Evaluation of Gentamicin Exposure Effects on Human gut Microbiota using the Simulator of Human Intestinal Microbial Ecosystem (SHIME)

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Research

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Abstract

Background: Antibiotics are emerging toxic contaminant that have potential public health risk worldwide. They may cause the human intestinal microbial disorder, as well as the spreading of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Most of the intestinal bacteria are not cultivable for the moment, tracking the special gene-labeled plasmid from the exogenous bacteria would help obtain the direct evidence of the horizontal transfer of ARGs in the intestinal flora. However, to date, there are only a few research reports applying the exogenous labeled bacteria to study the transfer of ARGs among intestinal bacteria. Therefore, for the first time, this study evaluated the *in vitro* ability of gentamicin on colonization of exogenous bacteria and plasmid in the simulated human gut.

Results: This study indicated that exposure to gentamicin may be conducive to the colonization of exogenous bacteria and plasmid, as well as the conjugation of plasmid to gut microbiota. Gentamicin exposure was also confirmed to reduce the gene numbers of human disease-related pathways and promote the drug resistance in the gut microbiota. The effects on the genetic level might attribute to microbiota shift, as co-occurrence patterns suggested that *Bacteroides* attributed to the ARGs enrichment and *Klebsiella* played a crucial role in human disease-related pathways reduction after gentamicin treatment.

Conclusion: These results may open up new perspectives for assessing the direct effects of antibiotics on the intestinal microbiota. These suggested side-effects should be considered for antibiotics prescription.

Background

The antibiotic therapies have been demonstrated paramount importance in the treatment of bacterial infections since its discovery in the 1940s. Nowadays the extensively used antibiotics are considered as toxic emerging contaminants, which pose severe threats to environmental ecosystems [1]. Humans are directly and indirectly exposed to different antibiotics through inhalation, ingestion of drugs, drinking water, and foods, and their risk assessments have attracted more attention, recently [2–4]. As a result, antibiotics may progressively enter into the human gastrointestinal tract and produce a large variety of antibiotic-resistant gut-micro flora [5]. In our previous research, it was found that both human and veterinary antibiotics were mostly detected in the gut of the Chinese population [6]. The stable intestinal microbial ecosystem has been demonstrated not only to provide essential nutrients for human health but also to modulate the immune function by protecting infectious pathogens [7, 8]. However, exposure to antibiotics may lead to the disrupt of the stable ecosystem and promote the spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the human gut, which may limit the treatment efficiency and resulting from the chronic and relapsing infectious diseases [9–11].

Gentamicin is one of the few thermostable antibiotics, and it is one of the great technological achievements that independently developed in China. It is also one of the most commonly used aminoglycoside antibiotics, which would bind to the 30S subunit of the ribosome to block bacterial

protein synthesis [12]. Some studies have shown that taking gentamicin may change the structure of the flora and promote the spread of ARB and ARGs [13, 14]. At present, most intestinal bacteria are not cultivable, tracking the special gene-labeled plasmid from the exogenous bacteria would help obtain the direct evidence of the horizontal transfer of ARGs in the intestinal flora. There are only a few research reports applying the exogenous labeled bacteria to study the transfer of ARGs among intestinal bacteria [15–17]. However, there is no report that studied the antibiotic selection pressure impacts on the spread of exogenous labeled bacteria and antibiotic resistant plasmids in human intestinal flora. Therefore, this study would evaluate the ability of gentamicin on colonization of exogenous bacteria and plasmid in human gut.

The simulator of the human intestinal microbial ecosystem (SHIME) model is known to be a useful tool for *in vitro* studies as (i) interactions between the microbiota; and (ii) the effects of prebiotics and other compounds on the microbial communities and metabolic activities [18]. To the best of our knowledge, there are only a few researches that added the antibiotics into this *in vitro* model, which mainly focused on the benefit of the mucosal environment, high-fiber diets, probiotic, and propionate-producing consortium in human intestinal microbiota [19–22].

Therefore, this study, for the first time, evaluated the *in vitro* ability of gentamicin on colonization of exogenous bacteria and plasmid in the simulated human gut, as well as the impacts on human gut microbiota and associated functional pathways. Considering the reasonable dosage of gentamicin for adult human study is about 320 to 850 mg/day, and only half volume of the adult gut can be simulated in used SHIME model, 300 mg/day of gentamicin was used in this study [23–25]. In this research, fluorescence quantitative PCR detection technology was applied to determine the concentrations of exogenous strain and plasmid in the samples. Then, the composition of human intestinal microbiota was analyzed by 16S rRNA gene high-throughput sequencing, the human disease-related pathways were predicted by functional predictions, and the ARGs were quantified by high-throughput quantitative PCR (HT-qPCR). This study achieved a systematic investigation and precise understanding of the direct effects of gentamicin on the intestinal microbiota, which may be valuable for directing future work.

Results

Fate of exogenous donor strain and plasmid

The marker genes *Td-Tomato* and Enhanced-*gfp* (*Egfp*) were quantified to determine the concentrations of exogenous donor strain and the plasmid, respectively. The relative abundance of these genes in samples from the SHIME model were assessed at different time points, which included samples collected after exogenous donor strain was added to the SHIME for one day, for three days and before the gentamycin administration (Control), samples collected after the administration of gentamycin for one day, for three days and seven days (Gentamycin), samples collected after the administration of inulin for three days, for one week and two weeks (Inulin).

As shown in Fig. 1, the fate of exogenous donor strain and the plasmid showed a similar decreased trend in the three colon regions over time. Compared with the initial value, the final relative abundance of *Td-Tomato* gene decreased by approximately 3.3–3.7 log units, and the *Egfp* gene decreased by approximately 3.4–4.0 log units. Another finding is that the decline of exogenous donor strain and the plasmid in samples from gentamicin treatment group showed a flat trend and even slightly increased. At this stage, the relative abundance of *Td-Tomato* gene just decreased by about 0.3–0.9 log units, and the *Egfp* gene only decreased by about 0.0–0.8 log units. These phenomena suggested that gentamicin exposure may be conducive to the colonization of exogenous donor strain and the plasmid. Similarly, the ratio of relative abundance of *Egfp* gene to *Td-Tomato* also increased in the gentamicin treatment group. Compared with the initial value, the highest point were about 0.2–0.4 log units higher, indicating the occurrence of conjugation of RK2 to gut microbiota promoted by gentamicin exposure.

ARGs

In this study, a total of 48 targets ARGs were detected from different group samples using a high-throughput-qPCR (HT-qPCR) technique. The heatmap showed that the relative abundances of ARGs such as aminoglycoside, beta-lactam, multidrug, tetracycline, and multidrug resistance genes were noticeably higher in the gentamicin exposure group as compared to the control group (Fig. 2). For instance, the relative log abundance of *aph3iia* and *aac3iia* (aminoglycoside) were 3.1 and 4.0 log units higher after gentamicin treatment (Gen_A3) than in control (Con_A3). Similarly, *tetw* (tetracycline), *ndm-1* (beta-lactam), and *mexf* (multidrug) were 1.7, 3.6 and 3.9 log units higher than the control group. After two weeks of recovery by inulin, the log abundances of these resistance genes decreased. For example, the log abundances of these ARGs in Inu_A3 were about 0.8–3.9 log units lower than Gen_A3.

Human disease-related pathways

The metagenomics study of the 16S rRNA gene sequence by PICRUSt revealed the gene numbers of human disease-related functional pathways in the bacterial communities of the three groups, and the genes were presented in the heatmap (Fig. 3). The heatmap showed that the gene numbers of human disease-related pathways, including cancer, immune system diseases, infectious diseases, metabolic diseases, and neurosurgery diseases, were more abundant in control group than that in gentamicin treatment group. For instance, the gene numbers of cancer pathway in the Con_A3 sample was 1.5–8.0 times as that of the Gen_A3 sample. Similarly, the gene numbers of the African trypanosomiasis, pertussis, and amyotrophic lateral sclerosis pathway in the Con_A3 sample were about 3.8–4.7 times as that of the Gen_A3 sample. After two weeks of inulin recovery, the number of these pathway genes related to human diseases increased to a certain extent (that in Inu_A3 were about 1.3–4.3 times as that of the Gen_A3).

Microbiota community composition

In this study, the effects of gentamicin on gut microbial communities' composition were also investigated. Based on the 16S rRNA gene sequence analysis, the most abundant taxonomic groups assigned at the phylum level were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Synergistetes*, which account for 90.3–98.2% of the total gut microbiota (Fig. 4a). However, an obvious increase in the abundance of *Bacteroidetes* (from 24.3–26.8% to 59.3–70.5%), and decrease in abundances of *Proteobacteria* (from 44.7–62.2% to 15.6–28.3%), *Firmicutes* (from 6.5–8.7% to 1.2–3.1%), and *Synergistetes* (from 3.0–16.4% to 0.4–4.1%) were seen after gentamicin treatment (Fig. S1b). After using inulin for two weeks, the abundance of intestinal flora was observed to be obviously restored at the phylum level: the abundance of *Bacteroidetes* decreased (from 59.3–70.5% to 36.1–39.6%), while the abundances of *Proteobacteria* (increased from 15.6–28.3% to 43.1–49.6%), *Firmicutes* (increased from 1.2–3.1% to 6.3–13.0%), and *Synergistetes* (increased from 0.4–4.1% to 1.5–5.0%) increased.

At the genus level, the gentamicin treatment subjects were shown to be substantially overgrown by *Bacteroides* (from 17.5–19.6% to 58.3–69.1%), while the percentage of *Klebsiella* (from 8.0–22.7% to 1.0–1.7%) and *Pseudomonas* (from 5.1–10.2% to 1.2–4.0%) was decreased (Fig. 3b). During the recovery period, it was observed that the intestinal flora also had a certain recovery, the abundance of *Bacteroides* decreased (from 58.3–69.1% to 22.3–29.9%), and the abundances of *Klebsiella* (increased from 1.0–1.7% to 2.1–7.3%) and *Pseudomonas* (from 1.2–4.0% to 3.8–7.5%) increased.

The linear discriminant analysis effect size (LEfSe) comparison analysis between the three groups is shown in Fig. S2. As shown in Fig. 4a, LEfSe analysis indicated that gentamicin exposure resulted in a significant decrease in the abundance of *Klebsiella* (LDA = 4.85) and *Pseudomonas* (LDA = 4.46), accompanied by a significant increase in *Bacteroides* (LDA = 5.35). As shown in Fig. 4b, after discontinuation of gentamicin and administration of inulin for two weeks, a reduction in *Klebsiella* (LDA = 4.76) was still identifiable compared to the control group, while the abundance of *Bacteroides* and *Pseudomonas* was not significant difference. These findings were all consistent with the above gut microbial communities' composition results.

Microbiota diversity

Meanwhile, the fecal microbiota of alpha diversity was assessed. The taxon richness (Chao1 index), evenness (Simpson index), and diversity (Shannon index) are shown in Fig. S3. Compared with the control group, the decline of microbial richness (Chao1, $P < 0.05$) and diversity (Shannon, $P < 0.001$), and increase of evenness (Simpson, $P < 0.01$) were observed in the samples after gentamicin treatment. After discontinuation of gentamicin and administration of inulin for two weeks, no obvious change was found in alpha diversity between inulin recovery group and control. Besides, the beta diversity of the microbiota communities was also affected by gentamicin treatment. As shown in Fig. S4, the beta diversity results suggested that all the samples collected after gentamicin treatment or inulin recovery differed from the control group. Moreover, the samples in the inulin recovery group gathered closer to control group than that between the gentamicin treatment group and control, which suggested that the beta diversity difference caused by gentamicin treatment may be reduced after inulin recovery for two weeks.

Correlations between microbial taxa and ARGs or human disease-related pathways

The network analysis of co-occurrence patterns between the microbial taxa and the ARG subtypes is shown in Fig. 5. It was seen that *Bacteroides* (significantly enriched bacteria after gentamicin treatment) was positively associated with aminoglycoside and tetracycline resistance genes. For example, the correlation coefficients of *Bacteroides* with *aph3iii* and *tetw* were about 0.98 ($P < 0.05$). *Klebsiella*, the significantly decreased bacterial genus in gentamicin treatment group, was also positively associated with several ARGs. For example, the strong correlations were found in *Klebsiella* with *bl2be_shv2/2bl2_len* and *yidy/mdtI* ($r = 0.99$, $P < 0.01$).

Figure 6 shows the results of co-occurrence patterns between the microbial taxa and human disease-related pathways. A very similar pattern of results was observed that significantly decreased bacteria *Klebsiella* and *Sartella* after gentamicin treatment were positively associated with most of those human disease-related pathways, such as cancer, immune system diseases, infectious diseases, metabolic diseases, and neurosurgery diseases. Specifically, the correlation coefficients of *Klebsiella* with bladder cancer, primary immunodeficiency, and *Vibrio cholerae* pathogenic cycle were about 0.97 ($P < 0.001$) and that of *Sartella* with primary immunodeficiency and tuberculosis were 0.94 ($P < 0.05$). *Bacteroides*, the significantly increased bacterial genus in gentamicin treatment group, was also positively associated with several human disease-related pathways. For example, the strong correlations were found in *Bacteroides* with pathways in cancer and huntington's disease ($r = -0.92$, $P < 0.05$).

Discussion

Gentamicin exposure promoted the colonization of exogenous strain and plasmid

In this research, fluorescence quantitative PCR detection technology was applied to determine the concentrations of exogenous strain and plasmid in the samples. The results indicated that exposure to gentamicin may be conducive to the colonization of exogenous bacteria and plasmid, as well as the conjugation of plasmid to gut microbiota. Recently, Lambrecht and associates confirmed that drug-resistant *E. coli* commonly found in food would quickly transfer its drug-resistant plasmid to the human intestinal flora by SHIME system [26]. Our study also suggested the ARB and ARGs may spread in the simulated intestinal system, which indicated that the gut is a reservoir for ARB and ARGs. The spread and long-standing of ARB and ARGs in the human intestine would pose a significant therapeutic challenge. As discussed earlier, some studies have revealed that taking gentamicin may promote the spread of ARB and ARGs [13, 14]. ARGs are commonly spread by plasmid-mediated horizontal conjugative transfer, which may lead the diffusion of drug resistance among bacteria and increase the number of resistant bacteria [27, 28].

Most of the intestinal bacteria are not cultivable for the moment, tracking the special gene-labeled plasmid from the exogenous bacteria would help obtain the direct evidence of the horizontal transfer of

ARGs in the intestinal flora. There are only a few research reports applying the exogenous labeled bacteria to study the transfer of ARGs among intestinal bacteria [15–17]. One study labeled two *E. coli* strains isolated from human gut and poultry meat, and confirmed the transfer of exogenous plasmid to indigenous bacteria by *in vitro* intestinal model [15]. Another paper assessed whether the residual levels of tetracycline affect the integrity of epithelial cells through *in vitro* cultivation methods, using the translocation of *gfp* gene-labeled bacteria from the intestinal apical to basal compartment as an indicator [16]. The exogenous bacterium selected in this study was kindly presented by Professor Junwen Li and Zhigang Qiu, who investigated the colonization of this strain in the gut of aquatic model organism zebrafish [17]. In their study, after stopping the addition of exogenous labeled bacteria, the concentrations of exogenous strain and plasmid showed a downward trend, which was in agree with our results. In addition, another study applied a similar exogenous bacterium *Pseudomonas putida* KT2442 to explore the spread of ARGs in the soil microenvironment, which also found that the exogenous bacteria and plasmid reduced gradually while the conjugation transfer of the plasmid persistently happened [29]. However, there is no report that revealed the antibiotic selection pressure effects on the spread of exogenous labeled bacteria in human intestine. Therefore, this study for the first to evaluate the effects of gentamicin exposure on exogenous labeled bacteria and plasmid in the simulated human intestine, suggesting that antibiotic exposure may be beneficial to their colonization and promotion of the conjugation transfer.

Gentamicin treatment impacted the composition of community and their diversity

The SHIME model was stably operated in this study because the predominant phyla of *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* in the gut microbiome was previously demonstrated by Yu's group [30]. *Firmicutes* and *Bacteroidetes* are usually dominate in the microbiota of a healthy subject. However, *Proteobacteria* is majoritarian in the control samples in this study. For *in vivo* studies, highest percentage of *Proteobacteria* had also been observed in fecal samples from healthy human and animals [31, 32]. Our previous *in vitro* study also discovered this phenomenon [33, 34]. At the genus level, gentamicin exposure significantly increased the abundance of *Bacteroides*, while the abundance of *Klebsiella* and *Pseudomonas* decreased. Gentamicin is one of the most commonly used aminoglycoside antibiotics especially for infections caused by gram-negative bacteria, which can bind the bacterial ribosomal 30S subunit and block bacterial protein synthesis [12]. Greenwood and associates investigated the effects of gentamicin on the gut microbiota of premature infants and indicated that gentamicin caused a significant increase in the abundance of *Enterobacter* [35]. Staley and associates revealed that intramuscular injection of gentamicin had no impact on intestinal flora [36]. Other studies have even shown that the abundances of *Bacteroides* and *Eubacterium* were significantly reduced after gentamicin exposure [37, 38]. Our study results were not consistent with these conclusions, which may due to the different subjects and administration route. In our previous study, amoxicillin or vancomycin exposure was found to significantly increase the abundance of the opportunistic pathogen *Klebsiella* [33, 34]. These inconsistent results may attribute to the different mechanisms of antibiotics and the ARGs carried

by different kinds of intestinal bacteria [12, 39, 40]. Therefore, when different type of antibiotic was administrated, the survival or growth status of intestinal bacteria would be different.

This study also indicated that gentamicin exposure significantly reduces microbial diversity. As demonstrated by several studies that administration of antibiotics is significantly associated with decrease in microbial community diversity and richness [41], one study also confirmed that gentamicin reduced the diversity of intestinal microflora of preterm infants [35]. These conclusions may support the results of this study. However, in the previous studies of our research group, amoxicillin was found to increase microbial diversity while vancomycin had little effect on microbial diversity [33, 34]. Some studies have also found that exposure to β -lactam antibiotics does not have a significant effect on microbiome diversity [42–44]. These different results may also stem from the different mechanisms of different type of antibiotic administrated.

Antibiotic effects on the genetic level may attribute to microbiota shift

Through functional prediction analysis and high-throughput-qPCR detection, this study discovered that the relative abundance of ARGs was increasing after gentamicin exposure while that of human disease-related pathways is significantly reduced. Consistent with the changes in the flora richness, the gene abundance of ARGs and human disease-related pathways in different samples also showed a certain trend of change. This phenomenon shows that the results observed at the group level can also be traced back to genomic and metabolic levels, which is consistent with our previous reports [45, 46]. Some studies have shown that taking gentamicin would promote the spread of drug-resistant bacteria and ARGs [13, 14]. This conclusion may explain the increase in the relative abundance of ARGs found in this study. Our earlier research found that amoxicillin would also promote the increase of the relative abundance of ARGs, while vancomycin exposure reduced them [45, 46]. For disease-related genes, there is no literature have discovered their decrease after taking gentamicin. However, in the previous studies, it has been confirmed that amoxicillin or vancomycin exposure would increase of these genes [45, 46]. These findings are contrary to the conclusion of this study, which may be derived from the composition changes of the gut flora.

The more important finding in this study is that there were strong correlations between the abundance of significant changed floras after gentamicin exposure and ARGs or human disease-related pathways. This finding suggested that the shifts in the flora caused by antibiotic exposure may contribute to the changes of these functional gene. Genes associated with reduced human disease-related pathways after gentamicin exposure were significantly positively correlated with the abundance of *Klebsiella*, and tetracycline or aminoglycoside resistance genes were positively associated with *Bacteroides*, the significantly increased bacterial genus after gentamicin exposure. The phenomena of these correlations were also consistent with our previous studies [45, 46]. *Klebsiella* is a symbiotic microorganism in human gut, but the increase of the relative abundance of intestinal *Klebsiella* genus has been reported to associate with diverse human diseases such as pneumonia, inflammation, Crohn's disease, colitis,

cystitis, liver abscess, and wound infections [47, 48]. Moreover, pathways related to human diseases (such as cancer, infectious diseases, metabolic diseases, and neurosurgical diseases) are positively correlated with the abundance of *Klebsiella* found in this study were also supported by several other studies [49–53]. For *Bacteroides*, a common probiotic, the decrease of this kind of probiotic may cause dysbiosis of gut microbiota and leads to human health problem [54–57]. There are some other studies showed that *Bacteroides* intrinsically carries tetracycline and aminoglycoside resistance genes [58–60]. All above research conclusions provide possible explanations to the conclusions above.

Perspectives

The findings in this study suggested several numbers of opportunities for additional study. First, expansion of the analysis to incorporate multiple-omics approaches of the metagenome, metatranscriptome, and metabolome would help us confirm the genome composition and expression of the gut microbiota and understand how antibiotic affected human disease and drug resistance. It is also of interest to inoculate the microbiota mixture from several patients to discover the different effects of antibiotic between healthy and sick individuals. Further *in vivo* studies aimed to verify whether the findings in this *in vitro* study reflect the reality would also be interesting, which will provide new insights to measure how antibiotic affects the gut microbiota and the associated disease.

Conclusion

This study indicated that exposure to gentamicin may be conducive to the colonization of exogenous bacteria and plasmid, as well as the conjugation of plasmid to gut microbiota. Gentamicin exposure was also confirmed to reduce the gene numbers of human disease-related pathways and promote the drug resistance in the gut microbiota. The effects on the genetic level might attribute to microbiota shift, which were explained well by the phenomenon that *Bacteroides* was positively associated with the ARGs and *Klebsiella* was positively related to the human disease-related pathways. These results might be valuable to direct the future work and opened up new perspectives to address the direct effects of antibiotics on the intestinal microbiota.

Methods

Exogenous bacterium and culture condition

Escherichia coli K12Td-Tomato:RK2(EGFP) was the exogenous bacterium used in this study with the Td-Tomato gene genetically integrated into the genome and the Enhanced-gfp (*Egfp*) gene inserted into the plasmid [17]. This exogenous bacterium was kindly presented by Professor Junwen Li and Zhigang Qiu, Academy of Military Sciences PLA China. This exogenous bacterium was grown aerobically under 180 rpm at 37 °C overnight by shaking of inoculated Luria-Bertani (LB) broth cultures supplemented with 50 mg/L apramycin. The bacterial cells were harvested by centrifugation at 8500 g for 15 min and the cell pellets were washed twice and re-suspended in 0.9% sterile saline solution, the concentration of inoculants was measured by colony-forming unit (CFU) counting [29].

Antibiotic treatment and samples collection

In this study, the SHIME was constructed using five double-jacketed reactors designated as the stomach, small intestine, ascending colon, transverse colon, and descending colon, respectively (Fig. S1). The last three reactors were inoculated with a mixture of fecal microbiota from one healthy adult volunteer, who did not suffer by any gastrointestinal disease or take antibiotics in the past six months, on account of the differences between individuals may be alleviated by same culture condition [61]. During the first three weeks of the experiment, nutritional medium was added to the reactors to stabilize the microbial community. After this period, nutritional medium + 10^9 CFU/ml K12Td-Tomato:RK2(EGFP) was added to the SHIME for one day, then nutritional medium was added for six days. The SHIME was sequentially exposed to nutritional medium + 300 mg/day gentamycin for one week, and nutritional medium + 2400 mg/day inulin for two weeks. The details of the SHIME system and the startup process are summarized in the Supplementary material.

Liquid samples (mixtures of fecal microbiota with SHIME feed) were collected from simulated ascending colon, transverse colon and descending colon vessels at nine time points, as detailed in Fig. S1. Based on the situation of antibiotic or inulin exposure, these samples can be classified into three groups. Specifically, the first group of samples were collected after K12Td-Tomato:RK2EGFP was added to the SHIME for one day (Con_A1, Con_T1, and Con_D1 from ascending colon, transverse colon and descending colon, respectively), for three days (Con_A2, Con_T2, and Con_D2) and before the gentamycin administration (Con_A3, Con_T3, and Con_D3). The second group sampled after the administration of gentamycin for one day (Gen_A1, Gen_T1, and Gen_D1), for three days (Gen_A2, Gen_T2, and Gen_D2) and seven days (Gen_A3, Gen_T3, and Gen_D3). Finally, the third group of samples were collected after the administration of inulin for three days (Inu_A1, Inu_T1, and Inu_D1), for one week (Inu_A2, Inu_T2, and Inu_D2) and two weeks (Inu_A3, Inu_T3, and Inu_D3). Each sample is a mixture of three samples collected at specific time intervals in a day. The samples were stored at -80°C for further analyses.

Quantitative real-time PCR (qPCR)

Total DNA was extracted from the samples using the E.Z.N.A. stool DNA Kit (Omega, USA) according to the manufacturer's protocols. The concentrations of exogenous strain and plasmid were measured by quantification of *Td-Tomato* and *Egfp* genes, respectively. 16S rRNA was used as the internal standard gene, which indicated presence of both cultivable and uncultivable bacteria. The data for *Td-Tomato* and *Egfp* genes were obtained from the ratio of targeted genes (copies) to the 16S rRNA gene (copies). Real-time PCR assays were performed in triplicate using thermal cycler (CFX96, BioRad Inc., USA) and the SYBR Green system. The details of DNA extraction and qPCR procedures are described in the Supplementary material.

16S rRNA gene sequencing and analysis

The V3-V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR). The raw reads of the sequences were deposited into the NCBI Sequence Read Archive (SRA) database under

the accession number SRR11945447-SRR11945455. The raw Illumina fastq files were de-multiplexed, quality-filtered, and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) [62]. The 16S rRNA gene sequences were further taxonomically classified using the Ribosomal Database Project (RDP) classifier 2.0.1 [63].

The effects of antibiotics on alpha diversity, including the taxon richness (Chao1 index), evenness (Simpson index), and diversity (Shannon index), were calculated for all the samples as previous did [33, 64]. In addition, beta diversity of the microbiota communities at baseline and after antibiotics were portrayed by nonmetric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) of weighted and unweighted UniFrac distances [65]. Linear discriminant analysis effect size (LEfSe) was performed to determine bacterial taxa that were significantly differed between the six groups using the Galaxy application tool [66]. Functional predictions of microbial community were performed to visualize the distribution of human disease-related pathways in the six groups using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [67]. The accuracy of PICRUSt for the detection of more challenging functional groups was good (min. accuracy = 0.82), suggesting that their inference of gene abundance across various types of functions was reliable, and PICRUSt predictions had high agreement with metagenome sample abundances across all body sites (Spearman $r = 0.82$, $P < 0.001$). These analyses were conducted by Guangzhou Gene Denovo Co., Ltd (Guangzhou, China). The details of 16 s rRNA gene amplification and sequencing, taxonomical classification, LEfSe analysis, and functional predictions are described in the Supplementary material.

High-throughput quantitative PCR (HT-qPCR) and analysis

High-throughput-qPCR reactions were performed using the Wafergen SmartChip Real-time PCR system as previous did [33, 64]. The reactions were conducted by Anhui MicroAnaly Gene Technologies Co., Ltd (Anhui, China). A total of 108 primer sets were chosen (Excel S1), which included 102 primer sets to target the almost all major classes of antibiotic resistance genes (ARGs) found in the microbiota of Chinese human gut [68], along with five mobile genetic elements (MGEs) and one 16S rRNA gene. The results were analyzed using the SmartChip qPCR Software. Data with multiple melting peaks or amplification beyond the range (0) were excluded and then screened with conditions that a threshold cycle (CT) must be < 31 , and positive samples should have three replicates simultaneously. The details of HT-qPCR analysis are described in the Supplementary material.

Data analysis

All the results were expressed as mean values and standard deviations. The statistical analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, Ill., U.S.A.). The T-test was conducted to compare the differences between the groups, and all the statistical tests were two-tailed. The statistical significance was set at three different levels ($*P < 0.05$, $**P < 0.01$, and $***P < 0.001$). Correlations between the microbiota and human disease-related pathways or ARGs were analyzed using the Spearman test in R with the 'vegan' package. The correlations between the pairs of variables were considered to be

significant at $r > 0.6$, and P values were < 0.05 . The Gephi (V 0.9.1) software was used to visualize the bipartite network graphs using the Force Atlas algorithm.

Declarations

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Availability of data and materials

The datasets and scripts developed and generated in this manuscript are included within the manuscript and its supporting files.

Consent for publication

Not applicable.

Authors' contributions

Lei Liu: Methodology, Investigation, Formal analysis, Writing - original draft, Software. Hongmei Qi: Investigation. Pengcheng Suo: Investigation. Huai Lin: Investigation. Siyi Wang: Investigation. Xiaojun Zuo: Funding acquisition, Writing - review & editing.

Ethics approval and consent to participate

The study was approved by the Biomedical Ethics Committees of Nankai University. The participant has given written, informed consent to understand the study's purpose, procedures, risks, benefits, and rights.

Competing interest

The authors declare that they have no competing interests.

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Figures

Figure 1

The decay of the exogenous donor bacterium and plasmid during incubation in ascending colon (a), transverse colon (b), and descending colon (c), respectively. Log values of the ratios of Td-Tomato, Egfp genes in each sample to 16S rRNA genes were shown on the left Y-axis. The right Y-axis showed the ratios of plasmid RK2 to donor.

Figure 2

Heatmap of antibiotic resistance genes (ARGs) in the three groups. Heatmap colors reflect relative abundance of ARGs from low (blue) to high (red).

Figure 3

Heatmap of human disease-related pathways in the three groups. Heatmap colors reflect gene numbers of human disease-related pathways from low (blue) to high (red).

Figure 4

Composition of microbial community at phylum (a) and genus level (b).

Figure 5

Network analysis revealing the co-occurrence patterns between microbial taxa and ARG subtypes. The nodes in Network were colored according to ARG types. The edges were colored according to positive (red) or negative (blue) correlation. A connection represents strong and significant (P value < 0.05, r > 0.6) correlation. The size of each node is proportional to the number of connections, that is, degree.

Figure 6

Network analysis revealing the co-occurrence patterns between microbial taxa and human disease-related pathways. The nodes in Network were colored according to human disease-related pathways types. The edges were colored according to positive (red) or negative (blue) correlation. A connection represents strong and significant (P value < 0.05 , $r > 0.6$) correlation. The size of each node is proportional to the number of connections, that is, degree.

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