The Alteration of Akkermansiaceae/Lachnospiraceae Ratio Is a Microbial Feature of Antibiotic-Induced Microbiota Remodeling

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Pei-Chen Chen[1*], Ming-Shian Lin[2*], Tien-Ching Lin[3], Ting-Wei Kang[1] and Jhen-Wei Ruan[1]

[1]Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan. [2]Division of Pulmonary and Critical Care Medicine, Chia-Yi Christian Hospital, Chiayi. [3]Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan.
*Equal contributions.

ABSTRACT: Antibiotic treatment has been shown to cause gut microbiota dysbiosis. However, lacking critical features defining gut microbiota dysbiosis makes it challenging to prevent. By co-occurrence network analysis, we found that despite short antibiotic courses eliminating certain microbial taxa, the *Akkermansia* genus played the role of a high-centrality hub to maintain microbiota homeostasis. When the antibiotic courses continued, the elimination of *Akkermansia* induced a significant microbiota remodeling of the gut microbiota networks. Based on this finding, we found that under long-term antibiotic stress, the gut microbiota was rearranged into a stable network with a significantly lower *Akkermansiaceae*/*Lachnospiraceae* (A/L) ratio and no microbial hub. By functional prediction analysis, we confirmed that the gut microbiota with a low A/L ratio also had enhanced mobile elements and biofilm-formation functions that may be associated with antibiotic resistance. This study identified A/L ratio as an indicator of antibiotic-induced dysbiosis. This work reveals that besides the abundance of specific probiotics, the hierarchical structure also critically impacts the microbiome function. Co-occurrence analysis may help better monitor the microbiome dynamics than only comparing the differentially abundant bacteria between samples.

KEYWORDS: Antibiotics, gut microbiota, dysbiosis, microbiota remodeling, network

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CORRESPONDING AUTHOR: Jhen-Wei Ruan, Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, No. 1, University Road, Tainan 701. Email: jhenweiruan@mail.ncku.edu.tw

Introduction

A mature ecosystem possesses high biodiversity and can maintain ecological stability and succession. The gut microbiota is a complex microbial community that can sustain a steady equilibrium against dynamic perturbations.[1] The ability to prevent severe pathogenic invasions, known as colonization resistance, is critical for healthy gut microbiota to maintain host homeostasis.[2] In addition, resilience is another essential capability for gut microbiota to tolerate pressure and maintain stability.[1,3] However, gut microbiota compositions can be influenced by various factors, including diets, external environments, infections, and antibiotics.[4] Gut microbiota plasticity has been considered to play an important role in helping the host adapt to the challenges.[5] Moreover, changes in the composition of gut microbiota are also associated with many diseases and cancers.[6]

Antibiotic treatment is an effective and efficient clinical therapy to eliminate life-threatening bacterial infections. However, antibiotics might cause negative effects on hosts, such as antibiotic-induced diarrhea and metabolic diseases.[7,8] These symptoms and syndromes have been found to be strongly associated with alterations in the gut microbiota composition.[9] In healthy people, short-term antibiotic exposure could even lead to an imbalanced and disrupted gut microbiota community that requires a long time to reconstitute.[10] Long-term or repeated antibiotic treatment usually results in gut microbiota dysbiosis, which also impacts other distal organs.[11] Dysbiosis is defined as a hard-to-recover disturbance of gut microbial homeostasis.[12] Multiple microbial features might be observed in a dysbiotic microbiota, including low diversity, increased abundance of harmful microbes, and reduced abundance of beneficial microbes.[12] In healthy individuals, the well-structured gut microbiota with high microbial diversity can prevent colonization and growth of opportunistic pathogens, such as *Clostridioides difficile*. The reduced diversity and increased proportion of aerobic pathobionts in gut microbiota were often observed in *C difficile* colonized or infected patients.[13] However, no clear feature of gut microbiota changes has been suggested to be sufficient for concurrently monitoring dysbiosis during the antibiotic treatment period.

Akkermansia muciniphila is a gram-negative and strictly anaerobic bacterium belonging to the *Verrucomicrobiae* phylum. With the ability to degrade mucins, it has been detected in the mucosa layer near epithelial cells and might be associated with the mucosal-to-luminal regulation of microbiota composition.[14,15] The abundance of *A muciniphila* in the gut has been demonstrated to negatively correlate with obesity and other metabolic disorders in humans and mice.[16-18] Furthermore, supplementation with *A muciniphila* has shown beneficial effects on

glucose tolerance and reducing fat mass in obese and diabetic mice and obese humans.[19-22] Cold exposure has been shown to diminish the representation of *A muciniphila* by increasing the intestinal absorption surface in mice.[23] As one of the most abundant bacteria in the human gut, the regulation of *A muciniphila* abundance may play a critical role in regulating compositional homeostasis and the function of the gut microbiota.

Previous studies have proven the negative effects of antibiotics on the gut microbiota in low-diversity dysbiosis.[9,10] However, the sequential changes of gut microbiota compositional succession remain unclear. In this study, we aimed to investigate how short-term and long-term antibiotic treatment courses influence gut microbiota using 16S rRNA sequencing. Our results revealed a significant shift in gut microbial diversity and composition during short-term and long-term antibiotic treatment. Co-occurrence network analysis sheds light on the loss of potential key microbes in short-term antibiotic treatment. Monitoring those candidates may be an early indicator to detect gut microbiota changes.

Methods

Sample collection

A total of 42 stool samples were collected longitudinally from 7 patients in intensive care unit (ICU) of National Cheng Kung University Hospital (NCKUH; IRB protocol number: A-ER-107-097). All patients were treated with broad-spectrum cepham antibiotics primarily due to urinary tract infection. The samples collected before the start of the antibiotic course were assigned to the untreated group. The samples collected at the time point less than 10 days during the antibiotic course were assigned to the short-term group. The samples collected at the time point of more than 10 days during the antibiotic course were assigned to the long-term group. The samples collected at the time point after the withdrawal of the antibiotic course were assigned to the recovery group. Fresh stool samples were preserved using DNA/RNA Shield-Fecal Collection Tube (Zymo Research, Irvine, CA, USA, Cat #R1101) and stored at −20°C before extraction.

Fecal DNA extraction

Fecal DNA was extracted from the mixture of stool and DNA/RNA shield (Zymo Research, Cat #R1100). The procedure was followed by the manufacturer's illustration of ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Cat #D4300). DNA concentration was detected using NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific).

16S rRNA sequencing analysis

The V3-V4 region of 16S rRNA was amplified using the primer set of 341F and 805R that had been reported to have a low bias on amplifying V3-V4 amplicons.[24] A 300 bp length of paired-end 16S rRNA amplicons was performed on an Illumina MiSeq platform. 16S rRNA sequencing data were analyzed using QIIME 2 platform (version: 2021.4). Raw reads were demultiplexed by the q2-demux plugin, and quality control was performed using DADA2[25] via the q2-dada2 plugin that generated amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs with classify-consensus-blast taxonomy classifier[26] through the q2-feature-classifier plugin using the SILVA database (release 138) with the 97% identity. Rarefaction was performed at 15 000 sequences per sample using R software. All samples were normalized to the minimum read count for differential sequencing depth among samples. Observed richness, evenness, phylogenetic diversity, and the Shannon diversity were calculated as indices of alpha diversity. For beta diversity, the weighted or unweighted-UniFrac distance and the Bray-Curtis distance were calculated within indicated groups using the vegan package (Community Ecology Package version 2.6-2) of R software. For the BugBase analysis, ASVs were clustered by the q2-vsearch plugin against Greengenes 13.8 database at a 97% identity threshold. For analysis of compositions of microbiomes with bias correction (ANCOM-BC) analysis,[27] the differential taxa were identified through the global test by false discovery rate (FDR) < 0.001. The global test showed differential taxa that were identified between at least 2 groups (Untreated vs Long-term or Short-term vs Long-term).

Co-occurrence network analysis

The SparCC[28] correlation coefficients were calculated between genera within 1 group. An edge was assigned if the correlation was higher than 0.6 with a *P*-value less than .05. Network features were estimated through degree centralization, betweenness centrality, and modularity using the igraph package of R software.[29] Betweenness centrality higher than 100 was colored by phylum. The network was visualized by Cytoscape software (version 3.9.1).

Statistical analysis

Statistical analysis, excluding ANCOM-BC, was performed on Prism 9 (GraphPad Software, CA, USA). Comparisons between 2 groups were analyzed using a Mann-Whitney test. Comparisons of more than 2 groups were performed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Differences were considered significant when *P*-value was smaller than .05.

Data availability

The 16S rRNA sequencing data have been deposited to National Center for Biotechnology Information (NCBI) under the following BioProject ID: PRJNA929331. This study does not report the original code. Any additional information required to reanalyze the data reported in this article is available from the lead contact on request.

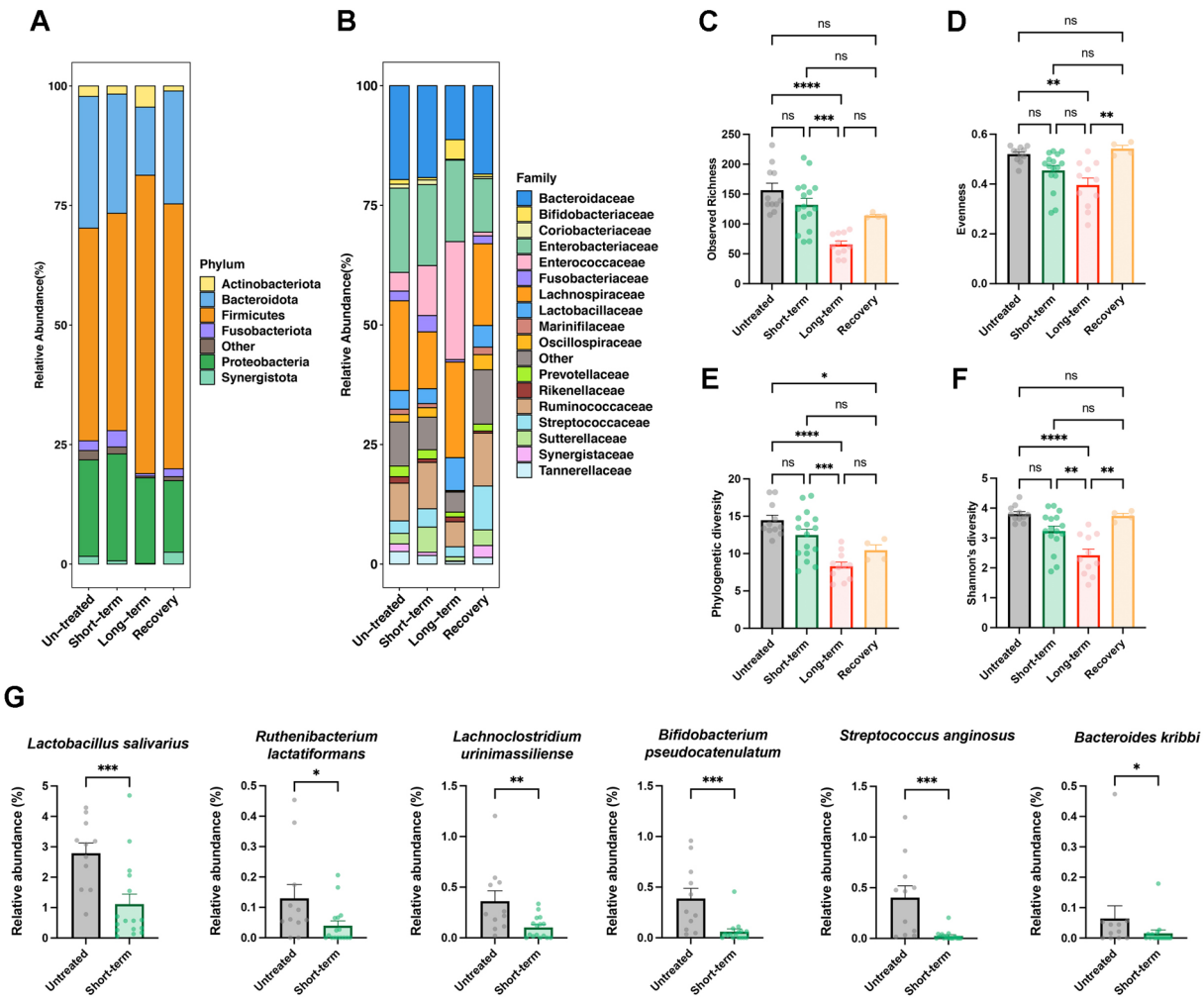

Figure 1. The time course of antibiotic treatment differentially alters gut microbiota composition: (A–B) composition and (C–F) alpha diversity of gut microbiota in untreated, short-term, long-term, and recovery groups. Bar plots showed microbial relative abundances at the phylum level (A) and at the family level (B). Alpha diversities were determined using (C) observed richness, (D) evenness, (E) phylogenetic diversity, and (F) the Shannon diversity. (G) The eliminated bacteria in short-term antibiotic treatment, including *Lactobacillus salivarius, Ruthenibacterium lactatiformans, Lachnoclostridium urinimassiliense, Bifidobacterium pseudocatenulatum, Streptococcus anginosus*, and *Bacteroides kribbi*. Data are presented by mean ± SEM. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$ according to the one-way ANOVA analysis and the Tukey post hoc test (C–F) and Mann-Whitney analysis (G). Untreated, N = 11; Short-term, N = 16; Recovery, N = 4. ns indicates non-significant; ANOVA, analysis of variance.

Results

The time course of antibiotic treatment differentially alters gut microbiota composition

Stool samples were collected from patients with urinary tract infections in the ICU of NCKUH. According to the collected antibiotic (cephems) treatment time points, 42 stool samples were divided into 4 groups, including the untreated group (samples collected before treatments, N = 11), short-term group (samples collected during the treatment period shorter than 10 days, N = 16), long-term group (samples collected during the treatment period longer than 10 days, N = 11), and recovery group (samples collected after the termination of a short-term antibiotic course, N = 4). By 16S rDNA sequencing, we found that both short-term and long-term antibiotic treatment showed effects on changing the compositions of the gut microbiota (Figure 1A and B and Tables 1 and 2). The most dramatic changes were observed in the long-term treated microbiota that has increased proportions of *Enterococcaceae* and *Lachnospiraceae* families (Figure 1B and Table 2). By measuring alpha diversity, we found that long-term exposure to cephems antibiotic significantly reduced the observed richness, evenness, phylogenetic diversity, and the Shannon diversity when compared with those of the untreated group, but samples in the short-term treatment group did not show significant changes in these 4 metrics of alpha diversity compared with the untreated group (Figure 1C to F). Despite this, we still observed that the abundance of certain species was reduced by short-term antibiotic treatment, including *Lactobacillus salivarius, Ruthenibacterium lactatiformans, Lachnoclostridium urinimassiliense, Bifidobacterium pseudocatenulatum, Streptococcus anginosus*, and *Bacteroides kribbi* (Figure 1G). This suggests that these reduced species during the short-term treatment stage might be more sensitive to cephams than other gut commensals.

Table 1. Microbiota composition at phylum level (related to Figure 1A).

	UNTREATED	SHORT-TERM	LONG-TERM	RECOVERY	UNTREATED VS SHORT-TERM	SHORT-TERM VS LONG-TERM	UNTREATED VS LONG-TERM	UNTREATED VS RECOVERY	SHORT-TERM VS RECOVERY	LONG-TERM VS RECOVERY
Actinobacteriota	2.19 ± 0.42%	1.71 ± 0.45%	4.43 ± 1.08%	1.06 ± 0.08%	*P* = .9479	*P* = .0200*	*P* = .1123	*P* = .8277	*P* = .9556	*P* = .0687
Bacteroidota	27.54 ± 1.42%	24.92 ± 2.16%	14.25 ± 1.85%	23.61 ± 2.01%	*P* = .7592	*P* = .0016**	*P* = .0003***	*P* = .7570	*P* = .9860	*P* = .1042
Firmicutes	44.49 ± 1.8%	45.46 ± 2.94%	64.42 ± 6.48%	55.41 ± 2.33%	*P* = .9978	*P* = .0156*	*P* = .0196*	*P* = .5273	*P* = .5685	*P* = .8169
Fusobacteriota	2.03 ± 0.45%	3.42 ± 0.92%	0.5 ± 0.14%	1.64 ± 0.71%	*P* = .4889	*P* = .0231*	*P* = .4780	*P* = .9928	*P* = .5767	*P* = .8609
Proteobacteria	20.18 ± 2.69%	22.36 ± 2.83%	17.94 ± 5.72%	14.95 ± 1.38%	*P* = .9729	*P* = .8181	*P* = .9768	*P* = .8987	*P* = .7350	*P* = .9786
Synergistota	1.61 ± 0.47%	0.68 ± 0.19%	0.1 ± 0.03%	2.49 ± 0.16%	*P* = .0719	*P* = .3986	*P* = .0029**	*P* = .3855	*P* = .0073**	*P* = .0005***

Data are presented by mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$ according to One-Way ANOVA analysis and the Tukey post-hoc test.

Table 2. Microbiota composition at family level (related to Figure 1B).

	UNTREATED	SHORT-TERM	LONG-TERM	RECOVERY	UNTREATED VS SHORT-TERM	SHORT-TERM VS LONG-TERM	UNTREATED VS LONG-TERM	UNTREATED VS RECOVERY	SHORT-TERM VS RECOVERY	LONG-TERM VS RECOVERY
Bacteroidaceae	19.62 ± 2.15%	19.21 ± 2.01%	11.27 ± 1.75%	18.48 ± 1.99%	$P = .9988$	$P = .0303$*	$P = .0385$*	$P = .9923$	$P = .9977$	$P = .3057$
Bifidobacteriaceae	0.97 ± 0.26%	0.49 ± 0.13%	4.11 ± 1.12%	0.52 ± 0.14%	$P = .9271$	$P = .0002$***	$P = .0036$**	$P = .9804$	$P > .9999$	$P = .0189$*
Coriobacteriaceae	0.83 ± 0.37%	0.97 ± 0.44%	0.17 ± 0.09%	0.42 ± 0.21%	$P = .9924$	$P = .4088$	$P = .6400$	$P = .9481$	$P = .8714$	$P = .9882$
Enterobacteriaceae	17.62 ± 2.98%	16.94 ± 2.52%	17.04 ± 5.73%	11.19 ± 2.39%	$P = .9991$	$P > .9999$	$P = .9996$	$P = .8235$	$P = .8510$	$P = .8606$
Enterococcaceae	3.9 ± 1.36%	10.43 ± 3.54%	24.66 ± 7.27%	0.78 ± 0.43%	$P = .7025$	$P = .1032$	$P = .0157$*	$P = .9856$	$P = .6800$	$P = .0540$
Fusobacteriaceae	2.03 ± 0.45%	3.42 ± 0.92%	0.5 ± 0.14%	1.64 ± 0.71%	$P = .4902$	$P = .0233$*	$P = .4787$	$P = .9929$	$P = .5782$	$P = .8607$
Lachnospiraceae	18.76 ± 1.43%	11.9 ± 1.4%	20.01 ± 2.42%	17.1 ± 3.2%	$P = .0363$*	$P = .0099$**	$P = .9651$	$P = .9674$	$P = .4484$	$P = .8527$
Lactobacillaceae	3.89 ± 0.23%	3.1 ± 0.87%	6.85 ± 1.84%	4.52 ± 0.81%	$P = .9529$	$P = .0796$	$P = .2911$	$P = .9922$	$P = .9115$	$P = .7324$
Marinifilaceae	1.1 ± 0.24%	0.85 ± 0.16%	0.1 ± 0.05%	1.56 ± 0.23%	$P = .7105$	$P = .0114$*	$P = .0016$**	$P = .5431$	$P = .1561$	$P = .0007$***
Oscillospiraceae	1.59 ± 0.35%	2 ± 0.5%	0.24 ± 0.11%	3.19 ± 0.41%	$P = .8784$	$P = .0149$*	$P = .1320$	$P = .2308$	$P = .4477$	$P = .0053$**
Prevotellaceae	2.24 ± 0.62%	1.93 ± 0.38%	1.01 ± 0.33%	1.45 ± 0.21%	$P = .9541$	$P = .4285$	$P = .2517$	$P = .8141$	$P = .9438$	$P = .9599$
Rikenellaceae	1.34 ± 0.34%	0.72 ± 0.28%	1.02 ± 0.5%	0.43 ± 0.08%	$P = .5842$	$P = .9271$	$P = .9291$	$P = .6021$	$P = .9767$	$P = .8529$
Ruminococcaceae	7.89 ± 0.98%	9.69 ± 1.79%	5.23 ± 1.09%	11.05 ± 2.61%	$P = .8247$	$P = .1625$	$P = .6527$	$P = .7424$	$P = .9681$	$P = .2604$
Streptococcaceae	2.61 ± 0.53%	3.83 ± 1.04%	2.1 ± 0.88%	9.15 ± 2.92%	$P = .8147$	$P = .6023$	$P = .9868$	$P = .0158$*	$P = .0509$	$P = .0083$**
Sutterellaceae	2.2 ± 0.64%	5.26 ± 2.58%	0.86 ± 0.32%	3.29 ± 2.02%	$P = .6519$	$P = .3483$	$P = .9647$	$P = .9925$	$P = .9519$	$P = .9245$
Synergistaceae	1.61 ± 0.47%	0.68 ± 0.19%	0.1 ± 0.03%	2.49 ± 0.16%	$P = .0719$	$P = .3986$	$P = .0029$**	$P = .3855$	$P = .0073$**	$P = .0005$***
Tannerellaceae	2.61 ± 1.04%	1.79 ± 0.42%	0.56 ± 0.13%	1.39 ± 0.3%	$P = .7474$	$P = .4382$	$P = .1123$	$P = .7477$	$P = .9857$	$P = .9016$

Data are presented by mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$ according to One-Way ANOVA analysis and the Tukey post-hoc test.

Table 3. The Verrucomicrobiota-related changes at phylum, class, and family levels..

MEAN ± SEM	UNTREATED	SHORT-TERM	LONG-TERM	RECOVERY	UNTREATED VS SHORT-TERM	UNTREATED VS LONG-TERM	UNTREATED VS RECOVERY
Phylum							
Verrucomicrobiota/Actinobacteriota	0.6399 ± 0.2248	0.5122 ± 0.1598	0.0516 ± 0.0225	0.1441 ± 0.1441	*P* = .8948	*P* = .0501	*P* = .3216
Verrucomicrobiota/Bacteroidota	0.049 ± 0.0192	0.0363 ± 0.0157	0.0054 ± 0.0017	0.0064 ± 0.0064	*P* = .8697	*P* = .1379	*P* = .3726
Verrucomicrobiota/Firmicutes	0.028 ± 0.0101	0.0176 ± 0.0059	0.0016 ± 0.0007	0.0037 ± 0.0037	*P* = .5337	*P* = .0280*	*P* = .1881
Verrucomicrobiota/Fusobacteriota	0.7859 ± 0.2651	0.6078 ± 0.2816	0.1473 ± 0.0364	0.0489 ± 0.0489	*P* = .9126	*P* = .2036	*P* = .3282
Verrucomicrobiota/Proteobacteria	0.0753 ± 0.0278	0.0414 ± 0.0148	0.0065 ± 0.0018	0.0115 ± 0.0115	*P* = .3655	*P* = .0309*	*P* = .1943
Class							
Verrucomicrobiae/Clostridia	0.0265 ± 0.009	0.0021 ± 0.0006	0.0046 ± 0.0046	0.0387 ± 0.0146	*P* = .6898	*P* = .0410*	*P* = .2216
Verrucomicrobiae/Gammaproteobacteria	0.0389 ± 0.0147	0.0057 ± 0.0016	0.0106 ± 0.0106	0.0742 ± 0.0277	*P* = .3334	*P* = .0310*	*P* = .1949
Family							
Akkermansiaceae/Lachnospiraceae	0.0532 ± 0.0158	0.0031 ± 0.0007	0.0065 ± 0.0065	0.656 ± 0.0254	*P* = .9137	*P* = .0459*	*P* = .2260
Akkermansiaceae/Enterobacteriaceae	0.0508 ± 0.0173	0.0059 ± 0.0017	0.0265 ± 0.0265	0.0981 ± 0.0379	*P* = .3152	*P* = .0263*	*P* = .3033

Data are presented by mean ± SEM. *$P < .05$ according to One-Way ANOVA analysis and the Dunnett's multiple comparisons test.

These results suggest that the gut microbiota has adaptive plasticity for the short period of antibiotic exposure, but long-term treatments of antibiotic treatments may cause a significant reduction in microbial diversity.

By unweighted- and weighted-UniFrac analyses, we confirmed that the long-term antibiotic-treated microbiota were significantly different from the gut microbiota in the untreated, short-term treated, and recovery groups (Figure 2A and B). To understand the microbial succession during antibiotic exposure, we compared the gut microbiota of the short-term or long-term group with that of the untreated group by dissimilarity analysis of unweighted or weighted-UniFrac distances. Short-term treated microbiota and long-term treated microbiota had an increased dissimilarity based on unweighted-UniFrac distances compared with untreated microbiota (Figure 2C). This suggests that both short-term and long-term treatments induced significant presence-absence changes in the microbiota. Interestingly, only long-term but not short-term treated microbiota showed a significantly higher dissimilarity compared with untreated microbiota based on weighted-UniFrac distances (Figure 2D). This implies that a critical remodeling of gut microbiota may occur between the short-term/long-term transition of antibiotic exposure.

Long-term antibiotic treatment remodels the microbial network

By ANCOM-BC global test, we confirmed that a global change of multiple genera occurred specifically in long-term treated microbiota (Figure 2E). When compared with the untreated and short-term groups, long-term antibiotic exposure significantly reduced the abundance of gut bacteria in *Clostridia, Bacilli*, and *Verrucomicrobiae* classes (Figure 2E). To further understand whether the long-term antibiotic treatments induce the network remodeling of the gut microbiota, we performed SparCC co-occurrence network analyses to determine the interactive complexity of bacteria in the microbiota by the number of nodes and edges.[30] We found that although the number of nodes (genus-level taxa) was similar between untreated microbiota (node number = 98) and short-term treated microbiota (node number = 95), the edge number of the short-term treated microbiota was decreased from 399 to 168 (Figure 3A and B). Higher modularity was also observed in the short-term treated network, suggesting that the antibiotic treatment disrupted the compact network connections observed in untreated microbiota (Figure 3B). Intriguingly, despite that the long-term treatments dramatically reduced the nodes (nodes number = 41) in the microbial network, an increased number of correlated edges compared with short-term treated microbiota was observed (edge number = 211) (Figure 3C). This indicates that the prolonged antibiotic treatment can remodel the gut microbiota into a newly formed network that rebuilt the interactions between microbes (Figure 3C).

The microbial hub theory suggests that specific species in the human gut microbiota may play a role as a regulatory hub in maintaining homeostasis.[31,32] Degree centralities and betweenness centralities are often used measurements for exploring the taxa with the hub-like property in a microbial network. Hence, we determined the microbial hubs in each network by setting a cutoff of betweenness centralities of nodes higher than 200 at the genus level. Before antibiotic treatment, the untreated network harbored multiple high-betweenness taxa with high degree centralities (Figure 3D). After the short-term treatment, most microbial hubs lost their betweenness and degree centralities to coordinate the network (Figure 3E). Instead, *Akkermansia* became the high-betweenness hub with the highest degree centrality in the network (Figure 3E). This suggests that *Akkermansia* plays a critical role in maintaining community stability during the antibiotic treatment period. However, in the long-term treated microbiota, no high-betweenness taxa were observed, suggesting that the hierarchical community structure was dramatically changed into a network with no regulatory hub (Figure 3F and G). Different from short-term treated microbiota, multiple genera in the *Clostridia* class showed high degree centralities in the network in long-term treated microbiota, including *Lachnospira, Tyzzerella*, and *Ruminococcus gauvreauii* groups (Figure 3F). Notably, the long-term treated microbiota dramatically rebuilt connections between microbes that significantly restore the degree centrality of the network (Figure 3H). This indicates that long-term antibiotic stress can remodel gut microbiota into a reorganized, stable community structure. The lack of microbial hub in the long-term treated network may also explain why dysbiotic microbiota is challenging to recover.

Akkermansiaceae/Lachnospiraceae *ratio is an important feature of antibiotic-induced microbiota remodeling*

Because the hierarchies of *Akkermansia* and *Lachnospiraceae* in microbiota networks were altered between the transition of short-term and long-term antibiotic treatments (Figure 3E and F), we further confirmed the changes of the abundance of *Akkermansia* and *Lachnospiraceae* in the short-term and long-term groups. Our results confirmed that *Akkermansia* was significantly eliminated in long-term treated microbiota, accompanied by a significant increase of *Lachnospiraceae* when compared with short-term treated microbiota (Figure 4A and B). To understand whether the features of *Akkermansia*-induced remodeling could be used for monitoring gut microbiota dysbiosis, we calculated ratios between *Verrucomicrobiota* and other phyla at different stages of the antibiotic course. At the phylum level, both *Verrucomicrobiota/Firmicutes* ratio and *Verrucomicrobiota/Proteobacteria* ratios were found to be significantly decreased in the long-term group compared with the untreated group (Table 3). At the class level, *Verrucomicrobiae/Clostridia* (V/C) and *Verrucomicrobiae/Gammaproteobacteria* ratios reflected the same trends (Table 3). At the family level, *Akkermansiaceae/Lachnospiraceae* (A/L) ratio and *Akkermansiaceae/Enterobacteriaceae* ratio were consistent with

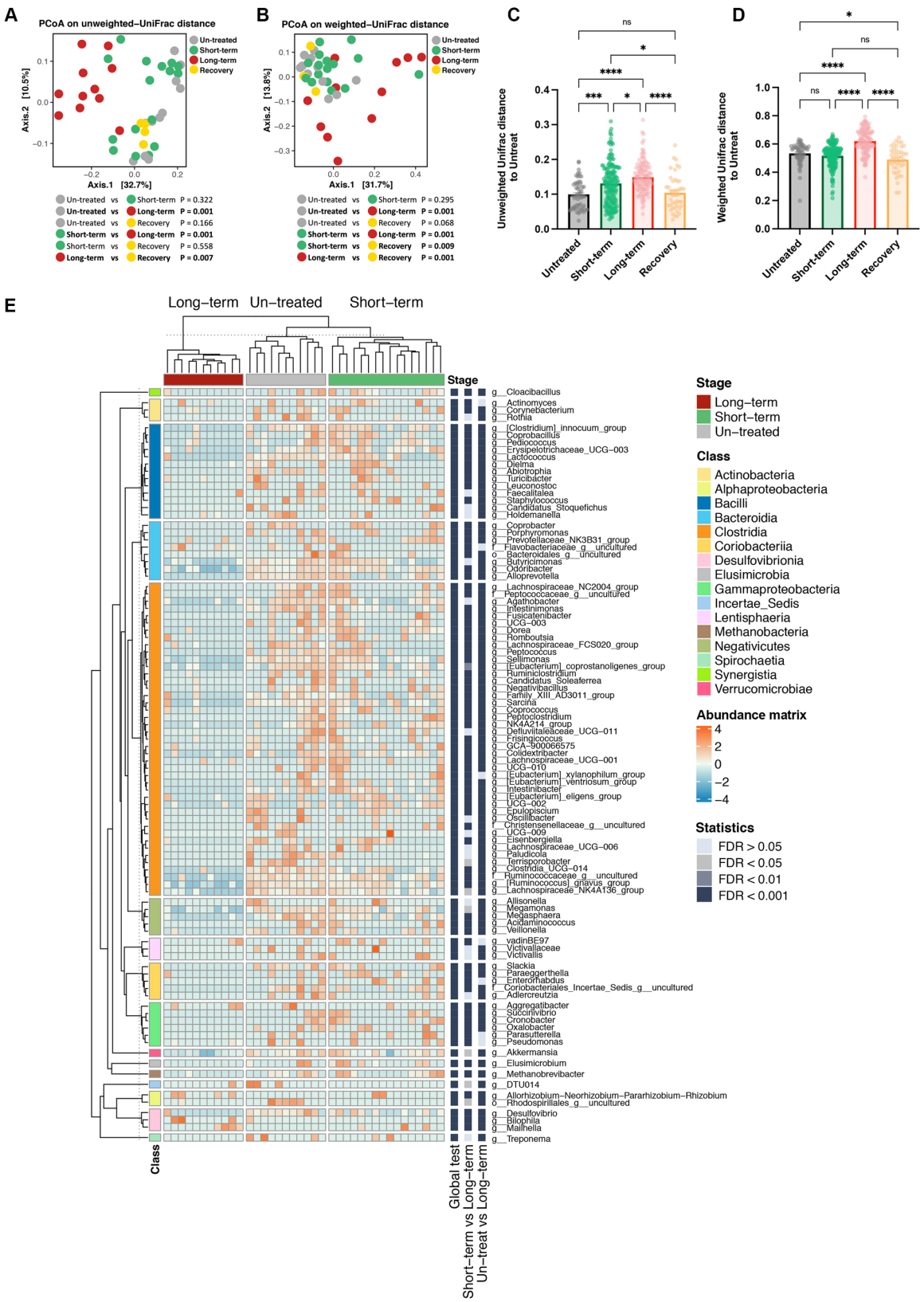

Figure 2. The ecological succession of antibiotic-treated microbiota. (A–B) Principal co-ordinates analysis (PCoA) of unweighted (A) and weighted (B) UniFrac distances of gut microbiota community in untreated, short-term, long-term, and recovery groups. Each dot represents a sample. Statistical analysis was performed using PERMANOVA pairwise test. (C–D) Dissimilarity analysis of unweighted (C) and weighted (D) UniFrac distances between gut microbiota of the untreated group and other indicated groups. (E) Heatmap of differentially abundant genera identified by ANCOM-BC analysis. Data are presented by mean ± SEM. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$ according to the one-way ANOVA analysis and the Tukey post hoc test. Untreated, N = 11; short-term, N = 16; long-term, N = 11; recovery, N = 4. ANCOM-BC indicates analysis of compositions of microbiomes with bias correction; ANOVA, analysis of variance; ns, non-significant; PERMANOVA, permutational multivariate analysis of variance.

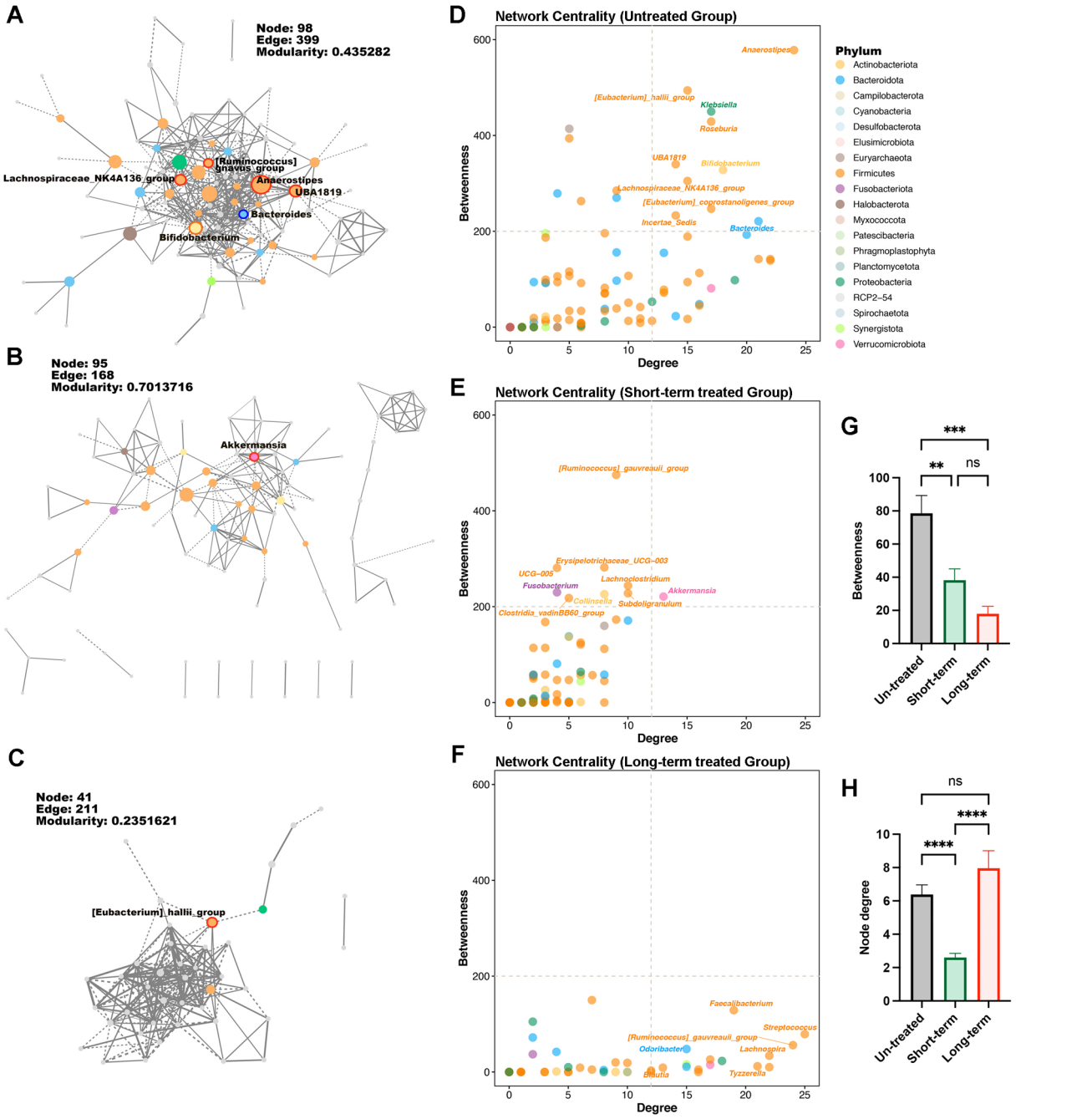

Figure 3. Long-term antibiotic treatment remodels the microbial network. (A–C) Co-occurrence networks are performed on SparCC correlations at the genus level of the (A) untreated microbiota, (B) short-term treated microbiota, and (C) long-term treated microbiota. Solid and dashed lines represent the positive correlation and negative correlation, respectively. Node sizes are characterized by betweenness centrality and betweenness centralities that are higher than 100 are colored by phylum. (D–F) Centralization measurement of degree and betweenness in the genus-level co-occurrence networks of the (D) untreated microbiota, (E) short-term treated microbiota, and (F) long-term treated microbiota. (G–H) Betweenness centralization (G) and degree centralization (H) in untreated, short-term treated, and long-term treated microbiota networks. Data are presented by mean ± SEM. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$ according to the one-way ANOVA analysis and the Tukey post hoc test. ANOVA indicates analysis of variance; ns, non-significant.

the results on phylum and class levels (Table 3). Based on the results of ANCOM-BC analysis, nearly half (37 out of 81) affected genera (long-term vs short-term) by long-term antibiotic treatment belong to the *Clostridia* class, and only 6 genera in the *Gammaproteobacteria* phylum were affected by long-term antibiotic treatment (Figure 2E). Therefore, these findings suggest that V/C and A/L ratios may be more sensitive than Verrucomicrobiota/Proteobacteria and *Verruco microbiae/Gammaproteobacteria* ratios to identify the occurrence of microbiota remodeling. Both A/L and V/C ratios were significantly decreased by long-term antibiotic exposure (Figure 4C and D and Table 3). These results suggest A/L ratio is an important feature of antibiotic-induced microbiota remodeling.

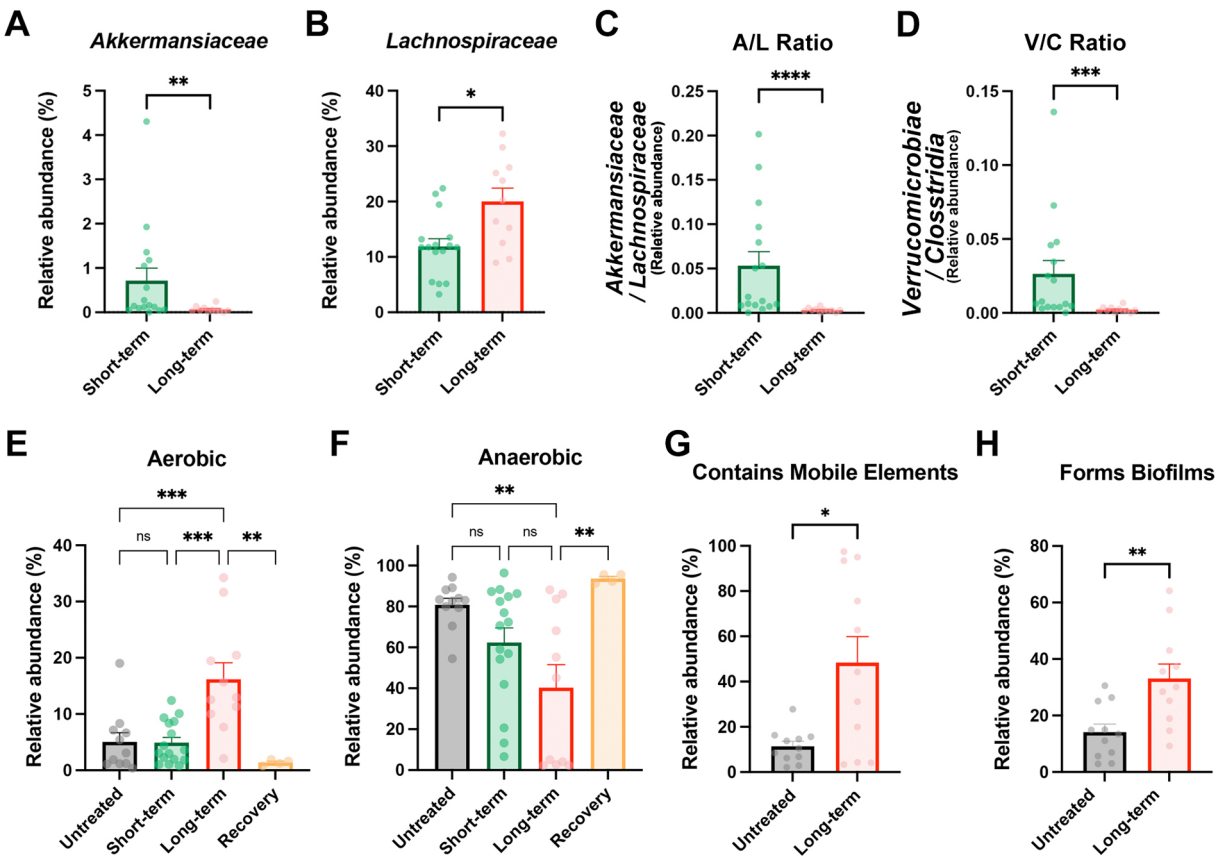

Figure 4. *Akkermansiaceae*/*Lachnospiraceae* ratio is an important feature of antibiotic-induced microbiota remodeling. (A–B) Relative abundances of the *Akkermansiaceae* family (A) and the *Lachnospiraceae* family (B) in short-term and long-term antibiotic treatment groups. (C–D) The *Akkermansiaceae*/*Lachnospiraceae* (A/L) ratio (C) and the *Verrucomicrobiae*/*Clostridia* (V/C) ratio (D) were calculated to perform the dysbiotic remodeling during antibiotic treatment. (E–H) Predicted phenotypes by the BugBase analysis, including aerobic (E), anaerobic (F), containing mobile elements (G), and biofilm formation (H). Data are presented by mean ± SEM. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$ according to the one-way ANOVA analysis and the Tukey post hoc test. Untreated, N = 11; short-term, N = 16; long-term, N = 11; recovery, N = 4. ANOVA indicates analysis of variance; ns, non-significant.

To understand whether the long-term antibiotic-induced remodeling changes microbiota functions, functional prediction analysis was performed using the BugBase database. The results showed that the proportions of aerobic bacteria and anaerobic bacteria were both changed specifically by the long-term treatment but not by the short-term treatment (Figure 4E and F). This result supports our finding that the microbiota remodeling occurred specifically in long-term antibiotic-treated patients.

In addition, the long-term treated microbiota harbored a significantly higher proportion of bacteria mobile elements and biofilm-formation functions that may increase the pathogenic and antibiotic resistance of the gut microbiota (Figure 4G and H). By the BugBase analyses, the increase in biofilm-forming population was primarily contributed by biofilm-related taxa in Proteobacteria phylum (untreated: 8.665 ± 2.149%; long-term: 26.847 ± 6.741%) and biofilm-related taxa in Actinobacteria phylum (untreated: 3.256 ± 1.118%; long-term: 6.228 ± 2.684%) (Figure 4H). These results suggest that long-term antibiotic-induced microbiota remodeling could promote biofilm formation and drive the transfer of mobile elements that contribute to the stabilization of newly formed microbial social networks.[33,34]

Discussion

Although antibiotic treatment may induce gut microbiota dysbiosis, it is an essential treatment to prevent or eradicate bacterial pathogenic infections. To date, there is no sufficient laboratory diagnostic method for the identification of gut dysbiosis. In this study, we monitored the effect of short-term and long-term courses of antibiotics on human gut microbiota. We found that although the short-term antibiotic course may temporally collapse the microbial network, most microbial changes that occurred in this stage were irreversible. However, the long-term treatment course could induce microbiota remodeling to construct a rearranged microbial network with enhanced biofilm-formation function. The A/L ratio identified in this study may serve as indicator of microbiota dysbiosis. Accordingly, we offer practical indicators to help physicians be aware of the occurrence of gut microbiota dysbiosis synchronously during the treatment period.

Dysbiosis is a difficult-to-recover state of the gut microbiota with an illness phenotype and reflects an imbalanced interaction between hosts and microbes.[1] The elimination of certain gut bacteria may not simply induce the occurrence of dysbiosis because a healthy gut microbiota usually possesses a strong

resilience to environmental stresses.[1] In this study, we found that besides the A/L ratio, the long-term antibiotic-treated microbiota also has significant alterations in the proportion of aerobes and anaerobes. Previous studies have demonstrated that oxygen influx is one of the critical factors needed to change microbial metabolic interactions, which can dynamically regulate the switching process.[35,36] Because incomplete modulation of oxygen influxes may irreversibly impact the gut microbiota dysbiosis, it is suggested that recording the intestinal oxygen influx or the anaerobe ratio might be an alternative method to track the intestinal condition. Further investigation will be required to understand whether the A/L dynamics are associated with intestinal oxygen regulation.

Notably, we observed a substantial increase in *Firmicutes* and *Actinobacteria* in comparison to others in the long-term antibiotic-treated patient. Similar changes were also observed in previous studies investigating the effects of broad-spectrum antibiotics on gut microbiota in humans and gorillas.[37,38] The potential explanation is that only a limited number of commensals have been found to colonize or adherent to the intestinal mucus layer, including *Lachnospiraceae, Ruminococcaceae, Bifidobacterium bifidum, Bifidobacterium longum*, and *A muciniphila*.[15] When prolonged antibiotic exposure eliminates the representation of *A muciniphila*, the diminishment of this dominant mucus-degrading species may subsequently support the expansion of other mucus-associated commensals, such as *Lachnospiraceae* (*Firmicutes*) and *Bifidobacterium* (*Actinobacteria*) identified in this study. This strengthens that *A muciniphila* is a critical microbial hub regulating gut microbiota remodeling.

Although no significant compositional change on phylum level was observed in short-term antibiotic-treated microbiota, the abundance of certain bacteria was specifically reduced by short-term exposure, such as *B pseudocatenulatum, R lactatiformans*, and *B kribbi*. All these 3 species are anaerobes that can produce lactic acid by glucose fermentation.[39,40] *B pseudocatenulatum* has been shown to alter the gut microbiota composition to protect mice against colitis and liver injury.[41,42] *R lactatiformans* has been shown to regulate intestinal immune responses.[43,44] Two of the most significantly reduced species in short-term treated microbiota, *B pseudocatenulatum* and *L salivarius*, are strong probiotics that may be the chief cogs of the following *A muciniphila*-induced remodeling. It is worth considering that losing these species at the early stage of the antibiotic course may impact long-term microbiota succession by interfering host-microbe interaction when the treatment continues.

Despite *Faecalibacterium, Akkermansia*, and *Lactobacillus* genera showing higher degree centralities than other genera in long-term treated microbiota, the betweenness centrality of gut microbiota was dramatically interrupted by the prolonged antibiotic treatment. These findings suggest that the microbial hub, such as *Akkermansia*, is critical for maintaining the hierarchical structure of gut microbiota. Antibiotics globally impact the gut microbiota, including pathobionts, commensals, and probiotics.[45,46] Decreased abundances of beneficial strains in the gut microbiota have been shown to be associated with intestinal permeability, inflammation, and metabolic disorders.[47] However, supplementation with probiotics after antibiotic treatment may interfere with the natural recovery of a healthy gut microbiome.[48] Hence, it suggests that the maintenance of a homeostatic gut microbiota may not simply depend on the presence or absence of specific probiotics. Carefully monitoring the dysbiosis-associated features of the gut microbiota in patients receiving long-term antibiotic treatment would be necessary for preventing the occurrence of dysbiosis and severe infections, such as *C difficile* infection.

Taken together, our results demonstrate that long-term antibiotic exposure induces substantial stress that causes remodeling and changing of the ecological functions of the gut microbiota. It is indispensable to identify the specific features of an imbalanced gut microbiota, which could prevent the occurrence of a difficult-to-recover dysbiosis.

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Author Contributions

T-CL and J-WR conceptualized this study. T-CL contributed to clinical sample acquisition and collection. T-WK isolated stool DNA. P-CC analyzed the sequencing data and wrote the original manuscript. T-CL, M-SL, and J-WR provided the funding to support this study. J-WR supervised the findings in the work. All authors reviewed the manuscript.

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