Effects of pulsed in-feed antimicrobials on chicken fecal resistome

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Research

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Abstract

Background: Antimicrobial resistance has become a global problem that poses great threats to human health. Antimicrobials are widely used in broiler chicken production and consequently affect the fecal resistome. Here we used metagenomic approach to investigate the effects of pulsed antimicrobial administration on chicken fecal resistome. Chickens received three 5-day-course of alone/combined antimicrobials at therapeutic dose, including amoxicillin, chlortetracycline and florfenicol. Chicken feces was collected on T0 (before treatment), T5 (after first treatment), T10 (after second treatment), T15 (after third treatment) at the chicken age of 40 days that are ready for slaughter.

Results: Tetracycline resistance gene and polymyxin resistance gene only responded to chlortetracycline and amoxicillin, respectively. The tetracycline resistance gene tetC significantly increased from T0 to T15 in chlortetracycline group. The multidrug resistance gene acrB, acrF, mdtA, mdtK and CRP only occurred significant increase in amoxicillin group ($P<0.05$). Klebsiella significantly decreased from T0 to T10 in florfenicol group, while significantly increased from T0 to T15 in chlortetracycline group ($P<0.05$). Amoxicillin did not lead significant change to Klebsiella. Escherichia was always the major host for multidrug resistance genes. ARG-harboring Escherichia increased mainly due to the antimicrobial selection for β-lactam, tetracycline, chloramphenicol resistance genes they harbored.

Conclusions: The results indicated that the effects induced by amoxicillin, chlortetracycline and florfenicol significantly shaped the fecal microbiome and resistome. We provided a comprehensive insight into antimicrobial-mediated alteration of chicken fecal microbiome and resistome. These findings will give suggests to veterinarian for combating antimicrobial resistance in broiler chicken production.

Introduction

The overuse or misuse of antimicrobials in medical treatment, veterinary or agriculture bring about the continuous release of antimicrobials into the natural environment and undoubtedly promoted the widespread of antimicrobial resistance. Antimicrobial resistance genes (ARGs), for instance, the recent emergence of tetX (plasmid-mediated tigecycline resistance genes) and MCR-1 (plasmid-mediated colistin resistance) [1, 2], have caused worldwide attention due to the significant implications for human health [3].

Abundant ARGs have been continually detected in different natural environment such as lake water [4], soil [5], wastewater treatment plants [6] and coastal industrial mariculture systems [7]. ARGs can persist in the natural environment in which the antimicrobial selective pressure has already disappeared [8]. Although the phenomenon of antimicrobial resistance is considered to be natural and immemorial, earlier than the modern selection pressure of practical use of antimicrobials [9], the high level and prevalence of antimicrobial resistance found so far is generally considered to be a modern phenomenon caused by human activities. Enormous quantity of antimicrobial resistance genes harbored by animal fecal

microflora can be transported into the environment settings via land application [10], leakage [11], fecal pollution [12].

The use of antimicrobials in animal industry is a contributor for the antimicrobial resistance crisis. Antimicrobial administration leads profound effects on indigenous microbes of animal feces, causing changes in microbial community structure and gut resistome. The use of antimicrobials in animal production can increase the richness and diversity of antimicrobial resistance genes in animal feces. Animal intestinal tract contains extremely complex and dense microbial communities, and the ability of coding genes like ARGs is at least 100 times greater than that of human genome [13].

The world is facing the challenge that feeds the world's increasing population of 9.7 billion in 2050 [14]. Sustainable meat production is prior to offering a protein source that is safe and high-grade for human consumption. The modern large-scale effected farming systems rely on antimicrobials to prevent and treat animal disease, and to enhance growth performance. It is estimated that the global consumption of antimicrobials in agricultural field will increase by 67% from 2010 to 2030 [15]. China, one of the largest consumers of antimicrobials in the world, applies a large proportion of antimicrobials in animal disease treatment and growth promotion. A previous report revealed that about 97,000 tons of antimicrobials which accounted for almost half of the annual antimicrobial production in China went into the animal industry [16]. Approximately 30–90% of the antimicrobials fed to animals can be excreted by feces or urine as parent compounds or metabolites [17].

Poultry farming is the most widely adopted animal farming practice in many developing countries including China. China is the world's second largest poultry producer and consumer [18, 19]. Amoxicillin, chlortetracycline and florfenicol are the most commonly used antimicrobials in livestock production for treating the disease such as pullorum disease with a treatment period of 3 to 5 days [20–22]. However, the changes in microbial community and antimicrobial resistance in the gastrointestinal tract of poultry and the resulting feces remain largely unknown, even under pulsed antimicrobial treatment. A broader view of the co-occurrence of the ARGs and bacterial hosts in the production chicken's feces would help evaluate this risk to the local environment and the human health when the poultry manure is discarded into the environment or applied in the agricultural industry.

Here we examined the changes of antimicrobial-induced fecal microbial structures and the bacterial resistome under the three 5-day-courses in pulsed administration of amoxicillin, chlortetracycline and florfenicol. Using the metagenomic sequencing approach, we conducted a comprehensive profiling of the changes in chicken fecal bacterial community, the ARG variations, and bacterial host abundance.

Methods

Chicken and antimicrobial exposure

Thirty 15-day-old chickens, obtained from the same batch which were raised under the standard commercial conditions with no history of antimicrobial use (Guangdong Wens Dahuanong Biotechnology

Co., Ltd., China), were adopted in the Laboratory Animal Center of South China Agricultural University. The chickens with identical characteristic of body weight and sex were adapted for five days prior to antimicrobial administration. The chickens were separated into five groups of three replicates. Each replicate had two chickens. Each chicken was raised in a cage separately and obtained the same supplement of food and water according to the standard commercial production conditions. One group set as the control group received no antimicrobial, while the other antimicrobial-treated groups received three 5-day-course of antimicrobial at therapeutic dose at age 15-20 day, 25-30 day, 35-40 day, including amoxicillin, chlortetracycline, florfenicol, and mixed (sequential courses of amoxicillin, chlortetracycline, florfenicol), respectively (Figure 1). The feces were cleaned every day and were collected on T0 (first time, before first treatment), T5 (second time, after first treatment), T10 (third time, after second treatment), T15 (forth time, after third treatment). The fresh feces from each cage was individually collected into sterilized tube by using a sterilized spoon, followed by immediately transferring to laboratory for processing.

DNA extraction

Feces from each replicate were mixed and homogenized. DNA from each replicate was extracted using MoBio PowerSoil DNA isolation kit following the protocol from the manufacturer (MoBio Laboratories, Carlsbad, CA, USA). Total DNA concentration and purity were determined by UV spectroscopy using a NanoDrop ND-2000 instrument (NanoDrop Technologies, Wilmington, DE, USA).

Metagenomic sequencing

Each fecal DNA was sequenced on Illumina Hiseq 4000 platform using a strategy of Index 150 PE (paired-end sequencing). We had removed primers and the sequences with ambiguous bases, and filtered low quality reads.

Antimicrobial resistance gene prediction

The reads got in this study were shown in Supplementary Table S1. After quality filtering, the clean reads were *de novo* assembled using the CLC Genomics Workbench (version 10.01, Aarhus, Denmark) with the default k-mer size. The prediction of open reading frames (ORFs) was conducted using Prodigal. The average coverage was calculated by mapping metagenomic reads to the ORFs with a minimum similarity of 95% over 95% of the read length. The nucleotide sequences of the predicted ORFs were searched against nonredundant structured Clean deepARG database for the identification of the ARG-like ORFs using BLASTP under an *E* value $\leq 10^{10}$ [23]. An ORF sequence with best BLASTP alignment to ARG sequences cutoff of $\geq$ 80% similarity and $\geq$ 70% query coverage was regarded as an ARG-like ORF.

Taxonomic classification

The predicted protein sequences of ORFs within the ARG-carrying contigs were annotated against NCBI NR database using BLASTP at an *E* value $\leq 10^{-5}$. The assignment of taxonomic genus was conducted by

annotating the search results *via* MEGAN6. The assignment of taxon required the portion of ORFs which were classified into the same taxon more than 50%. We submitted the sequences of ARGs-carrying contigs to NCBI by using BLASTN and obtained taxonomy information at the genus level.

Statistical analysis

The abundance of a particular ARG type, indicated by coverage (in units of "times per Giga base", ×/Gb), was calculated by summing the coverages of ARG-like ORFs belonging to that ARG type. Statistical comparisons were performed using nonparametric Kruskal-Wallis tests. A *P* value of < 0.05 was regarded as statistically significant.

Results

Antimicrobial resistance genes

A total of 325 ARG subtypes belonging to 21 ARG types were identified. The resistance genes for multidrug (40%), aminoglycoside (8.9%), polymyxin (7.4%), aminocoumarin (7.4%), and tetracycline (6.9%) were the 5 predominant ARG types (Supplementary Figure S1). The detected resistance genes represented all major resistance mechanisms including antimicrobial deactivation, efflux pumps and cellular protection. The total coverage found in the control group ranged from 2506-6224 ×/Gb, indicating that the chicken feces even though without antimicrobial treatment is the hotspot of ARGs (Supplementary Table S2). The coverage found in the four antimicrobial-treated groups was significantly higher than control group (*P*=0.00, n=60) (Figure 2). Tetracycline resistance gene significantly increased on T15 compared with T5 in chlortetracycline group (*P*=0.031, n=12), and polymyxin resistance gene significantly increased on T5 compared with T0 in amoxicillin group (*P*=0.039, n=12). Both florfenicol and chlortetracycline significantly increased the abundance of aminoglycoside resistance gene (*P* =0.013, n=12). Out of these detected ARG types, we found that 8 ARG types including bacitracin, β-lactam, fosfomycin, glycopeptide, macrolide-lincosamide streptogramin, streptothricin, triclosan resistance genes did not occur any significant change under either of the above antimicrobials administration. We further analyzed the pearson relationship between the abundance of 21 ARG types and the total coverage (Supplementary Table S3). As the most predominant ARG type in the feces, the high correlation (r^2 =0.81-0.98, *P*<0.05) of multidrug resistance gene with total coverage indicated the important role of multidrug resistance gene under the antimicrobial administration (Figure 2).

We further analyzed the ARG subtype variations to quantitatively compare the effects of the antimicrobials on the fecal resistome (Figure 2, Supplementary Table S4). A total of 325 ARG subtypes were detected among the chicken feces. The tetracycline resistance gene *tetA* was the most abundant ARG subtype in the florfenicol, chlortetracycline, mixed group on T15, while sulfonamide resistance gene *sul2* was the predominant gene in amoxicillin group. The multidrug resistance gene *acrB*, *acrF*, *mdtA*, *mdtK* and *CRP* only occurred significant increase in amoxicillin group (*P*<0.05). Beside amoxicillin group, *mdfE* occurred significant increase in chlortetracycline group, while *mdfM* occurred significant decrease

in florfenicol group (P<0.05). *FloR* significantly increased in florfenicol group (P=0.03, n=12). The tetracycline resistance gene occurred significant change in abundance only under chlortetracycline treatment. At subtype level, the tetracycline resistance gene *tetC* significantly increased from T0 to T15 (P=0.013, n=12). Importantly, the *mcr-1* gene occurred as high frequencies and significantly increased on T15 compared with T0 (P=0.012, n=12) in mixed group. Antimicrobial pressure also led some emergence of ARG subtype. The gene resistance to multidrug, β-lactam, chloramphenicol, tetracycline accounted for 60% (44 of 74) of the total newly detected ARG subtype during the administration period (Supplementary Table S5). However, there was no specificity in the emergence induced by the antimicrobial treatment. The above antimicrobials could not only result in the emergence of the respective antimicrobial resistance gene, but also the other types of resistance gene. For example, the antimicrobial pressure caused by florfenicol generated some chloramphenicol resistance genes (*catB10*) and the ARG subtypes belong to the rest of the detected ARGs.

Bacterial community

The change of ARGs is correlated with the structure and composition of the bacterial community [24]. We analyzed bacterial community structure using metagenomic sequence data. The four predominant taxonomic phyla in fecal microbiota of broiler chickens were *Proteobacteria* (70%), *Bacteroidetes* (15%), *Firmicutes* (11%), *Actinobacteria* (0.030%) (Figure 3, Supplementary Table S6).

The antimicrobials had profound influence on the bacterial community structure of the broiler chicken feces. This result was confirmed by the PCA in which PC1 accounted for 81% of the variations between samples (Supplementary Figure S2). We found that the significant compositional shifts of the two primary predominant taxonomic phyla *Proteobacteria* and *Bacteroidetes* in these antimicrobial-treated groups occurred after the second time of antimicrobial administration (Figure 3). Specific changes in the microbial community under amoxicillin, chlortetracycline, florfenicol treatment included the decreases (from 51%, 18%, 15% on T0 to 4.2%, 1.4%, 4.9% on T10, respectively) in the abundance of *Bacteroidetes* compared with the control group (P = 0.015, 0.026, 0.020, n= 33, respectively). On the contrary, the above antimicrobials led significant increases (from 37%, 67%, 60% on T0 to 84%, 83%, 86% on T10) in abundance of *Proteobacteria* compared with the control group (P = 0.022, 0.027, 0.009, n= 33, respectively). Due to the compound effect, doubtlessly, the mixed group led the *Proteobacteria* significantly increase from 50% on T0 to 90% on T10 (P= 0.009, n = 33), while the *Bacteroidetes* significantly decrease from 30% on T0 to 1.4% on T10 (P = 0.015, n = 33) (Figure 3). Furthermore, we characterized the difference of fecal microbiota between groups and discovered indicator taxa by using LDA Effect Size (LEfSe) (Figure 4). The predominant taxa *Proteobacteria* and *Bacteroidetes* were the main responders which confirmed by the LDA score (LDA>4, P<0.05). This indicated that a few key members of the community could be the drivers of community dynamics.

We further analyzed the microbial communities on genus level. *Escherichia* (68%) and *Bacteroides* (15%) were the two predominant genera in the chicken feces (Supplementary Table S7 and Figure S3). Although amoxicillin, chlortetracycline and florfenicol are against both Gram-positive and Gram-negative bacteria,

we found that there were some common points and different points in the effects caused by these antimicrobials on the genus community. On the common side, the variable trend of the two predominant genera mediated by the antimicrobials was the major contributor to the changes of taxonomic phyla of *Proteobacteria* and *Bacteroidetes* in the antimicrobial-treated groups observed above. As the predominant genus of *Proteobacteria*, *Escherichia* significantly increased in amoxicillin group (from 36% on T0 to 83% on T10, $P = 0.019$, n= 12), chlortetracycline group (from 45% on T5 to 80% on T10, $P = 0.013$, n=12), florfenicol group (from 60% on T5 to 85% on T10, $P = 0.013$, n = 12), mixed group (from 49% on T0 to 88% on T10, $P = 0.013$, n=12) (Supplementary Figure S4). In contrast with *Escherichia*, *Bacteroides*, the predominant genus of *Bacteroidetes*, significantly decreased in amoxicillin group (from 51% on T0 to 4.0% on T10, $P = 0.013$, n= 12), chlortetracycline group (from 40% on T0 to 1.4% on T10, $P = 0.013$, n=12), florfenicol group (from 15% on T0 to 5.0% on T10, $P = 0.013$, n = 12), mixed group (from 30% on T0 to 1.4% on T10, $P = 0.013$, n=12) (Figure 5). On the different side, we found that genus which accounted for more than 1% responded quite different to the antimicrobials. For example, *Klebsiella* significantly decreased from 2.7% on T0 to 0.15% on T10 in florfenicol group (P=0.028, n=12), while significantly increased from 0.67% on T0 to 3.7% on T15 in chlortetracycline group (P=0.028, n=12). However, amoxicillin did not lead significant change of *Klebsiella*. Furthermore, the distances and variations of genus among all the samples were visualized by principal component analysis (PCA) which PC1 accounted for 80% of the variations between samples, demonstrating similar microbial community composition (Figure 5). The antimicrobial-treated groups clustered clearly apart from the control group, indicating that therapeutic dose of the antimicrobials played important roles in shaping genus compositions in chicken feces.

Bacterial hosts of antimicrobial resistance genes

We annotated 10272 ORFs as ARG-like ORFs that were located in 7234 contigs (Supplementary Table S8). Understanding the composition of the bacterial host of ARGs is conducive to assess the impact induced by the antimicrobial on the chicken feces. Throughout our experiment, the genera harboring most of the ARGs detected were *Escherichia* (84%), followed by *Klebsiella* (5.1%), *Bacteroides* (3.9%), *Shigella* (2.9%), and *Clostridium* (1.7%) (Figure 6 and Supplementary Figure S5). Especially for *Escherichia*, the proportion of it in the antimicrobial resistance bacteria was 84%, indicating that *Escherichia* carried more resistance genes than other bacterial hosts. *Escherichia*, *Bacteroides*, *Shigella* harbored nearly all kinds of detected ARGs, including the genes resistant to multidrug, tetracycline, aminoglycoside, macrolide lincosamide streptogramin, β-lactam, and sulfonamides (Supplementary Figure S5). Especially for *Escherichia*, 93% of multidrug resistance genes, 91% of polymyxin resistance genes, 78% of tetracycline resistance genes, 61% of aminoglycoside resistance genes, 58% of β-lactam resistance genes, 86% of aminocoumarin resistance genes resided in *Escherichia*. During the experimental duration, *Escherichia* was always the major host for the multidrug resistance genes in all the antimicrobial-treated groups (Figure 6). We also found that some less abundant ARGs such as triclosan resistance genes mainly resided in *Pseudomonas* (100%) (Supplementary Table S9).

We found the results of ARG-harboring host also match the results in bacterial community. The major change under the antimicrobial administration pressure occurred at T10. In short, the ARG-harboring *Escherichia* increased at T10 in all the antimicrobial-treated groups, while *Bacteroides* decreased. These results can be explained by the ARGs they harbored. In amoxicillin group, the β-lactam resistance genes harbored by *Escherichia* increased from 22% on T0 to 77% on T10. In chlortetracycline group, the tetracycline resistance genes harbored by *Escherichia* increased from 6.0% on T5 to 97% on T10. In florfenicol group, the chloramphenicol resistance gene harbored by *Escherichia* increased from 0% on T0 to 100%. Interestingly, the host of chloramphenicol resistance genes changed from *Klebsiella* (100%) to *Escherichia* (100%) (Figure 6). In contrast, the β-lactam resistance genes, chloramphenicol resistance genes and the tetracycline resistance genes harbored by *Bacteroides* decreased after the second antimicrobial administration (Supplementary Table S10). Interestingly, we found that change in the mixed group led by the alternating treatment of the three antimicrobials seems like a combined result of antimicrobial-treated alone groups. For example, the major host for β-lactam resistance genes changed from *Bacteroides* on T0 into *Escherichia* on T10 when the mixed group had been feed with amoxicillin. The result found in the mixed group indicated effect caused by antimicrobials has accumulated consequence in shaping the ARG-harboring host composition.

Discussion

Here we defined effects of pulsed in-feed antimicrobials on chicken fecal resistome. Amoxicillin, chlortetracycline and florfenicol are the most common antimicrobials used in livestock industry for disease prevention and feed-efficiency improvement. Many antimicrobial resistance studies using traditional bacterial culture methods provided information about the current and emerging threat of antimicrobial resistance [25]. However, the limitations of the traditional methods impose restriction on the research of antimicrobial resistance at community-wide level [26]. Using high-throughput sequencing-based metagenomics approach, we firstly provided a comprehensive insight into the effects induced by pulsed antimicrobial treatment on chicken fecal resistome. We demonstrated that pulsed in-feed antimicrobials affected the composition of the fecal microbiota and aggravated the enrichment of particular ARGs. Antimicrobial-alter of microbial community was the dominant determinants of shaping the broiler fecal resistome.

Highly diverse and abundant ARGs were found in chicken fecal samples of the control and antimicrobial-treated groups. The finding of ARGs in control group supported the fact that the chicken feces are the reservoir of ARGs even in the absence of antimicrobial pressure. The genes conferring resistance to polymyxin, aminocoumarin, aminoglycoside, multidrug and tetracycline were the most abundant in the broiler feces. These abundant ARG types detected in the current study were also found in human feces [27], animal feces [28] and different kinds of environment settings such as river [29], landfill [30], wastewater [31]. Undoubtedly, the composition of top predominant ARGs were different from other kinds of animal such as mouse [13], swine [32], indicating the specificity of fecal resistome in animalia.

The coverage found in the antimicrobial-treated groups was significantly higher than control group, demonstrating that in-feed amoxicillin, chlortetracycline and florfenicol aggravated the enrichment of total ARGs in broiler feces. Similar results were described for fish where ARGs were enriched at least 4.5 times in the gut during florfenicol exposure [33]. More than that, the previous study had found that the total coverage of ARGs of chicken samples was significantly higher than that of human, ocean, pig and soil samples [34]. A small dosage of in-feed antimicrobials could not only increase the level of ARGs, but also pose a risk by selecting bacteria able to perform chemotaxis mechanism [35, 36]. The abundance of the most predominant multidrug resistance gene was strongly correlated with the total coverage in all the antimicrobial-treated groups, highlighting the impact of the antimicrobials in the enrichment of ARGs. Multidrug-resistant bacteria is one of the most threatening type of antimicrobial-resistant bacteria to public health, including superbug methicillin-resistant Staphylococcus aureus (MRSA), which is one of the most difficult to treat bacteria causing human infection [37]. Therefore, the multidrug resistance gene should be paid more attention to competing antimicrobial resistance.

The resistance genes found in our samples were not limited to the antimicrobials administered. Some types of resistance gene, for example, the aminoglycoside resistance gene increased in abundance with in-feed florfenicol and chlortetracycline, although they do not confer resistance to the antimicrobials therein. Similar result has also been found in pig where the abundance of aminoglycoside resistance gene increased with in-feed ASP250 which contained chlortetracycline, sulfamethazine, and penicillin [38]. The tetracycline resistance gene only occurred significant change in abundance under chlortetracycline treatment, while polymyxin resistance gene only occurred significant change under amoxicillin treatment. Moreover, this finding can also apply to ARG subtype level. The multidrug resistance gene mdtE occurred significant increase in the chlortetracycline group, while mdtM occurred significant decrease in the florfenicol group ($P < 0.05$). These findings indicated that particular antimicrobial resistance gene may have specific response to antimicrobial treatment due to co- and cross-selection. Our finding supported the theory that antimicrobial resistance has an indirect mechanism of selection on the in-feed antimicrobials [38].

It is believed that the influence of the in-feed antimicrobials on the fecal bacterial resistome was dependent on ARG subtype and not simply on the overall community level of ARG types [39]. The mcr-1, the gene resistant to colistin that is an antibiotic of last resorts for Gram-negative multidrug-resistant infections, was firstly found in pig in 2015 [40], we found its presence in all the groups. The high prevalence of mcr-1 detected in chicken feces poses a great risk to the use of colistin in poultry production. The increase in mixed group indicated the alternate use of antimicrobials may increase risk of mcr-1 propagation. The efflux pump gene tetA commonly changed into the most predominant ARG subtype in the antimicrobial-treated groups of florfenicol, chlortetracycline and mixed. The enrichment of tetA in florfenicol group may be related to the co-occurrence mechanisms. Florfenicol resistance can be produced by the gene floR, pp-flo, fexA, flo, cfrC, and poxtA [41, 42]. Out of all these genes, only two multidrug resistance genes floR and fexA were detected in florfenicol group. The floR gene significantly increased under the induce of florfenicol administration.

The previous studies had demonstrated that ARGs depended on the composition of the microbial community which could be an important conduit for transferring ARGs into the environment [43]. The three commonly used antimicrobials were administered continuously for three 5-days courses, constituting a continuous pulsed selective pressure rather than instantaneous disturbance to the microbiota. In this study, tracking the change of microbial community is central to estimate the impact induced by antimicrobials. Here, the four predominant taxonomic phyla in fecal microbiota of broiler chickens were Proteobacteria (70%), Bacteroidetes (15%), Firmicutes (11%), Actinobacteria (0.030%). However, our result was contrast with the previous study in which the three predominant phyla and their average abundances were Firmicutes (50%), Proteobacteria (37%), and Bacteroidetes (9.0%) for household chicken fecal samples, and Proteobacteria (65%), Firmicutes (17%) Bacteroidetes (16%) for production chicken fecal samples [18]. The most dramatic bacterial changes at phylum level were Proteobacteria and Bacteroidetes. The antimicrobials significantly affected the Proteobacteria and Bacteroidetes composition at the second pulsed antimicrobial treatment. LEfSe analysis further identified these two phyla as the bacterial biomarkers that responded to antimicrobials.

Although amoxicillin, chlortetracycline and florfenicol were against both Gram-positive and Gram-negative bacteria, the response of genus community to the in-feed antimicrobials may be different. Amoxicillin, chlortetracycline and florfenicol did not lead inhibition to Escherichia. The previous study had reported increase in E. coli prevalence in response to oral amoxicillin and chlortetracycline treatment in mammalian gut microbiota [44], indicating the antimicrobial effect on this genus is specific to the antimicrobial administered. We found that the significant increase of Proteobacteria in these antimicrobial-treated groups can be explained by the increase of Escherichia induced by the antimicrobial administration. Similarly, the decreased abundance of the phylum Bacteroidetes during the different phases of the experiment can be explained by the Bacteroides. However, the results also showed us the difference of effects caused by the antimicrobials on genus level. For example, Klebsiella significantly decreased from T0 to T10 in florfenicol group, while significantly increased from T0 to T15 in chlortetracycline group. However, amoxicillin did not lead significant change to Klebsiella. It should be noticed that the antimicrobials caused different collateral effects on microbial community. For example, the proportion of the genus Bacteroides significantly increased after ciprofloxacin administration in human study [45]. The human study found that the children have less stable communities after receiving 9 to 15 antimicrobial treatments in the first 3 years of life compared with the children never received antimicrobials [46]. According to our result, the pulsed administration of amoxicillin, chlortetracycline and florfenicol significantly decreased the genus Bacteroides which are generally associated with degradation of polysaccharides, especially starch and glucans and the formation of short-chain fatty acids [47]. These results provide precise administration strategy to treat Bacteroides-caused disease in poultry production.

Antimicrobial treatment corresponded with short-term instability of ARG-harboring bacterial host structure. One important consequence of antimicrobial usage is the change of distribution of ARG-harboring bacterial hosts. Longer samplings of the study may reveal more common long-term responses to antimicrobial therapy. Otherwise, the antimicrobial resistance genes occurred in diverse bacterial hosts

with different proportion. Consequently, the predominant antimicrobial resistance gene caused the host resistant to specific antimicrobial type. Take the chlortetracycline group as an example, we found that 40% of the gene resistant to tetracycline resided in Bacteroides, 53% resided in Lactobacillus on T5. At the termination of the second administration of chlortetracycline, the ARG-harboring Escherichia increased from 47–79%. It can be explained by the change of tetracycline resistant gene which 97% resided in Escherichia on T10. The increased tetracycline resistant gene resided in Escherichia made Escherichia survival under the continuous administration of chlortetracycline. The previous studies which performed the research on single antimicrobial or single administration limited the realization of the effect caused by antimicrobial use [48, 49]. This study administered continuously for three 5-days courses constituting a continuous pulse which is more similar with the practical application in poultry production, providing a wider perspective on influence of fecal bacterial community and fecal resistome caused by pulsed in-feed antimicrobials.

Conclusions

We used metagenomic approach to investigated changes in microbiome structure and variations of ARG contents responded to the pulsed in-feed antimicrobial administration. The result showed that three 5-day-course of the commonly used antimicrobials amoxicillin, chlortetracycline, florfenicol significantly influenced the fecal resistome and microbial community. These antimicrobials increased the total ARG level and the ARG-haboring bacterial hosts of Escherichia. The commercial broiler chickens are sold in food market at age about 40d. It means that the increased ARG levels caused by antimicrobials during the raising period will be stay when the broiler chicken sold. Further studies are needed to estimate the influence on human health and improve the strategy of antimicrobial use.

Abbreviations

ARGs: antibiotic resistance genes; OTU: operational taxonomic units.

Declarations

Ethics approval and consent to participate

Not applicable. All the samples originated from feces of the chickens.

Consent for publication

Not applicable.

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Availability of data and materials

The datasets supporting the conclusions of this article are available in the NCBI Sequence Read Archive (SRA) repository (accession no. PRJNA586747)

Authors' contributions

SYC analyzed the data and wrote the manuscript; JTY, JXZ analyzed the data and prepared figures; JH, RNZ collected samples; WGX and ZLZ conceived the study. All authors edited the manuscript and approved the final draft.

Competing interests

The authors declare that they have no competing interests.

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Figures

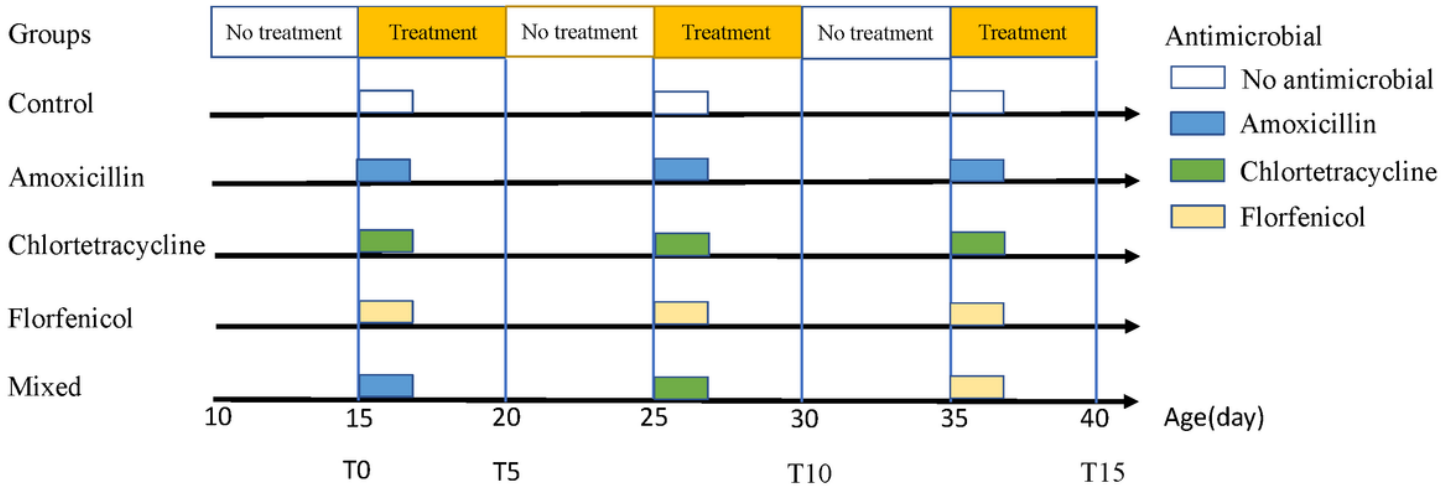

Figure 1

Animal groups with pulsed antimicrobial administration and sampling time points. The 30 broiler chickens were divided into five groups. Each group had three replicates. Three 5-day course of antimicrobials were pulsed administered at 0.06 g/L (amoxicillin, A), 0.1 g/L (chlortetracycline, T), 0.06g/L (florfenicol, F). One group (M) was administrated by above antimicrobials respectively. One group without antimicrobial administration was set as the control group (C). Fresh feces from each group were collected on T0 (day 15, before treatment), T5 (day20, after first treatment), T10 (day30, after second treatment) and T15 (day 40, after third treatment).

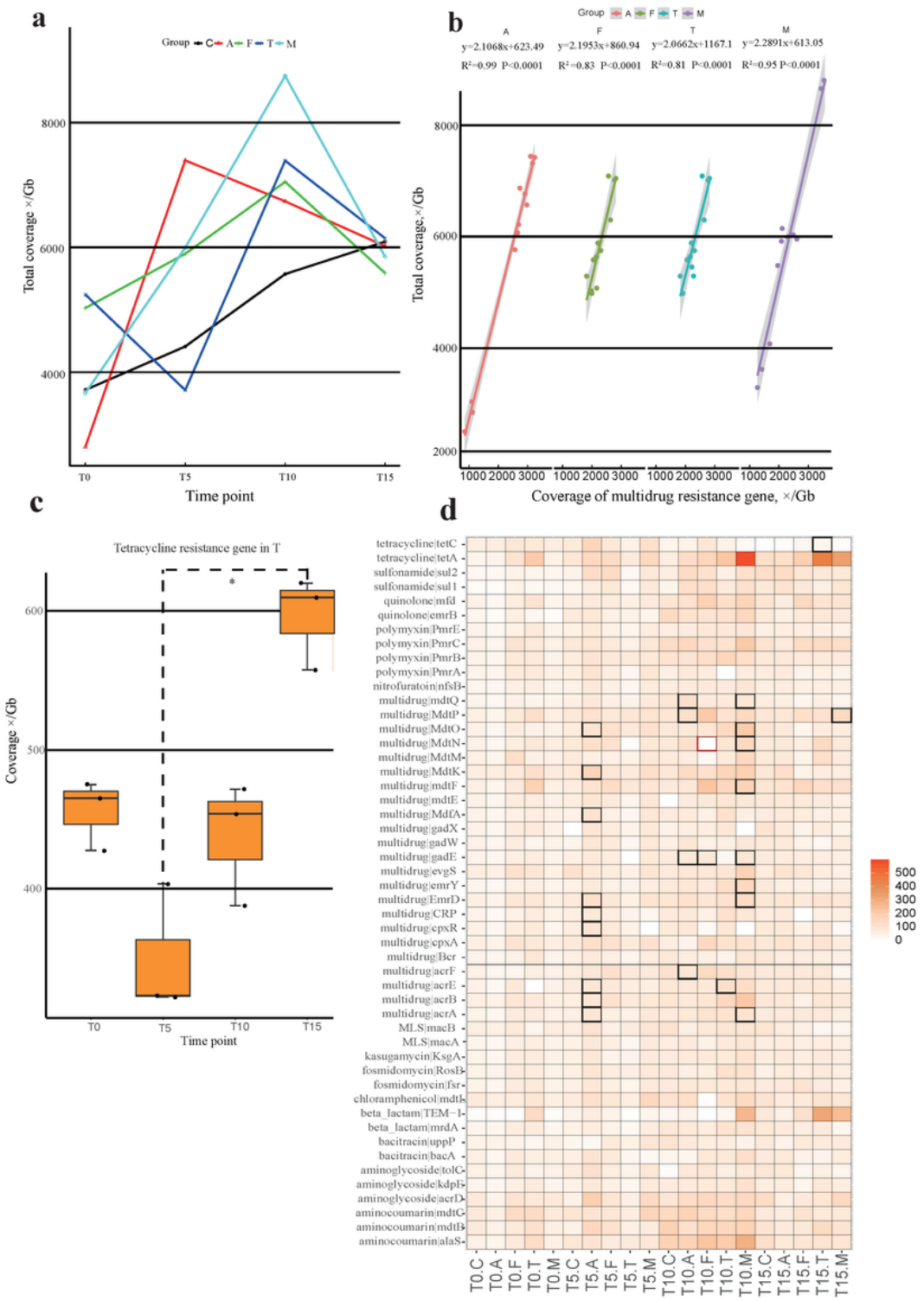

Figure 2

Variations of ARGs in different samples over the course of pulsed antimicrobial administration. a) Total coverage in different groups over time. b) The correlation between the abundance of multidrug resistance gene with total coverage in different groups. c) Changes in tetracycline resistance gene over time in the chlortetracycline group. d) Heatmap of variations of ARG subtypes based on the abundance of ARG

subtypes. Significant (P < 0.05) increases and decreases were shown by border lines of cells colored by black and red, respectively. ARG subtypes with an average abundance > 1% were shown.

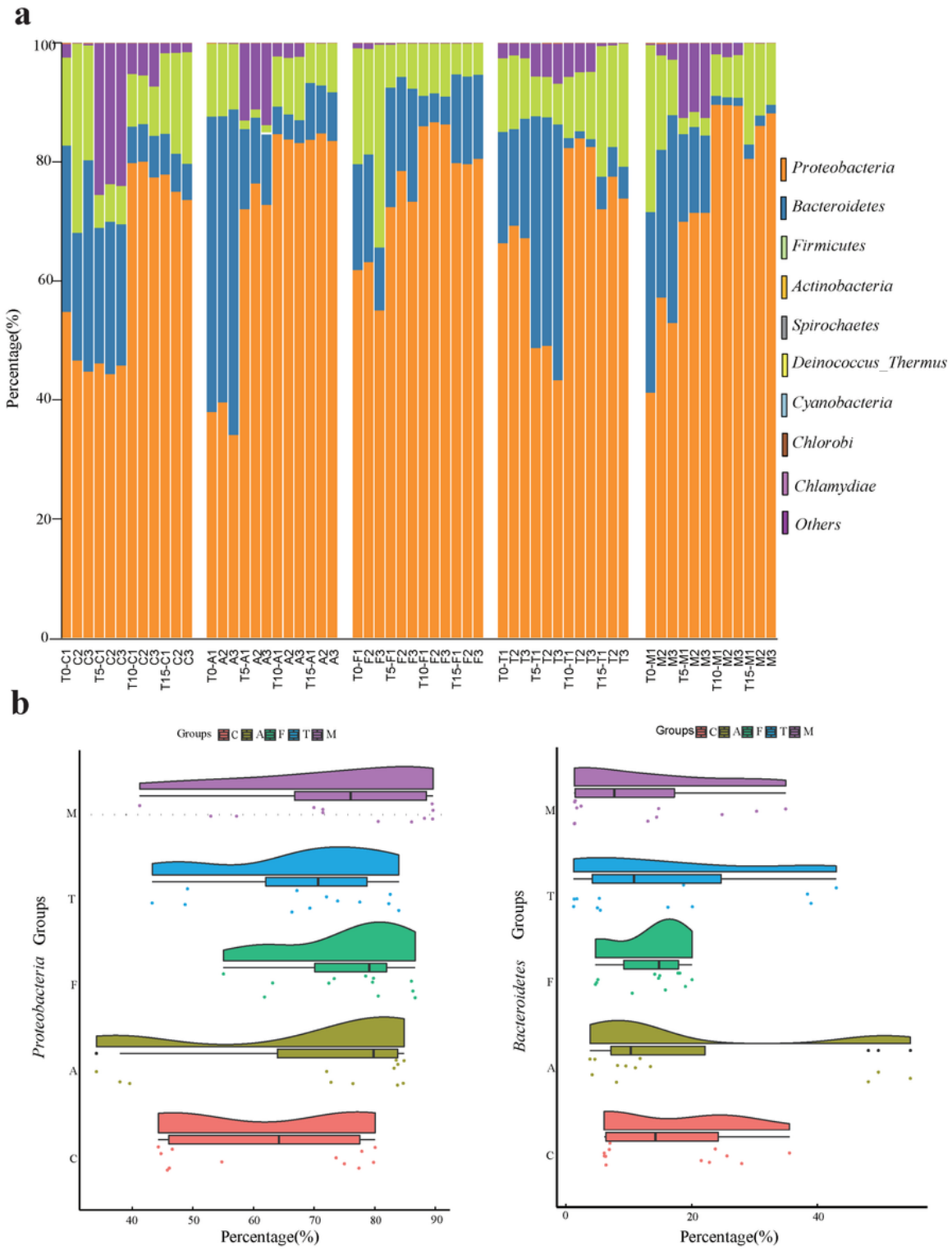

Figure 3

Changes in bacterial community structure. a) Changes in taxonomic phyla in the antimicrobial-treated groups compared with the control group. Others in Figure 3a meant the phylum which did not belong to Bacteria. b) Changes in taxonomic Proteobacteria and Bacteroidetes over time in the different groups.

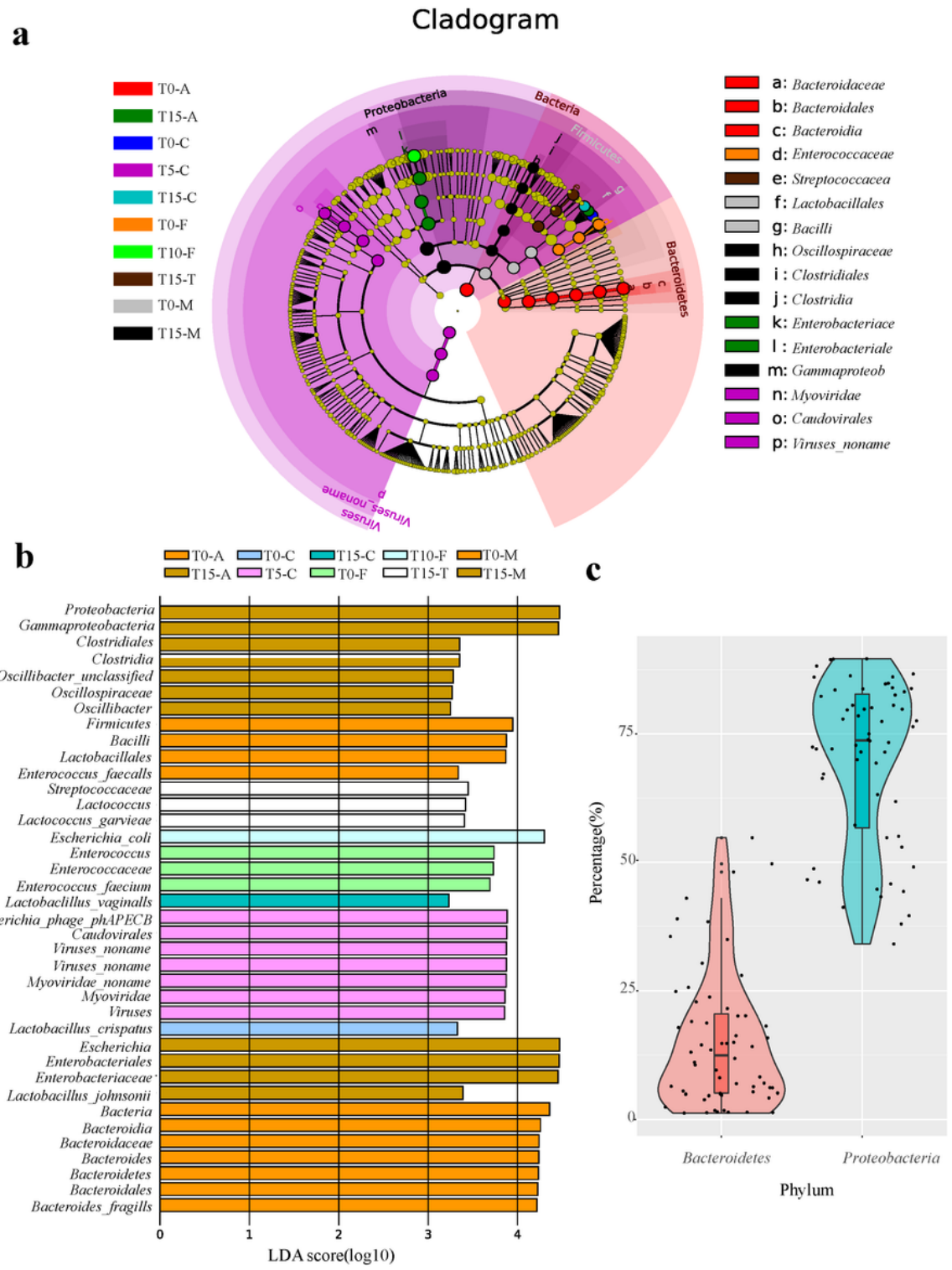

Figure 4

Main bacterial responders of the gut microbiome to pulsed antimicrobial administration. a) LDA score of the LEfSe analysis. b) Cladogram representing the bacterial biomarkers associated to the groups. The cutoff value of linear discriminant analysis >4.0. c) The distribution of Proteobacteria and Bacteroidetes in the groups.

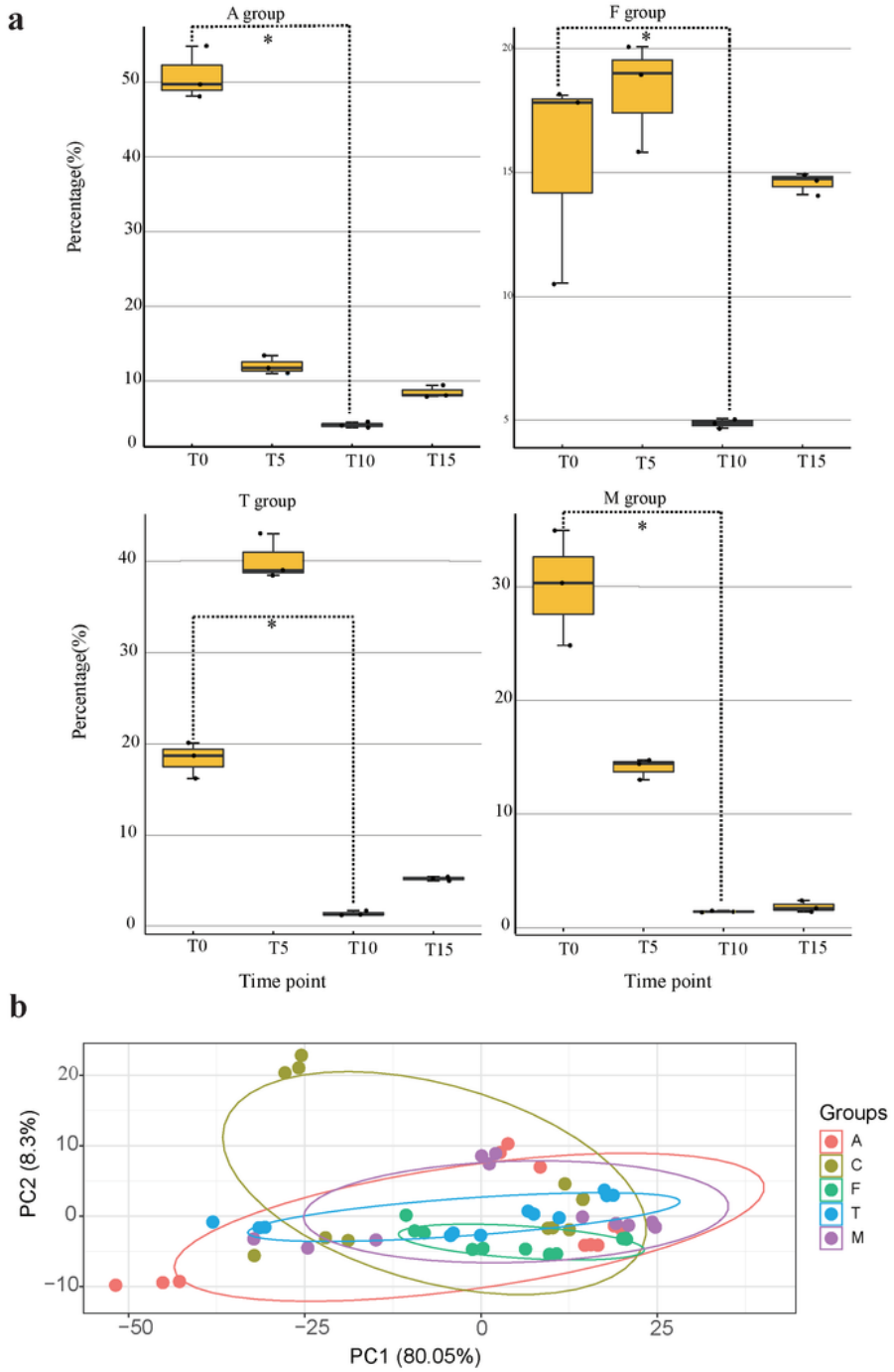

Figure 5

Changes in bacterial community structure at genus level. a) Changes in taxonomic Bacteroides over time in the antimicrobial-treated groups. b) Principal component analysis of taxonomic genera in the antimicrobial-treated groups and in the control group. A, the amoxicillin group. C, the control group. F, the florfenicol group. T, the chlortetracycline group. M, the mixed group.

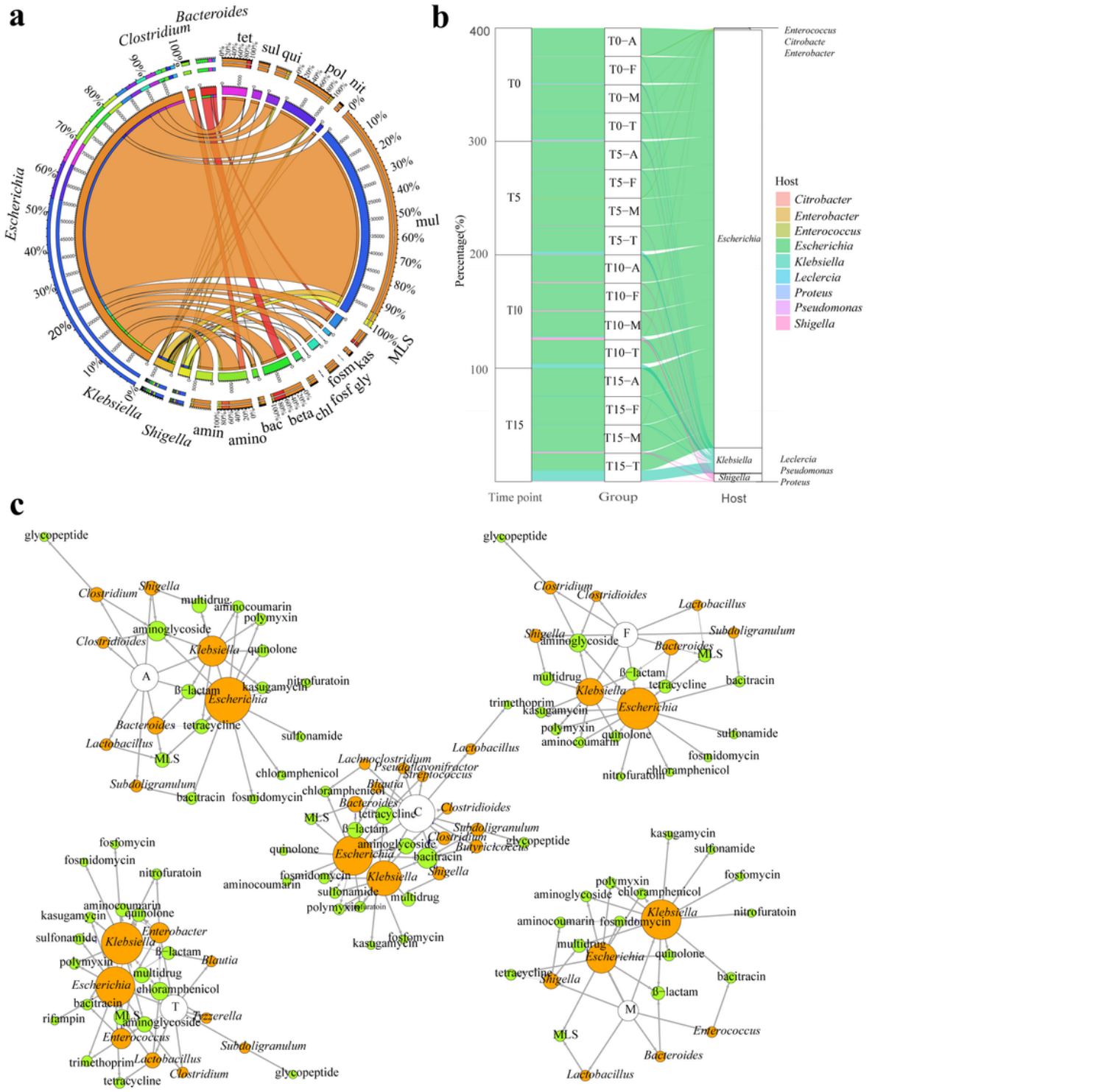

Figure 6

ARGs and their bacterial hosts. a) Percentages of ARG-carrying bacterial hosts. Mul multidrug, tet tetracycline, amino aminoglycoside, amin aminocoumarin, MLS macrolide-lincosamide-streptogramin, beta beta-lactam, bac bacitracin, sul sulfonamide, fosm fosmidomycin, pol polymyxin, kas kasugamycin, chl chloramphenicol, fosf fosfomycin, qui quinolone, gly glycopeptide, nit nitrofuratoin. b) Variations of the multidrug resistance gene carried by bacterial hosts over time in the antimicrobial-treated groups. c)

Changes of bacterial hosts carrying ARGs from the control group compared to the antimicrobial-treated groups on T10. A the amoxicillin group. C the control group. F the florfenicol group. T the chlortetracycline group. M, the mixed group.