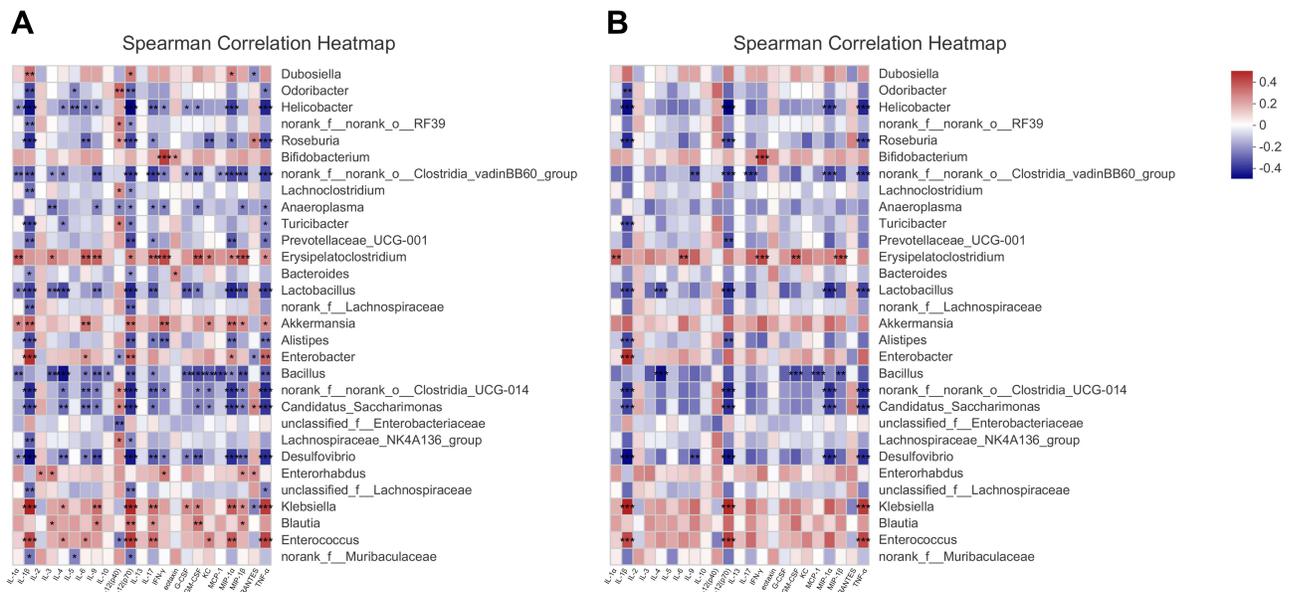


Figure 6 Correlations microbiome compositions (at the top 30 genus level) and the 23 serum cytokine levels of host immune markers on 0W, 1W and 4W. **(A)** Significance thresholds included absolute correlation coefficients higher than 0.1 and $P < 0.05$. **(B)** Significance thresholds included absolute correlation coefficients higher than 0.3 and $P < 0.05$. The number in color present Spearman correlation coefficient: red color indicates positive correlation; blue color indicates negative correlation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

immunity after 1 and 4 weeks of treatment, and attempted to find the correlation between the gut microbiome and immune response.

Consistent with previous studies, four broad-spectrum antibiotics significantly reduced the α - and β -diversity of gut microbiota.³¹ During the experiment, the fluctuating dominant bacteria were similar in the TGC, CRO and TZP groups. There was a decreased abundance of *Bacteroidetes*, *Muribaculaceae* and *Lachnospiraceae*, and an increased abundance of *Firmicutes*, *Proteobacteria*, *Enterobacteriaceae* (including *Klebsiella* and *Enterobacter*) and *Enterococcaceae* (including *Enterococcus*).

Continuous intervention with TZP, CRO and TGC significantly decreased the α -diversity of the gut microbiota and *Bacteroidetes* in short- and long-term exposure. Previous studies have demonstrated that substantial reductions in the abundance of *Bacteroidetes*, an important producer of short-chain fatty acids, have been associated with intestinal inflammation and various other pathological states.^{32,33} On the contrary, a normal gut microbiome dominated by *Firmicutes* and *Bacteroidetes* is unfavorable for Gram-negative bacterial colonization and can reduce the risk of corresponding bloodstream infections.³⁴ During antibiotic intervention, the microbial community shifted from a normal microbiota structure dominated by *Firmicutes* and *Bacteroidetes* to *Firmicutes* and *Proteobacteria*, which includes many potent pathogenic microorganisms. The significant increase in the abundance of *Proteobacteria* may be due to the antibiotic treatment disrupting the anaerobic environment in the gut.³⁵ Current research suggests that a high relative abundance of *Proteobacteria* is not beneficial to human health. For instance, patients with obesity, diabetes or nonalcoholic fatty liver disease often show a higher abundance of *Proteobacteria*.^{36–38}

When the intervention time was the same, the impact of different antibiotics on the gut flora of mice showed specific changes. Under the short-term effects of TZP, CRO and TGC, the abundance of *Muribaculaceae* and *Lachnospiraceae* (a family of butyrate-producing bacteria) significantly reduced compared with the control group.³⁹ The reduction of normal flora can provide a breeding ground for the growth of opportunistic pathogens in the gut.⁴⁰ However, a significantly higher relative abundance of opportunistic pathogens ($p < 0.01$) was observed in the antibiotic groups except for LEV. *Enterococcus* was significantly increased in the TZP and TGC groups. Although *Enterococcus* constitute only a small proportion ($< 0.1\%$) of the intestinal microbiota of healthy individuals, they are also common pathogens. Excess *Enterococcus* can lead to abdominal and pelvic infections and can cause intestinal inflammation by stimulating antigen-presenting cells and $CD4^+ROR\gamma^+$ T cell infiltration.⁴¹ In addition, in our study, the abundance of bacilli such as *Bacillus*, *Enterobacter* and

Klebsiella significantly increased in CRO. *Enterobacter* and *Klebsiella* are representative pathogenic bacteria genera of the *Enterobacteriaceae*. Studies have shown that the presence of *Klebsiella* spp. in the gut is a serious risk factor for infection.⁴² *Klebsiella* also plays a key role in causing pathological damage in patients suffering from Crohn's disease and diabetes.^{42–44}

After long-term intervention with antibiotics, the abundance of *Muribaculaceae* was still decreased in the TZP, CRO and TGC groups. But *Lachnospiraceae* was not decreased in the CRO and TGC groups because of an increase of *Blautia*. *Klebsiella* was significantly increased and replaced *Enterococcus* as the main pathogen in the TZP group. *Klebsiella* spp. can develop resistance to β -lactam antibiotics by producing β -lactamase. Therefore, both TZP and CRO, both of which belong to β -lactam antibiotics, can cause *Klebsiella* to increase during the intervention process.⁴⁵ *Enterococcus* and *Enterobacter* became the dominant pathogenic bacteria in the CRO and TGC groups. The dominant pathogenic bacteria were constantly changing during the course of treatment.

Compared with the control, LEV treatment did not induce any significant changes in the bacterial composition. The minor changes were observed as a decrease in *Desulfobacterota* and *Lachnospiraceae_NK4A136_group* in the first week, and a decrease in *Saccharimonadaceae* and *Candidatus_Saccharimonas* and an increase in *Verrucomicrobiota_Akkermansiaceae* and *Akkermansia* in the fourth week. *Akkermansia* is a promising probiotic that can help patients with inflammatory bowel disease and diabetes by regulating intestinal homeostasis and basal metabolism.⁴⁶ It has also recently been found to reduce intestinal injury and mortality in mice infected with *Clostridium difficile*. But whether a simple antibiotic-mediated *Akkermansia* increase is beneficial to host is not clear.⁴⁷ Studies have also found that vancomycin-treated mice had a gut compositional shift to *Verrucomicrobia* and its species *Akkermansia muciniphila*, and *Proteobacteria* and its species *Escherichia coli*. The increased abundance of L-aspartate mediated by *Akkermansiaceae* could be associated with inflammation in the host, which was partially confirmed in our correlation results.⁴⁸ Correlation results found that *Akkermansia* is positively correlated with IL-1 α , IL-1 β , IL-6, IL-12(p70), eotaxin, KC, MIP-1 α , MIP-1 β , and TNF- α , but the specific mechanism of action needs to be further explored.

Previous studies have found that antibiotic-induced microbiota perturbations are accompanied by modulation of host immune status. For example, antibiotic treatment for up to 4 weeks, especially with vancomycin, increases susceptibility to invasive candidiasis by reducing IL-17A and GM-CSF and promoting non-inflammatory escape of gut bacteria, leading to systemic coinfection and death.⁴⁹ This immune response is associated with a decrease in some bacterial families such as *Bacteroidales*, *Deferribacterales*, *Erysipelotrichales*, and *Clostridiales*, and their short-chain fatty acid-producing species, such as *Ruminococcus*, *Roseburia*, and *Butyricimonas*.⁵⁰ These findings were partially confirmed by our study results. Long-term treatment with TZP, CRO and LEV can decrease the level of serum IL-17 and GM-CSF. In addition, long-term effects of TZP and CRO, both belonging to the β -lactam class, include reducing the levels of IL-1 α , IL-9, IL-12(P40), eotaxin, IFN- γ , MIP-1 α , MIP-1 β , RANTES, and TNF- α . Serum IL-1 β , IL-10, and RANTES cytokine concentrations were also significantly decreased with the quinolone levofloxacin ($p < 0.05$). However, in the case of TGC, there was no significant change in the level of cytokines. Immunodeficiency is not the only immune regulation disorder caused by antibiotics. A number of studies have shown that antibiotics cause excessive inflammation in the host. Zhang et al found that serum IFN- γ , IL-13, and IL-17A levels in vancomycin-treated mice and IL-13 and IL-17 levels in polymyxin B-treated mice were significantly increased compared with control mice after 8 weeks of treatment.¹² Combining our own findings with previous reports, we postulate that immune function is not only related to the timing of antibiotic treatment, but also the class of antibiotic used.

In addition to the timing and type of antibiotic treatment, which may affect the host's immune function, it is currently believed that the influence of host immunity is closely related to antibiotic-mediated changes in the microbiota. Through correlation analysis, it was found that some bacteria had a certain correlation with cytokines. *Klebsiella* and *Enterobacter* were strongly positively correlated with proinflammatory factors such as TNF- α , IL-12p70 and IL-1 β . One mechanism underlying the microbiome–cytokine interaction may be mediated by the metabolites originating from gut microbiota. It is known that *Klebsiella* and *Enterobacter* belong to the *Enterobacteriaceae* family, and can increase the concentration of lipopolysaccharides (LPS) to promote the release of proinflammatory cytokines. *Desulfovibrio*, *Lactobacillus*, *Roseburia*, *Candidatus_Saccharimonas* were strongly negatively correlated with these cytokines. Almost all of them are butyrate-producing bacteria.⁴⁹ Studies have demonstrated that short chain fatty acids (SCFAs), have a role in reducing inflammatory immune responses.⁵¹

Conclusion

Our study demonstrated that different antibiotics have different effects on the intestinal flora and immune response of mice. Compared to TZP, CRO and TGC, LEV had minimal impact on the gut microbiota, both in short-term and long-term interventions. In terms of the influence of intestinal microecology, LEV is more recommended clinically than β -lactam antibiotics (TZP and CRO) and tetracycline antibiotics (TGC). In addition to TGC, long-term CRO, TGC, and LEV treatment may compromise immune response through a decrease in serum cytokine levels, and this change may be related to gut microbiota, the class of antibiotic, and the timing of antibiotic treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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