Antibiotic treatments to mothers during the perinatal period leaving hidden trouble on infants

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Abstract

Antibiotic application during the perinatal period is unavoidable in clinical, but the potential effects on mothers and infants remain unknown. Herein, 25 breast milk samples from mothers who received cefuroxime (CXM) or CXM+cefoxitin (CFX) treatments and fecal samples from their infants were collected to investigate antibiotics induced undesirable effects on the microbiota of mothers and neonates. Furthermore, five fecal samples of infants, whose mothers had antibiotic treatments, were collected in a six-month postpartum follow-up visit to evaluate the long-term effects on infants' gut microbiota. Moreover, the relative abundance of antibiotic resistance genes (ARGs) in fecal samples was compared to investigate the transfer of ARGs in infants' gut microbiota. The results indicated that the antibiotic treatments had no influence on the microbiota of breast milk. The dominant bacteria phyla in the fecal samples had changed to *Firmicutes* and *Proteobacteria* after antibiotic treatments; while the bacterial community showed a recuperative trend in follow-up visits. Besides, the abundance of ARGs of infants' gut microbiota demonstrated declining trend in CXM- and CXM+CFX-treated groups, while ARGs abundance presented a significant increasing trend after a 6-month recovery period.

Conclusion: Antibiotic treatments to mothers during perinatal period would disturb the gut microbiota in neonates. The infants' gut microbiota would partly return to their initial state after rehabilitation, but the transfer of ARGs would leave the hidden trouble of antibiotic resistance. Overall, data presented here can help to guide the scientific use of antibiotics during the perinatal period and provide potential approaches to mitigate the negative consequences.

What Is Known

Antibiotic application during the perinatal period is unavoidable in clinical.

Misuse of antibiotics can cause various unintended consequences especially for antibiotic resistance.

What Is New

Antibiotic treatments had no influence on the microbiota of breast milk but greatly disturbed the gut microbiota composition in infants.

The gut microbiota in infants would partly return to their initial state after rehabilitation but the transfer of ARGs would leave the hidden trouble of antibiotic resistance.

Introduction

Antibiotics are a large group of prescribed pharmaceuticals that most commonly used to prevent and treat infectious diseases with humans, animals, and crops. Antibiotic usage on a global scale would certainly result in more and more frequent detections of antibiotics in the environment. It had been reported that the highest antibiotic residue concentration in surface waters was up to 300 ng/L of

erythromycin; other antibiotics identified in the soil had a content of 15 μg/kg dry weight even seven months after application [1]. The application of antibiotics not only brings about improved performance in health and animal husbandry but also frequently results in negative effects. Concerns about antibiotic residues in the environment arise from two aspects: the first is the potential threat of direct toxicity to human beings, and the second is whether low-level antibiotic exposure would result in microbial mutation with higher lethality or the possible development of resistant strains. The widespread use of antibiotics (about 10^6-2×10^6 tons annually worldwide in 2002) has dramatically increased concerns about the negative effects on human gut microbiota [2]. Additionally, the transfer of antibiotic resistance genes (ARGs) from and between mutualists to pathogens would assist in the spread of antibiotic resistance [3, 4]. Accumulative evidences had revealed that gut microbiota was involved in the metabolism, pathogen resistance, immunomodulation, and even neural functions in the host [5]. Thus, a balanced compositional signature of gut microbiota is critical to the host's health [6]. As a result, antibiotics induced disturbance on gut microbiota would definitely result in adverse effects on the host.

The rapid spread of antibiotic-resistant pathogens and incidental emergencies have drawn public attention to the issue of antibiotic resistance. Between 2014 and 2016, more than one million people died due to antibiotic-resistant microbial strain infections, and the death toll is anticipated to rise in the future [7]. Bacteria have developed various resistance mechanisms to withstand antibiotic exposures [8]. Except for mutational events, antibiotic resistance is most likely the result of lateral transfer of genes called ARGs from other bacteria [9]. Antibiotic-resistance selection may also occur within the gut microbiota, acting as a primary avenue for developing resistance in bacterial pathogens and subsequently transferring to pathogenic bacterial strains [3, 10]. Therefore, it is envisaged that the transfer of ARGs into the gut microbiota might weaken the efficacy of antibiotic treatments.

Clinically, antibiotics are often used throughout the perinatal period for treating urinary and bacterial vaginosis, as well as preventative measures during intrapartum/peripartum to reduce the risk of infection in the mother and newborn [11–13]. Under such circumstances, newborns are under great probability to antibiotic exposure. On the other hand, many previous studies had reported the presence of antibiotic residues in breast milk [14, 15], thus the breastfeeding infants were exposed to antibiotics throughout the lactation phase. Human breast milk, which traditionally were considered sterile, has been discovered to provide a continual supply of commensal bacteria to the infant gut tract [16]. Furthermore, neonates are unavoidably subjected to antibiotic treatments due to their high vulnerability to bacterial infections during the neonatal period [17]. The composition of infants' gut microbiota is also influenced by environmental factors such as diet, diseases, and antibiotic treatments [18]. However, the colonization of the sterile fetal gut tract by bacteria starts at birth when the neonate comes into contact with the vaginal and gut microbiota of the mother [19]. Each time an antibiotic is delivered, antibiotic-resistant gut microbiota may be selected irrespective of the health status of the host. Perturbations to the gut microbiota, such as antibiotic applications, would often result in altered colonization by various gut pathogens [20]. Contemplating all these, it is unsurprising that antibiotic treatments during parturition and the neonatal period has dramatic and long-lasting negative effects on infants' gut microbiota. What is worse, the

transfer of ARGs into the gut microbiota of infants would leave the hidden trouble of antibiotic resistance. Applications of antibiotics during pregnancy has been previously connected with an increased incidence of food allergy, hay fever, and asthma in infants [21, 22]. However, little is known about the increasing risks of abnormal development in infants induced by maternal modification of the gut microbiota with clinical antibiotic treatments.

Herein, breast milk samples from mothers and fecal samples from neonates were collected and evaluated for the purpose to probe possible effects of antibiotic treatments on the normal microbiota of mothers and neonates. Additionally, fecal samples from infants whose mother received antibiotics were collected in a six-month postpartum follow-up visit to investigate the long-term effects of perinatal antibiotic treatments on the gut microbiota of infants. In addition, the abundance of ARGs in fecal samples from different periods was also compared to analyze the transfer of ARGs in infant gut microbiota. Data presented here can shed light on how prenatal antibiotic treatments disrupt the homeostasis of gut microbiota and the development of antibiotic resistance in infants. All these could provide useful direction for the clinical perinatal antibiotic applications.

Material And Methods

1. Study Population and Sample Collection

The breast milk and fecal samples were collected between May 7th to October 28th, 2020, at Women's Hospital, School of Medicine, Zhejiang University. The inclusion criteria for the study were defined as those mothers who were healthy, were of childbearing age, did not receive antibiotics three months before parturition, and were not consuming probiotics two weeks before parturition. Similarly, those subjects who had a diagnosis or history of serious infectious disease or gastrointestinal disease during pregnancy, inability to breastfed, neonates with health problems at the time of birth were excluded from the study. Among the 25 participants, nine women received no antibiotic treatment during the perinatal period were labeled as control group; 13 women received cefuroxime (CXM; 1.5 g each time, and the defined daily doses were based on infection degree) were labeled as CXM treated group; while three women received CXM and cefoxitin (CFX; 1.5 g for CXM and 2 g for CFX each time, and the defined daily doses were based on infection degree) and were labeled as CXM + CFX treated group. After parturition, the breast milk samples from mothers and the first fecal samples from neonates were collected. The follow-up visits were scheduled about six-month after parturition before taking supplementary food. The inclusion criteria at this stage of the study were defined as those mothers who only received CXM antibiotic treatments during the perinatal period, those mothers who did not receive any antibiotic treatment or consumed any probiotics after parturition, those infants who were developing normally, and infants were breastfed. Meanwhile, those infants who either received antibiotics or probiotics, received any supplementary food, and those infants who had serious infectious diseases or gastrointestinal diseases were excluded from the study. Five fecal samples of infants from CXM-treated mothers (CXM-FV) were collected in the follow-up visits. All breast milk and fecal samples were stored at -80°C until further use. The breast milk samples were labeled as B-Con, B-CXM, and B-CXM + CFX groups based on antibiotic treatments given to mothers.

Similarly, the fecal samples were labeled as F-Con, F-CXM, and F-CXM + CFX groups based on antibiotic treatments given to mothers; and fecal samples from follow-up visits were labeled as F-CXM-FV group.

2. DNA Extraction

The extraction of global bacterial genome was conducted according to previous studies with slight modification [23]. Briefly, breast milk or fecal samples were thawed and suspended in 790 μL of sterile lysis buffer (4 M guanidine thiocyanate, 10% N-lauroyl sarcosine, 5% N-lauroyl sarcosine-0.1 M phosphate buffer) with adding 1 g of glass beads (0.1 mm, BioSpec Products, Inc., USA). The samples were subjected to vigorous vortexing and mixtures were then incubated for 1 h at 70°C and beaten for 10 min at maximum speed. The bacterial DNA was extracted according to manufacturer's protocols using E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek, Inc., GA). The extracted DNA from each sample was used as the template for PCR amplification.

3. PCR Amplification and Sequencing

To analyze the microbial community of breast milk and fecal samples, the V3-V4 regions of the bacterial 16S rRNA gene were amplified using the universal primers supplemented with Illumina sequencing adapters and sample-specific barcodes according to Illumina's instructions. The primer sequences of universal primers (*16S rRNA*) are shown in **Table S1**. PCR reactions were run on an EasyCycler 96 PCR system (Analytik Jena Corp., AG). The PCR reaction system consisted of 1 μL of DNA template, 5 μL of 5× *TransStart® FastPfu* Buffer, 0.5 μL of 10 mM dNTPs, 0.5 μL of forward and reverse primer, 0.5 μL of *TransStart® FastPfu* DNA Polymerase, and 17 μL of ddH_2O. The PCR program was set as follows: denaturation at 95 ℃ for 3 min; 21 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, elongation at 72 ℃ for 30 s; and extension at 72 ℃ for 5min. The PCR products were indexed and mixed at equal ratios for Illumina sequencing by Shanghai Mobio Biomedical Technology Co. Ltd. using Miseq platform (Illumina Inc., USA) according to the manufacturer's instructions.

To analyze the abundance of ARGs in fecal samples, the total DNA extractions were amplified using high-throughput quantitative PCR (HT-qPCR). The primer sequences of target ARGs are shown in **Table S1**. The HT-qPCR reaction was run on an Applied Biosystems ViiA TM 7 Real-Time PCR System (Wcgene Biotechnology, Shanghai). The PCR reaction system consisted of 1 μL of DNA template, 5 μL of 2×TB Green Premix Ex Taq II (Tli RNaseH Plus), 0.4 μL of forward and reverse primer, 0.2 μL of 50×ROX Reference Dye, and 3 μL of ddH_2O. The PCR program was set as follows: denaturation at 95 ℃ for 30 s; 40 cycles of denaturation at 95 ℃ for 5 s, annealing at 60 ℃ for 30 s. A final melt curve, ranging from 60 ℃ to 95 ℃, was then conducted to confirm the specificity of amplification.

5. Data Processing

Raw data from Miseq sequencing were merged into one sequence based on the overlapping region of paired end reads. In the meantime, quality-filter for the raw reads and merged sequences were conducted according to the index and primer sequences on both ends of the sequences, and the sequence direction

were corrected as well. Quality-filtered sequences were clustered into Operational Taxonomic Units (OTUs) based on 97% similarity using Usearch (version 11, http://drive5.com/uparse/) and chimeric sequences were omitted in this step. The acquired representative OTUs sequences were mapped with all optimized sequences to screen sequences with over 97% similarity of representative OTUs sequences. To acquire the classified information of each OUT, representative OTUs sequences with 97% similarity were conducted with taxonomic analysis based on Silva reference database (Release 138, http://www.arb-silva.de) and the microbiota composition were summarized at the level of phylum, class, order, family, genus, and species, respectively.

The richness and diversity of microbial community were reflected by a-diversity indices using Mothur (version v.1.42.1, http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity). The microbial community richness was assessed using the Chao1 estimator (http://www.mothur.org/wiki/Chao) based on the following equation:

$$S_{chao1} = S_{obs} + \frac{n_1\left(n_1 - 1\right)}{2\left(n_2 + 1\right)}$$

Where S_{chao1} represents the estimated OTUs; S_{obs} represents the observed OTUs; n_1 represents OTUs with one sequence; n_2 represents OTUs with two sequences.

The microbial community diversity was assessed using the Shannon index (http://www.mothur.org/wiki/Shannon) based on the following equation:

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} \ln \frac{n_i}{N}$$

Where S_{obs} represents the observed OTUs; n_i represents the sequence number of the *i*th OUT; N represents the total sequence number.

The comparisons of the relative abundance of microbial community were performed using rank-sum test. Comparison between two independent groups was done using a non-parametric Wilcoxon rank-sum test; comparison of multiple groups was done using a nonparametric Kruskal-Wallis rank-sum test. The differences in microbial community composition of multiple groups were reflected by b-diversity indices in Qiime. Principal Co-ordinates Analysis (PCoA) based on unweighted-UniFrac dissimilarity and permutational MANOVA (Adonis) were generated in R package (version 3.6.0) vegan 2.5-7 using 10,000 permutations. The linear discriminant analysis (LDA) effect size (LEfSe) was used to detect taxa with differential abundance among groups (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload). Random forest conducted in Qiime (http://qiime.org/scripts/supervised_learning.html?highlight=random%20forest) was used to screen markedly different OTUs.

The relative abundances of ARGs were calculated using the method of ΔCt based on the following equation [24]:

$$F = 2^{-\Delta(Ct_{ARG} - Ct_{16SrRNA})}$$

Where Ct_{ARG} and $Ct_{16SrRNA}$ represent the threshold cycles of target *ARG* and *16S rRNA* gene.

6. Statistical Analysis

R package (version 3.6.0) and SPSS (IBM Corp., NY, USA.) were used for statistical analysis. The comparisons of the relative abundance of microbial community were performed using rank-sum test with Wilcoxon rank-sum test for the comparisons of two independent groups and Kruskal-Wallis rank-sum test for the comparisons of multiple independent groups. The comparisons of the relative abundance of ARGs were performed using one-way ANOVA with a Tukey posthoc analysis. Statistical significance was set at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

1. Overview of Illumina sequencing

In general, 55 samples (25 breast milk samples and 30 fecal samples) were sequenced using Miseq platform and 1,482,182 optimized sequences were acquired after quality-filter. Overall, 161 OUTs were classified for the following analysis on the basis of 97% similarity.

2. Microbiota in breast milk after perinatal antibiotic treatments

The disturbance to the microbiota in breast milk after perinatal antibiotic treatments was assessed based on the α-diversity indices. As illustrated in Fig. 1A-C, no significant difference in microbial community richness or diversity was observed in the breast milk samples from CXM treated or CXM + CFX treated mothers comparing with the control group. According to the unweighted-UniFrac dissimilarity based PCoA scores (Fig. 1D), there was no significant difference in the microbial community composition between the control group and CXM treated group ($p > 0.05$) according to the Adonis analysis.

The microbiome composition in breast milk at the phylum and genus levels across different groups are depicted in Fig. 2A, B. The bacterial community composition in breast milk was relatively simple, regardless of antibiotic treatments. *Firmicutes* were the most prevalent bacteria, followed by *Actinobacteria*. As to genus level, the most abundant bacterial genera were in order as follows: *Streptococcus*, *Staphylococcus*, and *Rothia*. The most vulnerable bacteria were identified by analyzing the differences in bacterial communities between groups. Generally, no dominating bacteria at the phylum or genus levels was identified in breast milk samples from CXM or CXM + CFX treated groups when compared to the control group using Kruskal-Wallis rank-sum test. LEfSe analysis had reflected the significantly affected bacteria in breast milk (Fig. 2C, D). According to the LDA score at the genus level, the abundance of *Pseudomonas* was significant higher in breast milk samples from CFX-treated group (*p*

< 0.05, LDA > 2), whereas *Clostridiaceae-sensu-stricto* was the most abundant bacteria in breast milk from CFX + CXM-treated group ($p < 0.05$, LDA > 2). Additionally, 25 significantly disturbed bacteria OTUs were identified in the microbiota of breast milk (Fig. 2E). In summary, CXM treatment upregulated 18 OTUs and downregulated 3 OTUs in breast milk microbiota comparing with that of the control group. In breast milk from CFX + CXM-treated group, 8 OTUs increased while 5 OTUs decreased comparing with control group.

3. Gut microbiota in neonates

Antibiotic treatments to mothers during the perinatal period seemed to induce more profound effects on the gut microbiota in neonates. Shannon index indicated no significant difference in the microbial community diversity in the fecal samples from control group and CXM-treated group, but the microbial community diversity in CXM + CFX treated group was significantly lower than that in control group and CXM-treated group. However, no significant difference was observed about the community richness of the gut microbiota in neonates from different groups (Fig. 3A-C). As shown in Fig. 3D, PCoA and Adonis analyses also revealed a huge discrepancy existed in the gut microbiota composition between control group and CXM + CFX-treated group ($p < 0.001$).

Meanwhile, the dominant bacterial community in fecal samples were more abundant than that in breast milk samples (Fig. 4A, B). The most abundant bacteria phyla in the fecal samples from control group followed the order of *Bacteroidota*, *Proteobacteria*, and *Firmicutes*. In antibiotic treatments groups (both CXM and CXM + CFX treatments), the most dominant bacteria phylum was *Firmicutes* followed by *Proteobacteria*. At the genus level, the most abundant bacteria genera in fecal samples from control group were *Bacteroides*, *Escherichia-Shigella*, and *Clostridium-sensu-stricto*. The dominant bacteria genera in CXM-treated group were *Streptococcus*, *Clostridium-sensu-stricto*, *Escherichia-Shigella*, *Enterobacteriaceae-unclassified*, and *Clostridioides*. In CXM + CFX-treated group, the dominant bacteria genera were slightly different, which were *Streptococcus*, *Enterobacteriaceae-unclassified*, and *Enterococcus*. Kruskal-Wallis rank-sum test reflected that CXM treatment enhanced the abundance of *Firmicutes* while decreased *Bacteroidetes* abundance at the phylum level. As for genus level, CXM treatment reduced the abundance of *Bacteroides*, *Escherichia-Shigella* but increased the abundance of *Clostridioides*, *Enterobacteriaceae-unclassified*, *Lactobacillales-unclassified*, *Streptococcus*, *Veillonella* (Fig. 4C, D). The significance in bacterial community differences of CXM + CFX-treated group was subject to the limited clinical samples. As the Fig. 4E, F shows, further LEfSe analysis revealed that *Bacteroides*, *Escherichia-Shigella*, and *Lactobacillus* were the most different bacteria genera in fecal samples from control group ($p < 0.05$, LDA > 2). In CXM-treated group, the most abundant bacteria genera were *Clostridioides*, *Faecalibacterium*, *Lactobacillales-unclassified*, and *Veillonella* ($p < 0.05$, LDA > 2). As to CXM + CFX-treated group, the most susceptible bacteria genera were *Enterobacteriaceae-unclassified* and *Streptococcus* ($p < 0.05$, LDA > 2). Besides, the significantly disturbed bacteria OTUs by antibiotic treatments in fecal samples were up to 31 (Fig. 4G). Among these, OTU91 (*Faecalibacterium*), OTU19 (*Pseudomonas*), OTU17 (*Veillonella*), OTU4 (*Clostridiaceae-sensu-stricto*), OTU1 (*Streptococcus*), OTU76 (*Pelomonas*), and OTU11 (*Enterobacteriaceae-unclassified*) exhibited the consistent phenotype in breast milk and fecal samples.

4. Gut microbiota alternations in infants

The gut microbiome composition in infants showed a substantial recuperative trend in the follow-up visits. The microbial community richness and diversity of gut microbiota in the fecal samples of CXM-FV group were significantly higher than that of CXM-treated group (Fig. 5A-C). As illustrated in Fig. 5D, PCoA analysis in conjunction with Adonis analysis revealed a significant change in the microbial community composition between CXM-FV group and CXM-treated group ($p < 0.001$).

The dominant bacterial community in fecal samples of CXM-FV group was also prone to revert to the initial states. The most abundant bacteria phyla in the fecal samples from CXM-treated group were *Firmicutes* and *Proteobacteria*; while the dominant bacteria phyla in CXM-FV group followed the order of *Actinobacteria*, *Firmicutes*, and *Proteobacteria* (Fig. 6A). At the genus level, *Escherichia-Shigella*, *Streptococcus*, *Clostridiaceae-sensu-stricto*, *Enterobacteriaceae-unclassified*, *Clostridioides* were the most abundant bacterial genera in CXM-treated group, whereas *Escherichia-Shigella* and *Bifidobacterium* were the most abundant bacterial genera in CXM-FV group (Fig. 6B). The Wilcoxon rank-sum test revealed that *Actinobacteria* were considerably more abundant in CXM-FV group at the phylum level. At the genus level, CXM-FV group had higher abundance of *Bifidobacterium* and another six bacteria genera, but CXM-treated group had lower abundance of *Clostridioides*, *Pseudomonas*, and *Firmicutes-unclassified* (Fig. 6C, D). As shown in Fig. 6E, F, LEfSe analysis further confirmed that *Clostridioides*, *Comamonas*, *Parabacteroides*, *Pseudomonas*, and *Firmicutes-unclassified* were the most distinct bacteria genera in CXM-treated group ($p < 0.05$, LDA > 2), whereas *Bifidobacterium* and the remaining 18 bacterial genera were more abundant in CXM-FV group ($p < 0.05$, LDA > 2). In addition, 24 significantly altered bacteria OTUs were identified between CXM-treated group and CXM-FV group with only OTU19 (*Pseudomonas*) and OTU6 (*Clostridioides*) exhibiting lesser abundance in CXM-FV group (Fig. 6G).

5. ARGs abundance of gut microbiota in neonates

The ARGs abundance in neonatal fecal samples was examined to evaluate the antibiotic resistance in gut microbiota. The results of HT-qPCR demonstrated that antibiotic treatments to mothers during the perinatal period made a difference to the antibiotic resistance in neonatal gut microbiota (Fig. 7A). The relative abundances of ARGs were substantially higher in fecal samples from the control group than those from CXM-treated group. Over 50% of ARGs demonstrated declining trend in CXM-treated group (particularly bla_{OXA10}, bla_{SHV}, *cfxA*, *tnpA-04*, and *tnti1*), while most ARGs were considerably down-regulated in CXM + CFX-treated group.

6. ARGs transfer in gut microbiota of infants

The ARGs transfer in gut microbiota of infants were investigated by comparing the obtained results with the fecal samples from the follow-up visits. As illustrated in Fig. 7B, the relative ARGs abundance of infants' gut microbiota presented a significant increasing trend (bla_{GES}, bla_{TEM}, *tnpA-04*, and *tnti1*) after a 6-month recovery period.

Discussion

Antibiotics are highly successful in controlling susceptible bacterial infections and undoubtedly provide remarkable benefits to human beings; however, the dark side of antibiotics also comes along with the abuse. The excessive application of antibiotics not only caused the universal residue but also induced great disturbance to the microbial community in host. Increasing evidences have confirmed that antibiotic treatments would perturb the indigenous gut microbiota [5, 25, 26]. Therefore, there are increasing concerns regarding to the adverse impacts of antibiotics on the human gut microbiota. One is the altered gut microbiota composition would interfere with the human-microbe interactions, which are vital to human health [27], and the other is the spread of antibiotic resistance among pathogens due to the transfer of ARGs from and between mutualists [3, 4]. The adverse outcomes of antibiotic abuse cover various aspects [26], and among which, antibiotic resistance is the most worrying issue that accounts for the lower efficacy of antibiotic as well as the development of resistant strains. Antibiotic treatments are indispensable in clinical treatments of parturition for the purpose of anti-infection. Meanwhile, antibiotic treatments would alter the microbial community in breast milk and antibiotics also could transfer to the infants during lactation [16, 28, 29]. Therefore, antibiotic treatments during perinatal period could induce direct exposure to neonates as well as indirect effects *via* breast milk. The neonatal period is one of heightened susceptibility to external invasion and the disturbed gut microbiota in neonates would greatly aggravate health risks [17]. More worryingly, transfer of ARGs in the gut microbiota of infants would leave the hidden trouble of antibiotic resistance in infants. Given all these, it is of great importance to investigate the perinatal antibiotic treatments induced adverse effects on infants.

Breast milk, which traditionally considered sterile, has been found to provide an ongoing supply of commensal bacteria to the infant gastrointestinal tract [16]. Previous studies also indicated that there was a transfer of bacterial strains, at least, to the genera of *Lactobacillus*, *Staphylococcus*, *Enterococcus*, and *Bifidobacterium*, belonging between mother and infant through lactation [30–33]. This is consistent with the most abundant bacteria phyla identified in breast milk from different groups (Fig. 2A). Hermansson et al., reported that perinatal antibiotic treatments were significantly associated with changes in milk microbial composition ($p = 0.001$), and higher breast milk microbiota α-diversity were considered to be associated with perinatal antibiotic treatments [34]. Thus, alteration of maternal breast milk microbiota during lactation could have a direct effect on infant health. Results of Illumina sequencing indicated that antibiotic treatments during the perinatal period had limited effects on the microbiota in breast milk. Only CXM + CFX treatment resulted in a significant disruption of the microbiota in breast milk, whereas CXM treatment showed a declining trend ($p > 0.05$). Regardless of the insignificant alteration in breast milk, the continuous disturbance to the gut microbiota of infants due to the long-term lactation period is too dangerous to ignore.

On the other hand, antibiotics could directly transfer to infants through breast milk. Existing researches had reported the detection of antibiotic residues in consumer milk, as well as other foods [35–37]. Smadi et al. had reported an approximately 5% presence of antibiotic residues in breast milk samples from Syrian refugee nursing mothers (n = 120), who even had no antibiotic history [15]. Antibiotic residues in

the mothers' milk were believed to be a result of their daily diet [38]. The antibiotic ingestion by breast milk would definitely induce direct effects on the gut microbiota of infants. According to previous study, administration of non-absorbable antibiotics to pregnant dams altered the relative abundances of *Lactobacillales* order and *Clostridium subcluster XVIII* family in antibiotic-treated dams as well as in the offspring [5]. Specific bacterial lineage blooms (*Akkermansia*, *Blautia*, *Enterococcus*, and *Faecalibacterium* genera) also occurred after antibiotic intervention [39]. Herein, the most dominant bacteria phylum in antibiotic treatments groups (both CXM and CXM + CFX treatments) was *Firmicutes* followed by *Proteobacteria*. Additionally, the consistent OTUs identified in breast milk and fecal samples (Fig. 2E and Fig. 4G) suggested that those bacteria OTUs could represent the critical microbiota correlated with antibiotic treatments. Mouse studies had indicated a causal role of the early-life antibiotic use disrupted microbiota with elevated risks for metabolic and immunological diseases [40]. Therefore, the hygiene hypothesis asserts that increasing rates of health issues in the infants are related with the disruption of gut microbiota induced by antibiotics.

It is undoubted that antibiotic resistance has become a global problem due to diminished therapeutic effects and increased risks of complications and catastrophic outcomes [41]. ARGs are commonly regulated by sophisticated mechanisms that trigger gene transcription in response to antibiotic treatments [42]. CXM and CFX are typical cephalosporin-like antibiotics which are highly resistant to hydrolysis by β-lactamase [43, 44]; whereas bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M} were identified as the genes encoding extended-spectrum β-lactamases phenotype [45]. In this study, ARGs also exhibited a decreasing trend in CXM-treated group (bla_{OXA10}, bla_{SHV}) and CXM + CFX-treated group (bla_{OXA10}, bla_{SFO}, bla_{TEM}, bla_{SHV}). These findings indicated that that cephalosporin-like antibiotic treatments would drastically disturb the gut microbiota in neonates but also introduce the specter of antibiotic resistance. Subirats et al., suggested that daily ingestion of antibiotics might expose the gut microbiota to antibiotic concentrations far exceeding the minimal selective concentration boundaries, which would favor the growth of potential resistant bacteria [46]. Thus, antibiotic treatments to mothers throughout the perinatal period would disturb the gut microbiota in infants by promoting resistant bacteria, and the disturbed gut microbiota would further impair infants' health.

Although the microbiota can be affected by antibiotic treatments, little is known about their responses comparing with baseline temporal variation. Measurements within individuals over time may reveal the range of variation conceivable in a system defined by the same set of interactions [25]. According to our findings, the diversity of gut microbiota in infants demonstrated a recuperative trend in follow-up visits after six months. Dethlefsen et al., also reported that gut microbiota began to return to their initial states one-week following antibiotic treatments, but the recovery was often incomplete [25]. As with other ecosystems, the gut microbiota at baseline is a dynamic system with a stable average state. Antibiotic treatments may cause a shift to an alternative stable state, but the full consequences of which remain unknown. In this study, the relative abundance of ARGs was dramatically elevated in follow-up visits, implying that ARGs were transferred to newborns' gut microbiota. Among the varied biochemical mechanisms of antibiotic resistance, acquisition of ARGs from the resistance genes pool of other

microbial genera and antibiotic-producing organisms were believed to be the most probable resistance determinants [47, 48]. The transfer of ARGs from bacteria to human pathogens is bound to pose menace to human health and public environmental sanitation. Given the observed transfer of ARGs in infant gut microbiota, antibiotic treatments to mothers during perinatal period would definitely leave the hidden trouble of antibiotic resistance in their infants, therefore, compromise therapeutic efficacy in the future.

In conclusion, our study found that antibiotic treatments to mothers during perinatal period would disturb the gut microbiota in neonates by lactation. In addition, the gut microbiota in infants would partly return to their initial state after rehabilitation, but the transfer of ARGs in the gut microbiota of infants would leave the hidden trouble of antibiotic resistance. We are aware that these results have been established with limited patients and will require further confirmation with a larger group of individuals and with other antibiotics. But data presented here can help to guide the scientific use of antibiotics during perinatal period and provide viable approaches to mitigate the negative consequences.

Abbreviations

ARGs: antibiotic resistance genes; B: breast milk samples; CFX: cefoxitin; Con: control; CXM: cefuroxime; DNA: deoxyribonucleic acid; FV: follow-up visits; F: fecal samples; HT-qPCR: high-throughput quantitative PCR; LDA: linear discriminant analysis; LEfSe: linear discriminant analysis effect size; OTUs: operational taxonomic units; PCoA: principal co-ordinates analysis; PCR: polymerase chain reaction; 16S rRNA: 16S ribosomal RNA.

Declarations

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Competing interests

All authors declare that they have no competing interests.

Author Contributions

Chenyang Ji: Writing-original draft, Software, Writing-review& editing; **Geer Zhang**: Resources; **Siyuan Xu**: Resources, Investigation; **Qingyi Xiang**: Resources, Investigation; **Meishuang Huang**: Resources; **Xiaoxia Bai**: Supervision, Project administration, Funding acquisition; **Meirong Zhao**: Conceptualization, Supervision.

Ethics approval

All experiments were conducted in accordance with approved Medical Ethics Committee of Women's Hospital, School of Medicine, Zhejiang University (IRB-20200140-R).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1-7.

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Figures

Figure 1

α and β-diversity indices of the microbiota in breast milk samples from control group (B-Con, n=9), CXM-treated group (B-CXM, n=13), and CXM+CFX-treated group (B-CXM+CFX, n=3). A: Observed OTUs of the microbiota in breast milk samples; B: Cloud plot of the Chao1 estimator regarding to the microbial community richness in breast milk samples; C: Cloud plot of the Shannon index regarding to the microbial community diversity in breast milk samples; D: Multiple samples PCoA analysis regarding to the difference in the microbial community composition in breast milk samples. Red cycles represent sample of B-Con group; purple squares represent sample of B-CXM group; green triangles represent sample of B-CXM+CFX group. Each box plot represents the median, interquartile range, minimum, and maximum values. *The data are statistically significantly different from B-Con group (p<0.05).

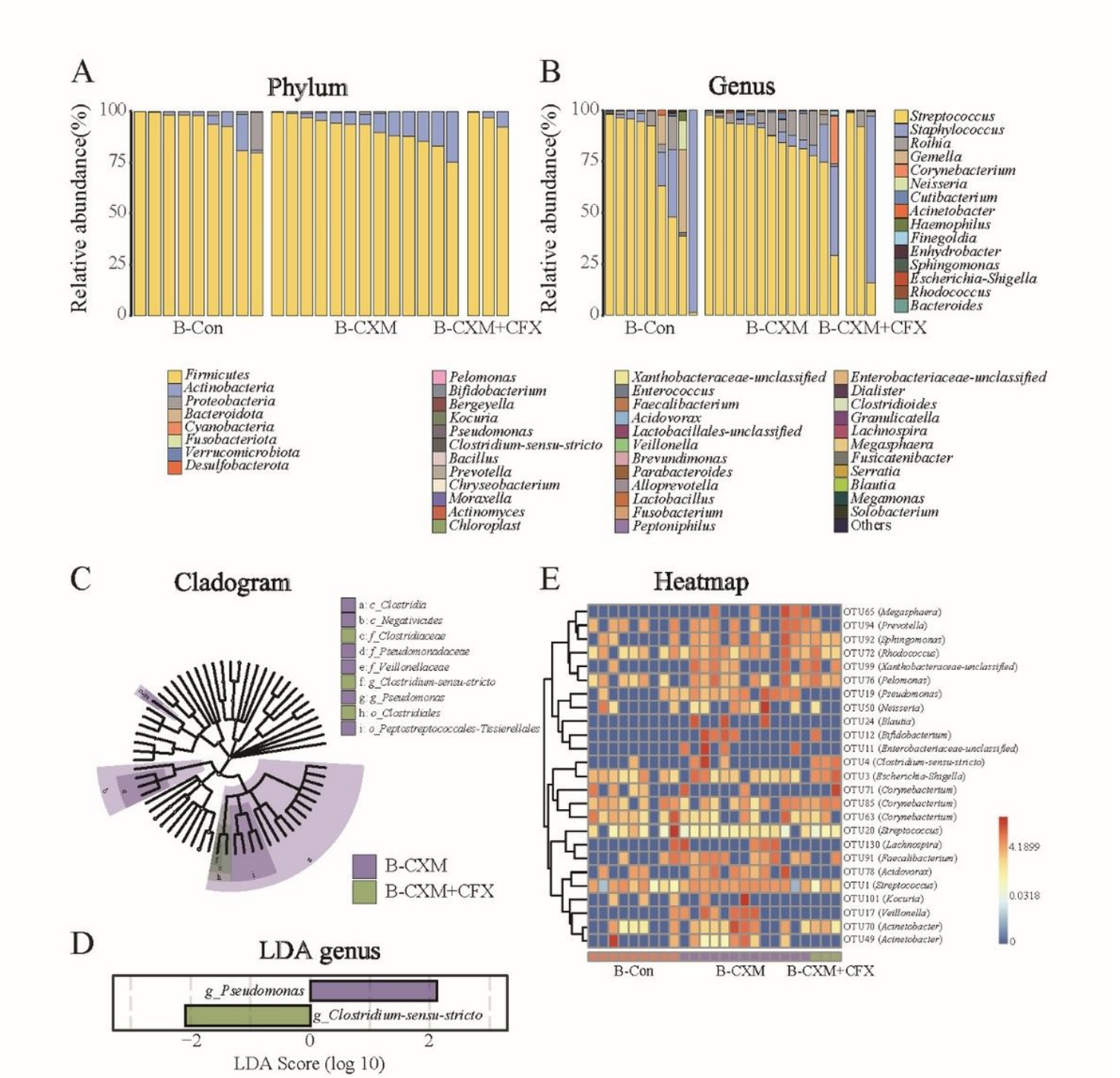

Figure 2

Comparison of the microbiota in breast milk samples from B-Con group (n=9), B-CXM group (n=13), and B-CXM+CFX group (n=3). A and B: Microbial community bar plot of the microbiota in breast milk samples at phylum and genus levels; C and D: Kruskal-Wallis rank-sum test of the microbiota abundance in breast milk samples at phylum and genus levels; E: LEfSe analysis cladogram of distinct bacteria in breast milk samples at phylum levels; F: LDA score of distinct bacteria in breast milk samples at genus levels; G:

Microbial community heatmap regarding to the microbiota abundance in breast milk samples. Red cells indicate increased; blue cells indicate decreased.

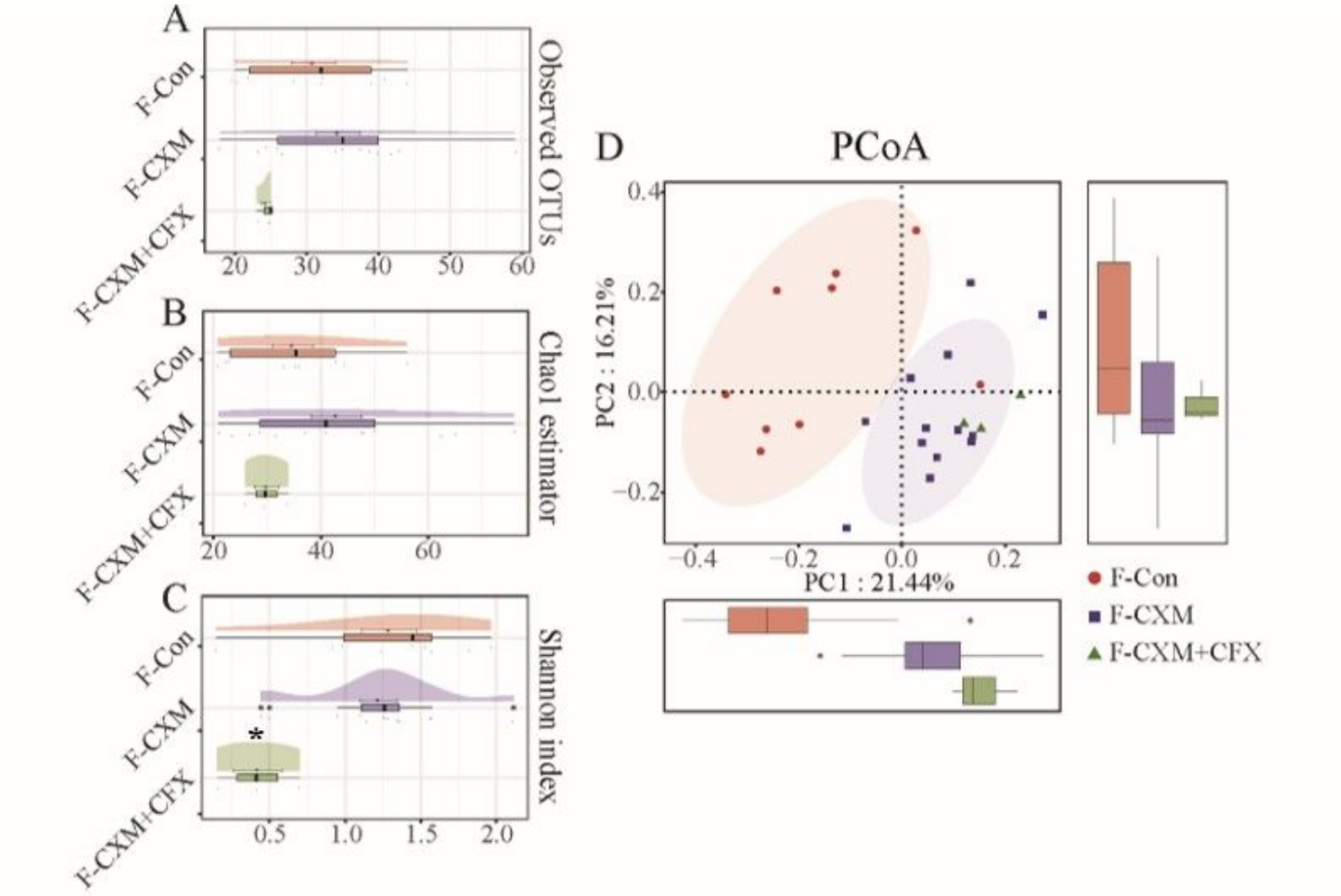

Figure 3

α and β-diversity indices of the gut microbiota in fecal samples from control group (F-Con, n=9), CXM-treated group (F-CXM, n=13), and CXM+CFX-treated group (F-CXM+CFX, n=3). A: Observed OTUs in the gut microbiota in fecal samples; B: Cloud plot of the Chao1 estimator regarding to the gut microbial community richness in fecal samples; C: Cloud plot of the Shannon index regarding to the gut microbial community diversity in fecal samples; D: Multiple samples PCoA analysis regarding to the difference in the microbial community composition in fecal samples. Red cycles represent sample of F-Con group; purple squares represent sample of F-CXM group; green triangles represent sample of F-CXM+CFX group. Each box plot represents the median, interquartile range, minimum, and maximum values. *The data are statistically significantly different from F-Con group (p<0.05).

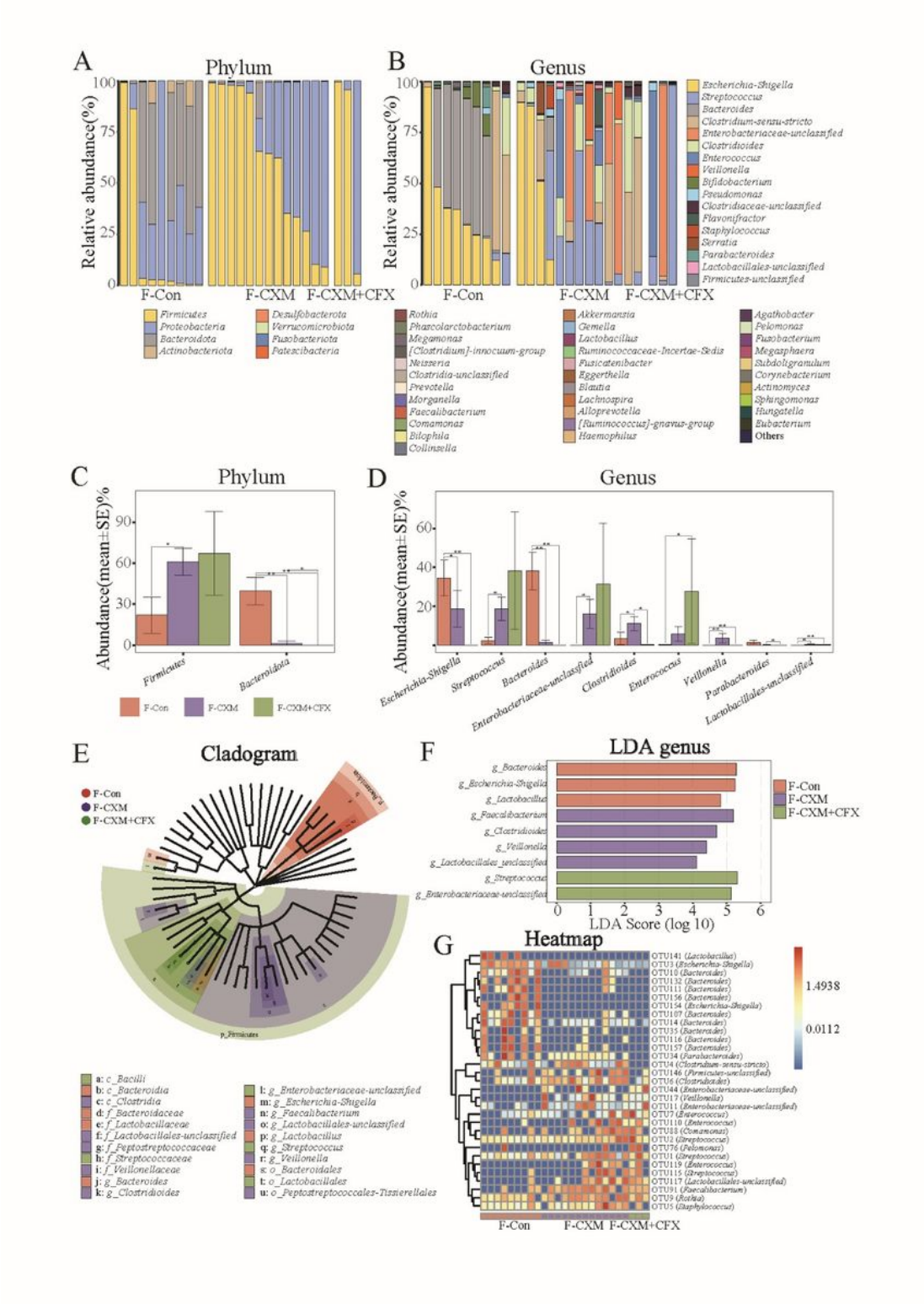

Figure 4

Comparison of the gut microbiota in fecal samples from F-Con group (n=9), F-CXM group (n=13), and F-CXM+CFX group (n=3). A and B: Microbial community bar plot of the gut microbiota in fecal samples at phylum and genus levels; C and D: Kruskal-Wallis rank-sum test of the gut microbiota abundance in fecal samples at phylum and genus levels; E: LEfSe analysis cladogram of distinct bacteria in fecal samples at phylum levels; F: LDA score of distinct bacteria in fecal samples at genus levels; G: Microbial community

heatmap regarding to the gut microbiota abundance in fecal samples. Red cells indicate increased; blue cells indicate decreased.

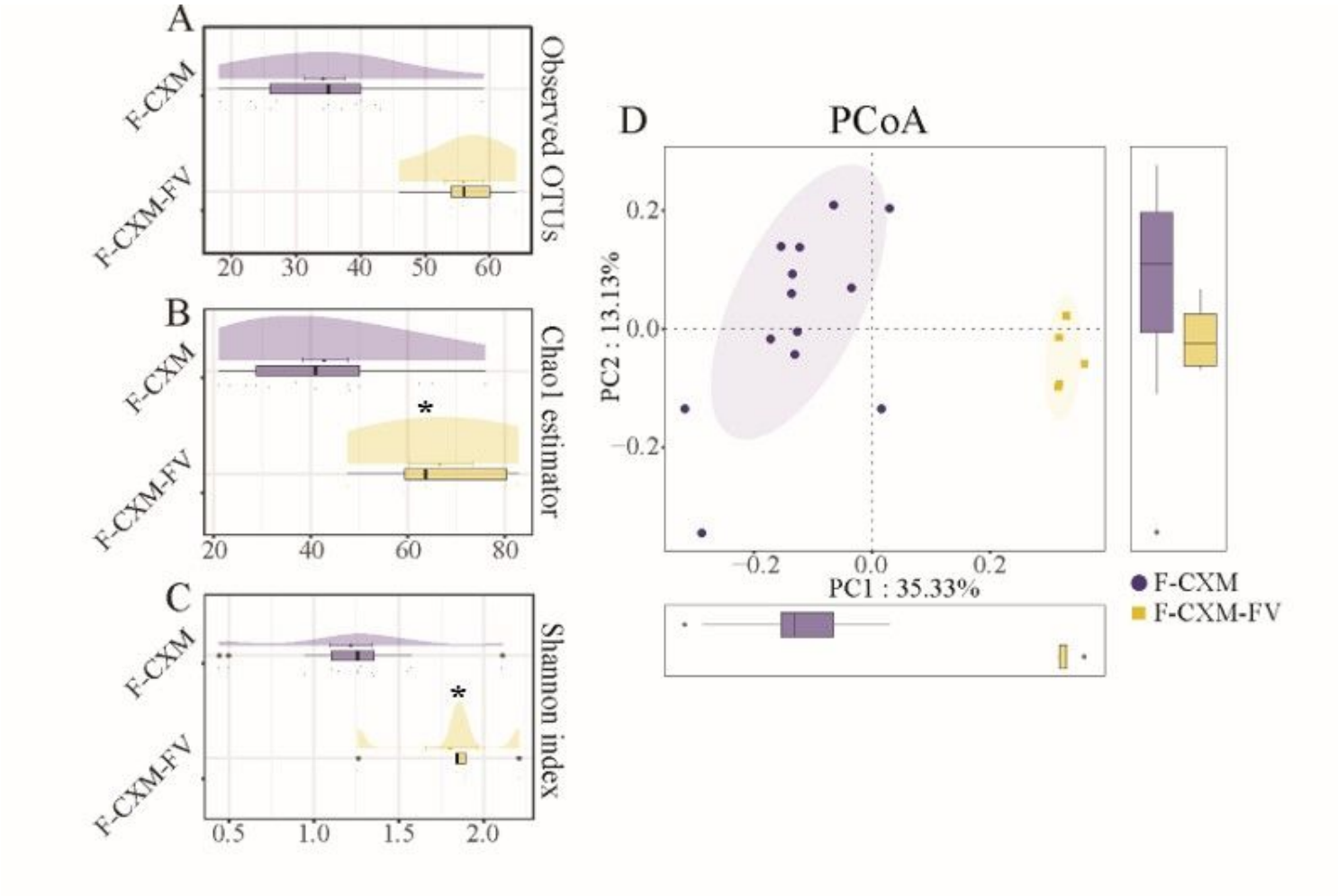

Figure 5

α and β-diversity indices of the gut microbiota in fecal samples from CXM-treated group (F-CXM, n=13), and CXM-treated group of follow-up visits (F-CXM-FV, n=5). A: Observed OTUs in the gut microbiota in fecal samples; B: Cloud plot of the Chao1 estimator regarding to the gut microbial community richness in fecal samples; C: Cloud plot of the Shannon index regarding to the gut microbial community diversity in fecal samples; D: Multiple samples PCoA analysis regarding to the difference in the microbial community composition in fecal samples. Blue cycles represent sample of F-CXM group; yellow squares represent sample of F-CXM-FV group. Each box plot represents the median, interquartile range, minimum, and maximum values. *The data are statistically significantly different from F-CXM group (p<0.05).

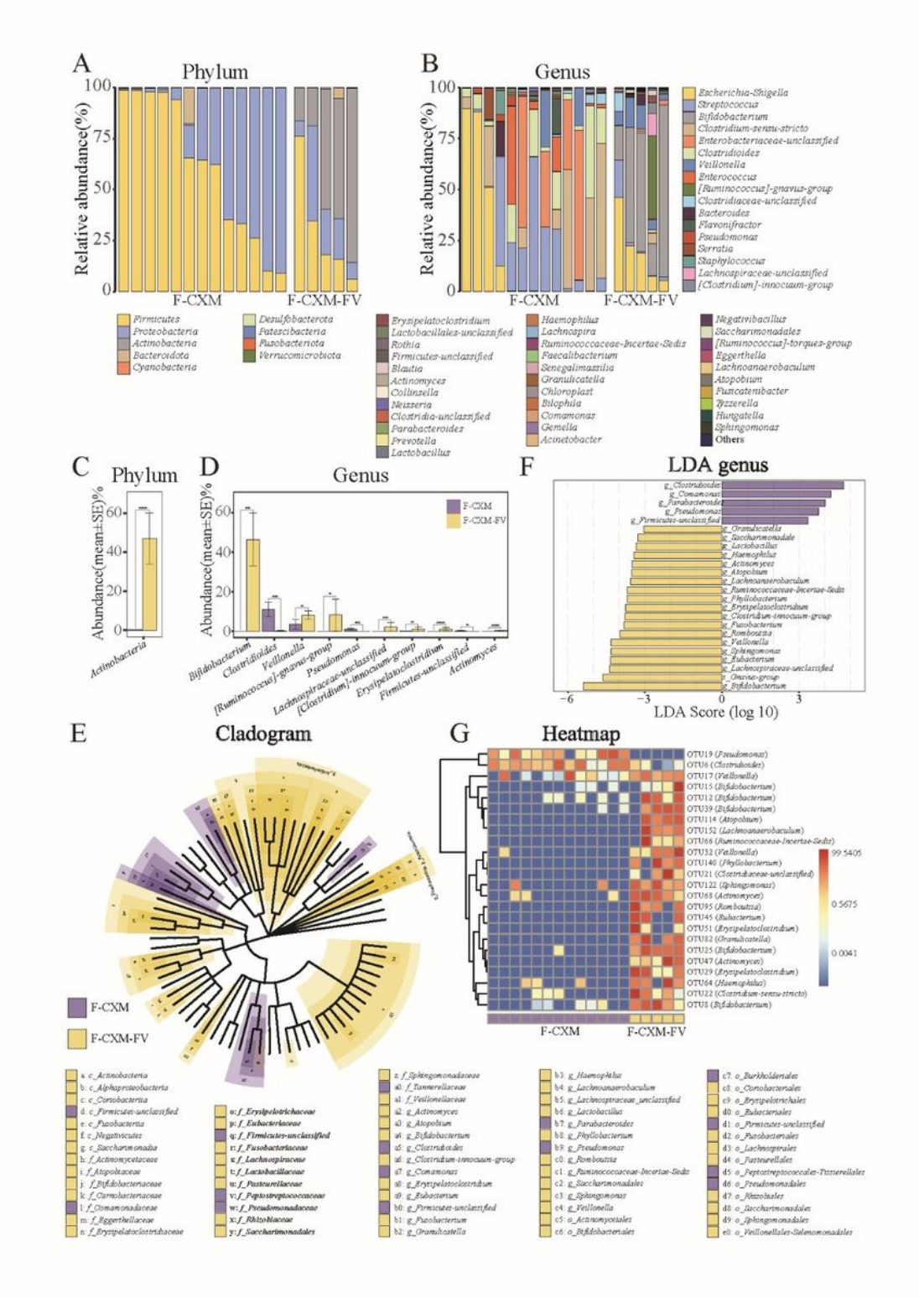

Figure 6

Comparison of the gut microbiota in fecal samples from F-CXM group (n=13) and F-CXM-FV group (n=5). A and B: Microbial community bar plot of the gut microbiota in fecal samples at phylum and genus levels; C and D: Wilcoxon rank-sum test of the gut microbiota abundance in fecal samples from at phylum and genus levels; E: LEfSe analysis cladogram of distinct bacteria in fecal samples at phylum levels; F: LDA score of distinct bacteria in fecal samples at genus levels; G: Microbial community

heatmap regarding to the gut microbiota abundance in fecal samples. Red cells indicate increased; blue cells indicate decreased.

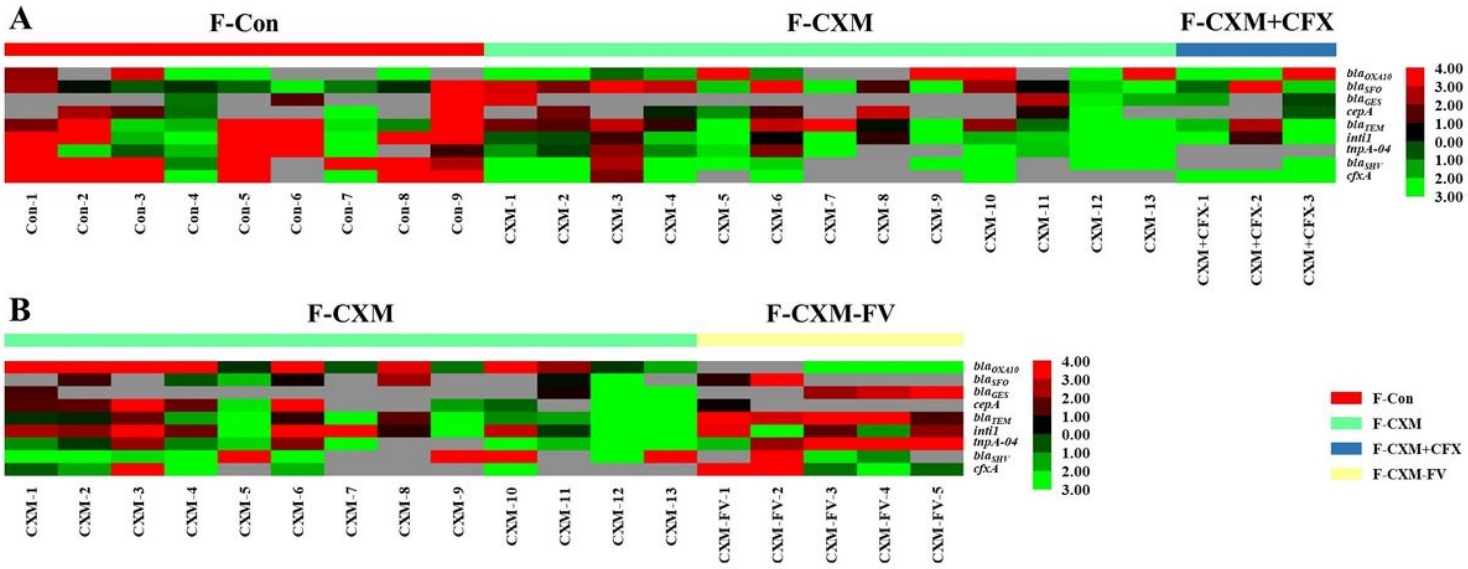

Figure 7

Heatmap regarding to the relative ARGs abundance of gut microbiota in fecal samples. A: Relative ARGs abundance of gut microbiota in fecal samples from F-Con group (n=9), F-CXM group (n=13), and F-CXM+CFX group (n=3); B: Relative ARGs abundance of gut microbiota in fecal samples from F-CXM group (n=13) and F-CXM-FV group (n=5). Red cells indicate increased; green cells indicate decreased.

Supplementary Files

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