ORIGINAL RESEARCH

Intestinal Flora Imbalance Induced by Antibiotic Use in Rats

Guojun Tong [iD][1,2], Hai Qian[1], Dongli Li[2], Jing Li[2], Jing Chen[2], Xiongfeng Li[3], Zhenhua Tan[1]

[1]General Surgery, Huzhou Central Hospital, The Affiliated Central Hospital of Huzhou University, Huzhou, People's Republic of China; [2]Central Laboratory, Huzhou Central Hospital, The Affiliated Central Hospital of Huzhou University, Huzhou, People's Republic of China; [3]Orthopedic Surgery, Huzhou Central Hospital, The Affiliated Central Hospital of Huzhou University, Huzhou, People's Republic of China

Correspondence: Zhenhua Tan, General Surgery, Huzhou Central Hospital, The Affiliated Central Hospital of Huzhou University, Sanhuan North Road 1558#, Huzhou, People's Republic of China, Email Tanzh99@163.com

Aim: This study aims to explore the effect of different doses of antibiotics on rats in order to observe alterations in their fecal microbiota, inflammatory changes in the colonic mucosa and four types of inflammatory markers in blood serum.

Methods: Our methodology involved separating 84 female Sprague Dawley rats into groups A-G, with each group consisting of 12 rats. We collected the rat feces for analysis, using a distinct medium for bacterial cultivation and counting colonies under a microscope. On the 11th and 15th days of the experiment, half of the rats from each group were euthanized and 5 mL of abdominal aortic blood and colon tissues were collected. Inflammations changes of colon were observed and assessed by pathological Hematoxylin Eosin (HE) staining. Enzyme-linked immune sorbent assay (ELISA) was adopted for detecting C-reactive protein (CRP), IL-6, IL1-β and TNF-α.

Results: Our findings revealed that the initial average weight of the rats did not differ between groups ($p>0.05$); but significant differences were observed between stool samples, water intake, food intake and weight ($p=0.009$, <0.001, 0.016 and 0.04, respectively) within two hours after the experiment. Additionally, there were notable differences among the groups in nine tested microbiota before and after weighting methods (all $p<0.001$). There were no difference in nine microbiota at day 1 (all $p>0.05$); at day 4 A/B ($p=0.044$), A/D ($p<0.001$), A/E ($p=0.029$); at day 8, all $p<0.01$, at day 11, only A/F exist significant difference ($p<0.001$); at day 14 only A/D has difference ($p=0.045$). Inflammation changes of colon were observed between groups A-G at days 11 and 15. Significant differences between all groups can be observed for CRP, IL-6, IL1-β and TNF-α ($p<0.001$).

Conclusion: This study suggests that antibiotics administration can disrupt the balance of bacteria in the rat gut ecosystem, resulting in an inflammatory response in their bloodstream and inducing inflammation changes of colon.

Keywords: antibiotics, imbalance of intestinal flora, Sprague Dawley rats, inflammatory bowel disease, IBD, colitis model

Introduction

Antibiotics first appeared in the 1940s and were regarded as a miracle of modern medicine.[1] The health and life expectancy of humans have been improved because of antibiotics.[2] After over a century, antibiotics have developed properties and susceptibility profiles for microorganisms. However, changes to microbial organisms and the struggle to fight microbiota infections with antibiotics has not stopped. The intestines are where bacterial flora, beneficial and pathogenic organisms, gather, and the intestinal flora is balanced in a healthy population. When using antibiotics, this balance is often altered, leading to intestinal diseases, such as antibiotic-associated diarrhea, inflammatory bowel disease (IBD),[3] and pseudomembranous colitis.[4] The gut microbiota is closely related to IBD;[5–7] furthermore, it is associated with colorectal cancer.[8–11] The intestine is a complex organ necessitating sophisticated and comprehensive animal models to study its function and associated diseases.[12] The immune system within the intestine is also complex, comprising coordinated responses between the innate and adaptive immune systems within the intestinal mucosa.[13,14] The immune system can stimulate cellular and liquid immunity to respond to infections in the intestinal mucosa, resulting in acute inflammation. Further, changes in the gut microbiota cause an inflammatory response in the intestinal mucosa, resulting in an inflammatory response in the blood. The main lymphocytic cellular components of the adaptive immune

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Graphical Abstract
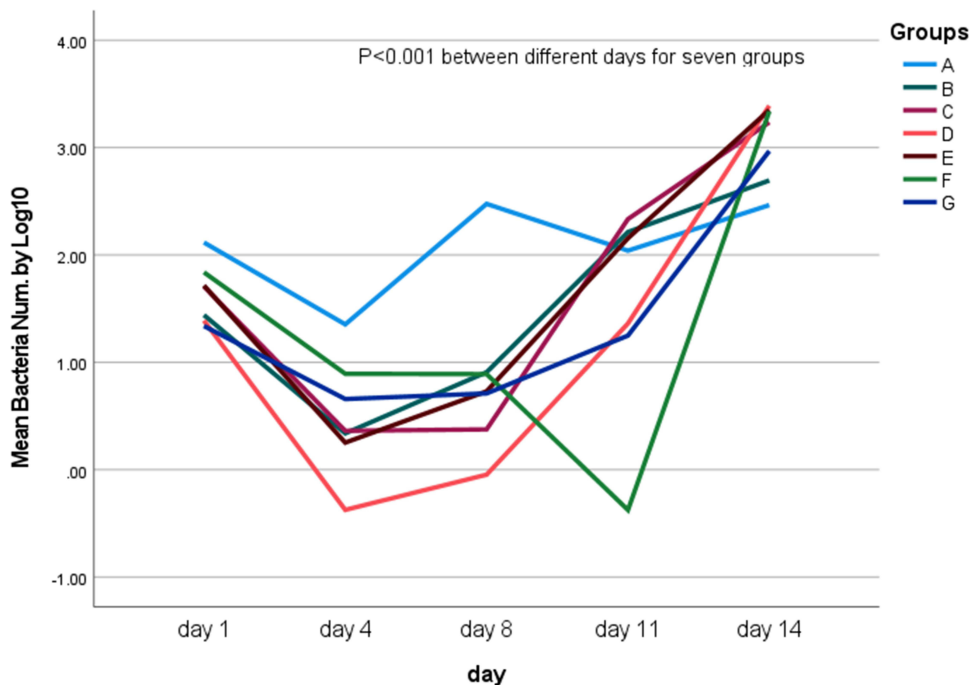
system comprise CD8+ and CD4+ T-cells. Cytotoxic CD8+ T-cells can also enhance the release of effector cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which collectively promote macrophage activation and cell death. Other inflammatory factors and proteins also exhibit high expressions, such as interleukin-1β (IL1-β) and C-reactive protein (CRP).[12,15] Sprague Dawley (SD) rats are easily raised and controlled because of their mild temperament. They have been used to establish various disease models, such as skin photoaging, glioma, pancreatico-duodenal transplantation, heart transplantation, and intestinal flora imbalance.[16,17] For example, the 2,4,6-trinitrobenze-nesulfonic acid (TNBS) model was initially developed for rats, but it is now widely used in other organisms.[12,18]

Changes in the intestinal microbial composition can cause intestinal inflammation. Studies have shown that the addition of probiotic bacteria in diets causes changes in the metabolic profiles of specific bacteria, thus supporting the growth of carbohydrate-reducing bacteria.[19,20] Because the gut microbiota is important to intestinal diseases, such as IBD and colorectal cancer, we aimed to study some common microbiota-related disorders using antibiotics; this is the highlight of the study. In this study, different doses of antibiotics (single and combination) were intragastrically administered to rats to show the changes in their intestinal flora and blood inflammatory responses. The target flora included *Staphylococcus aureus, Bifidobacterium, yeast, Bacteroides, Clostridium,* anaerobic bacteria, *E. coli, Enterococcus*, and *Lactobacillus*. We examined TNF-α, IL1-β, IL-6, and CRP since they are common inflammation factors expressed after immune responses.[12,15] This study aims to explore the effect of different doses of antibiotics on rats in order to observe alterations in their fecal microbiota, inflammatory changes in the colonic mucosa and four types of inflammatory markers in blood serum.

Materials and Methods
Rats
From Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China) (Experimental Animal Production License No.: SCXK (Liao)), 84 five- to six-week-old female Sprague Dawley rats with weights ranging from 172.4 to 179.5 g were obtained and randomized to seven groups (each group n = 12). The animals were examined for their health within 24

hours of arrival and grouped before beginning the experiment. Only healthy rats were used in the study, which allowed them to have free access to normal rat feed (Liaoning Changsheng, CS-102) and sterile water during the experiment. All animals underwent quarantine and adaptation to specific pathogen-free conditions for a total of 9 days. The experimental environment consisted of a time cycle of 12-hour light/12-hour dark, air was exchanged ≥15 times/h, temperatures was kept between 20°C and 26°C, pressure gradients of ≥10 Pa, and humidity levels between 40% and 70%. All procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23; 1996 version). All effort was made to minimize the number of animals used in the study.

Experimental Drugs, Reagents, and Instruments

The drugs employed comprised clindamycin hydrochloride (a 99% pure sample available from Shanghai Maclean Biotechnology Co., Ltd.), ampicillin (a 98% pure product from Hebei Bailingwei Superfine Material Co., Ltd.), and streptomycin (a ≥ 90% pure substance from Tianjin Guangxia Fine Chemicals Institute).

Rat CRP, TNF-α, IL-6 and IL1-β were procured from Bioswamp (Wuhan, China). Dichloran Rose Bengal Chloramphenicol (DRBC) agar, Lactobacillus selective agar and Bacteroides-Bile-Enterprise (BBE) agar were acquired from Qingdao Science and Technology Industrial Park Haibo Zhang Biotechnology Co., Ltd. (Qingdao, China). Mannitol sodium chloride agar medium, Trypticasephytone yeast extract (TPY) agar medium and Anaerobic bacteria agar were obtained from Beijing Luqiao Technology Co., Ltd. (Beijing, China). Eosin Methylene Blue (EMB) agar was purchased from Beijing Road and Bridge Technology Co., Ltd. (Beijing, China). Citrate Azide Tween® Carbonate (CATC) agar was sourced from Qingdao High-Tech Industrial Institute Haibo Zhang Technology Co., Ltd. (Qingdao, China). Reinforced Clostridium Culture Medium was procured from Beijing Luqiao Rapid Co., Ltd. (Beijing, China).

An electric day constant temperature incubator was acquired from Tianjin Taisite Instrument Co., Ltd. (Tianjin City, China). The Lab systems Multiskan MS microplate reader was purchased from Thermo Fisher (Pittsburgh, PA, USA). A low-speed condensation centrifuge was procured from Shanghai Luxiangyi Centrifuge Instrument Co., Ltd. (Shanghai, China). An electronic balance was obtained from YuyaoJinnuoTianping Instrument Co., Ltd. (Zhejiang Sheng, China). An upright microscope was acquired from Japan Nikon Guangxuan Microscope Manufacturing Co., Ltd. (Tokyo, Japan).

Animal Grouping and Drug Delivery

Based on previous investigations[21] and our own prior study,[22] different doses of streptomycin, ampicillin and clindamycin dissolved in saline solution (2 mL) were administrated to a group of 12 SD rats in both an experimental and control setting. The doses were listed below. Group A: only saline solution (control); Group B: clindamycin at a low dose of 250 mg/kg (single); Group C: clindamycin at a medium dose of 500 mg/kg (single); Group D: clindamycin at a high dose of 750 mg/kg (single); Group E: clindamycin at a low dose of 250 mg/kg, ampicillin at a dose of 272.1 mg/kg, and streptomycin at a dose of 136.1 mg/kg (triple); Group F: clindamycin at a medium dose of 500 mg/kg, ampicillin at a dose of 563.7 mg/kg, and streptomycin at a dose of 281.8 mg/kg (triple); Group G: clindamycin at a high dose of 750 mg/kg, ampicillin at a dosage of 835.8 mg/kg, and streptomycin at a dose of 417.9 mg/kg (triple).

The experiment consisted of two phases: the modeling period (days 1–7) and the recovery period (days 8–15). The administration (10 mL/kg) was carried out between 8:30 and 10:00 AM daily via stomach feeding through an oral needle during the modeling phase, which ceased during the recovery period.

Collected and Analyzed Indicators

On the 1st, 3rd, 5th, 7th, 9th, 11th, and 14th days within 2 hours, data of stool samples taken, water intake volume, food intake volume and weight were recorded. On the 1st, 4th, 8th, 11th, and 14th days, fecal microbial flora was tested in each rat. *Lactobacillus, Enterococcus, E. coli, anaerobic bacteria, Clostridium, Bacteroides*, yeast, *Bifidobacterium and Staphylococcus aureus* were examined. Five milliliters of Abdominal aortic blood of 5mL were collected from rat, following that (ELISA) was carried out to detected CRP, IL-6, IL1-β and TNF-α in undiluted blood serum using detecting kit from Shanghai Enzyme Union Biotechnology Co. Ltd.

Experimental Protocol

In a meticulous manner, 1g of fecal matter was combined with 9 mL of tryptone soy broth to establish an appropriate concentration. Subsequently, 20 μL of this sample was uniformly spread onto the agar medium using a coating bar. Nine distinct microbial species were isolated using nine unique media. These microorganisms were then inoculated onto mannitol sodium chloride agar medium plates, EMB, CATC agar plates under aerobic conditions at 37°C for a duration of 48 hours. In addition, these organisms were cultured under anaerobic conditions at 37°C on TPY agar medium plates, Bacteroides-Bile-Enterprise (BBE) agar plates, reinforced *Clostridium* medium plates, anaerobic agar plates, and *Lactobacillus* selective agar plates for an identical period of time. The methodology employed is akin to that published previously.[22] At 28°C in aerobic conditions, flora inoculated on DRBC agar plates were cultured for 5 days. Nine colonies were counted under the microscope. Colonies were enumerated using Equation 1.

$$number\ of\ colonies\ (CFU/g) = number\ of\ plate\ colonies \times 50 \times dilution\ factor \qquad (1)$$

Where the dilution factor was 10^6 (E6) as a uniform unit.

Half of the rats were dissected on the 11th day, and the other half were dissected on the 15th day after euthanization. The rats were anesthetized via intraperitoneal injection with 2% pentobarbital sodium (40mg/kg) and dissected via abdominal incisions. Five milliliters of abdominal aortic blood were collected under anesthesia to detect inflammation factors. TNF-α, IL-1β, IL-6, and CRP were detected in undiluted blood serum via ELISA. Under anesthesia with 2% pentobarbital sodium (40mg/kg), SD rats were euthanized by cutting the abdominal aorta. Animals were adjudged dead when no pulse was detected in the abdominal aorta and both pupils were dilated.

Tissue H&E Staining

Distilled water was added to the slices for several minutes in an aqueous solution of hematoxylin for dyeing. Color separation in both acid water and ammonia water for a few seconds was conducted. The slices were then rinsed with running water for 1 h and put in distilled water for a while. Dehydration was performed with 70% and 90% alcohol for 10 minutes each. Alcohol and eosin staining solution were then added for 2–3 minutes for staining.

Enzyme-Linked Immune Sorbent Assay (ELISA)

We took out the whole box ELISA kit (store in 4°C refrigerator) and put it in 37 incubator for 0.5 h to normal temperature, adjust the rocker temperature to 31°C, (150 rpm/min); MQ H2O, sterilized were Diluted to 10 XHRP wash buffer to 1 X; Put wells on the 96-hole floor, 300ul/well of 1X HRP wash buffer were slowly dropped along the wall and slightly shocked for 5s by hands; In the blank and standard wells, 20ul Matrix solution were put in. We set blank control as 1 hole, standard 5.2.5,1.25,0.625,0.3125,0.15625 ng/mL as six wells; Then we added 10 ul Assay Buffer, to the standard wells during the blank and sample wells, added 30 ul Assay Buffer; After that we added 20 ul standard samples to standard wells, added 20 ul treated samples in sample wells; 50ul/well of detection antibody were added, the sticker were covered then the wells were gently shaken for 5 s by hand, then they were put in a flat rocker to respond for 2h, (31°C,150 rpm/ min); We gently removed the sticker, got rid of the wells fluid, and slapped them on a tissue, with 1 X wash buffer washed them 3 times (300 ul each); Enzyme solution 100ul/well was mixed gently. The sticker was covered, and they were put on a flat rocker to respond for 30 min (31°C,150 rpm/min); The sticker was gently removed and we got rid of the wells fluid, Washing with 1× wash buffer 6 times were performed (300 ul each time); Substrate solution 100ul/well were added and mixed gently, and we covered the sticker, then we put them on a flat rocker to respond for 15 min (31°C,150 rpm/min); Stop solution 100ul/well were added. It is obvious that the liquid in the hole changed from blue to yellow. After adding, we gently shook and mixed them to make them fully responsive. We then read absorbance at wave length of 450 nm and 590 nm by enzyme labeling within 5 min; Blank values are 450 readings for blank well minus 590 readings. For the remaining wells, the reading value were 450 readings minus 590 readings and then minus the blank value. After making the standard curve, the formula was used to calculate the content of factors in the sample.

Ethics Approval and Consent to Participate

This study follows the Basel Declaration of 2010. Most of the authors of this article have been trained in animal experiments and have obtained a certificate of competency. We used animals to a minimum following animal welfare principles without affecting the accuracy of the experiment. The commissioned experimental unit has qualifications and right to use animals, approved by the Institution Animal Care and Use Committee (IACUC) of Xi'an United Nations Quality Detection Technology CO., Ltd (no.20220722). All applicable international, national, and/or institutional guidelines for care and use of animals were followed.

Statistical Analysis

SPSS v27 (IL, USA) was used for data analysis. ANOVA and F-test were used to measure and analyze the data between groups. Pairwise comparisons within groups were performed using the least significant differences (LSD) test if the variance was homogeneous and Dunn's test was used for uneven variance. Gut microbial counts are logged by 10 and visualized using SPSS27. A log10 of 0 is defined as a missing value which were replaced by serial mean. $p<0.05$ was considered statistically significant.

Results

Comparison of General Indicators Between Groups

The SPSS software used was legitimate. The average starting weight of all rats before the experiment was 172.6 ± 2.49 g, and there were no significant differences among groups A–G ($p> 0.05$). There were significant differences in groups A (control) to G (treated) for weight, food intake, water intake, and stool samples 2 hours after antibiotic use on all involved days (1st, 3rd, 5th, 7th, 9th, 11th, and 14th days) ($p= 0.04, 0.016, <0.001$, and 0.009, respectively). Means and standard deviations are shown in Table 1 and Figure 1A–D).

Comparison of Total Microbiota Between Groups

Nine intestinal floral bacterial species (*Staphylococcus aureus, Bifidobacterium*, yeast, *Bacteroides, Clostridium, Anaerobic bacteria, E. coli, Enterococcus*, and *Lactobacillus*) were cultured using a special medium for each (shown in Table 2) and counted with an upright microscope (Ni-U, Japan) (Figures 2 and 3). Microbial loads for these nine organisms at the 1st, 4th, 8th, 11th and 14th days for each animal group were compared. The means, standard deviations, and 95% confidence interval (CI) of each group are as follows: (A): 1376.7 ± 3683.8(95% CI: 562.15–2191.26); (B): 687.06 ± 1498.74 (95% CI: 355.65–1018.45); (C): 454.89 ± 2187.53 (95% CI: 548.96–2400.83); (D): 478.17 ± 1758.11 (95% CI: 65.03–891.31); (E): 664.50 ± 1567.91(95% CI: 317.80–1011.19); (F): 403.77 ± 1171.99 (95% CI: 144.62–662.92); (G): $3609.76 \pm 21,206.52$ (95% CI: −1079.38 ± 8298.91) (CFU/g). After being performed by Log10 method, there were significant differences among groups A/D ($p < 0.001$), A/F ($p < 0.001$) and A/G ($p = 0.013$) comparing the

Table 1 Comparison of Basic Status for Rats on All Involved Days $(g, \overline{X} \pm S)$

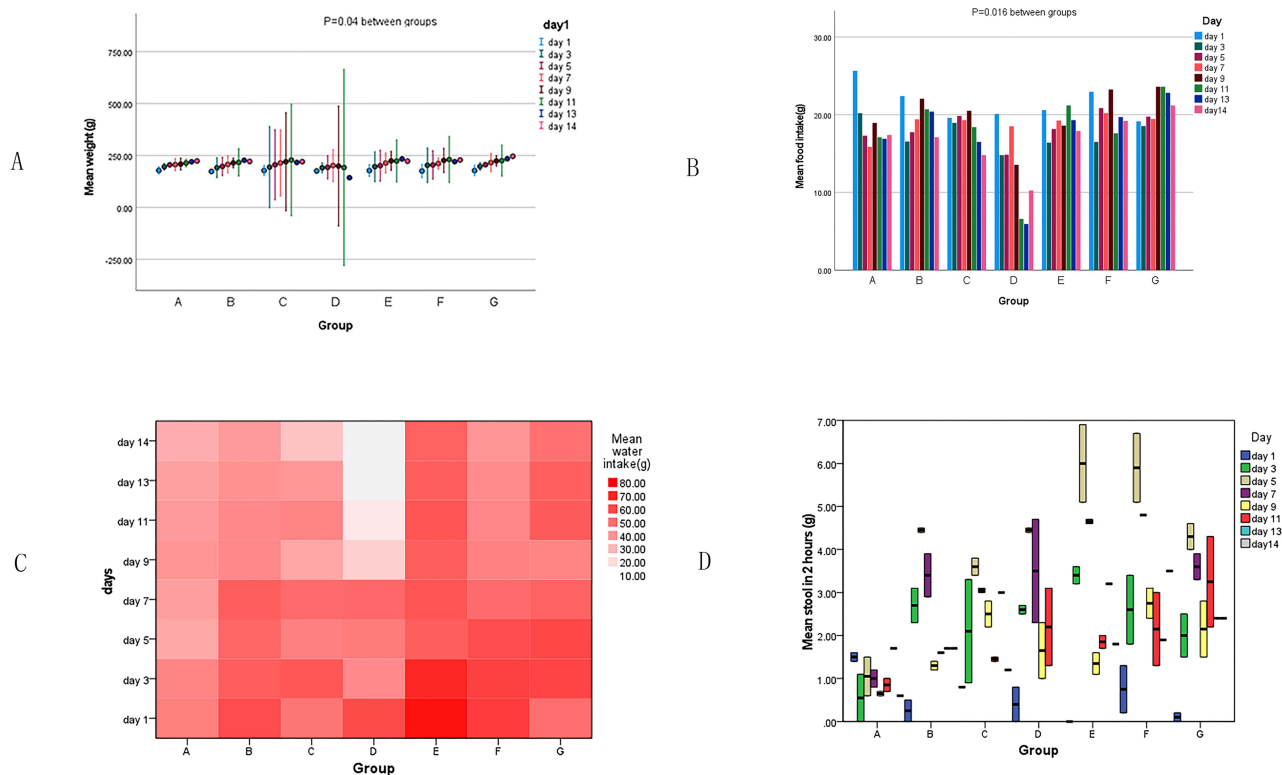|	Weight	Food Intake	Water Intake	Stool in 2h
A	204.02±13.5	19.02±3.63	38.86±4.73	0.96±0.48
B	203.31±17.2	19.58±2.83	49.76±11.31	2.20±1.38
C	208.09±22.15	18.93±2.75	44.40±9.24	2.23±1.09
D	188.13±24.05	15.47±5.66	41.96±18.95	2.47±1.53
E	209.00±18.58	18.80±2.11	60.33±9.02	2.82±2.01
F	210.70±19.18	20.31±3.57	52.68±10.23	3.09±1.79
G	212.12±20.31	20.66±3.13	53.47±7.84	2.33±1.55
F	2.313	2.807	6.213	3.043
P	0.04	0.016	<0.001	0.009

Figure 1 Basic indice of the rat. (**A**) Error bar graph of body weight in different groups on days 1, 3, 5, 7, 9, 11, 13, 14, there is significant between groups (P = 0.04); (**B**) Bar graph of food intake of rats in different groups on days 1,3,5,7,9,11,13,14, there is significant between groups(p = 0.016); (**C**) Heatmap of water intake of rats in different group on days 1, 3, 5, 7, 9, 11, 13, 14, there is significant between groups (P < 0.001); (**D**) Boxplot graph of stools in different groups of rats for 2 hours on days 1, 3, 5, 7, 9, 11, 13, 14, there is significant between groups (P = 0.009). Groups: A Control; B Low single dose; C Middle single dose; D High single dose; E Low triple dose; F Middle triple dose; G High triple dose.

total nine microbiota on all collected days, whereas there were significant differences between groups (F = 3.811, p < 0.001). Details are shown in (Figure 4A).

The microbial loads for all nine organisms were performed by Log10 and compared among groups A–G daily, using before and after weighted methods and F-test. All comparisons of nine microbiota variables between groups (from A to G) were significantly different (p < 0.001) (Figure 4B and C).

Comparison of Microbiota by Log 10 on Different Days Between Groups

Further analysis was conducted to explore the differences in the nine microbial species existed between group A (Control) and groups B–G (Experiments) on days 1, 4, 8, 11 and 14 by Log10 methods. On the 1st day, there were

Table 2 Culture Method for Nine Microbiota Species

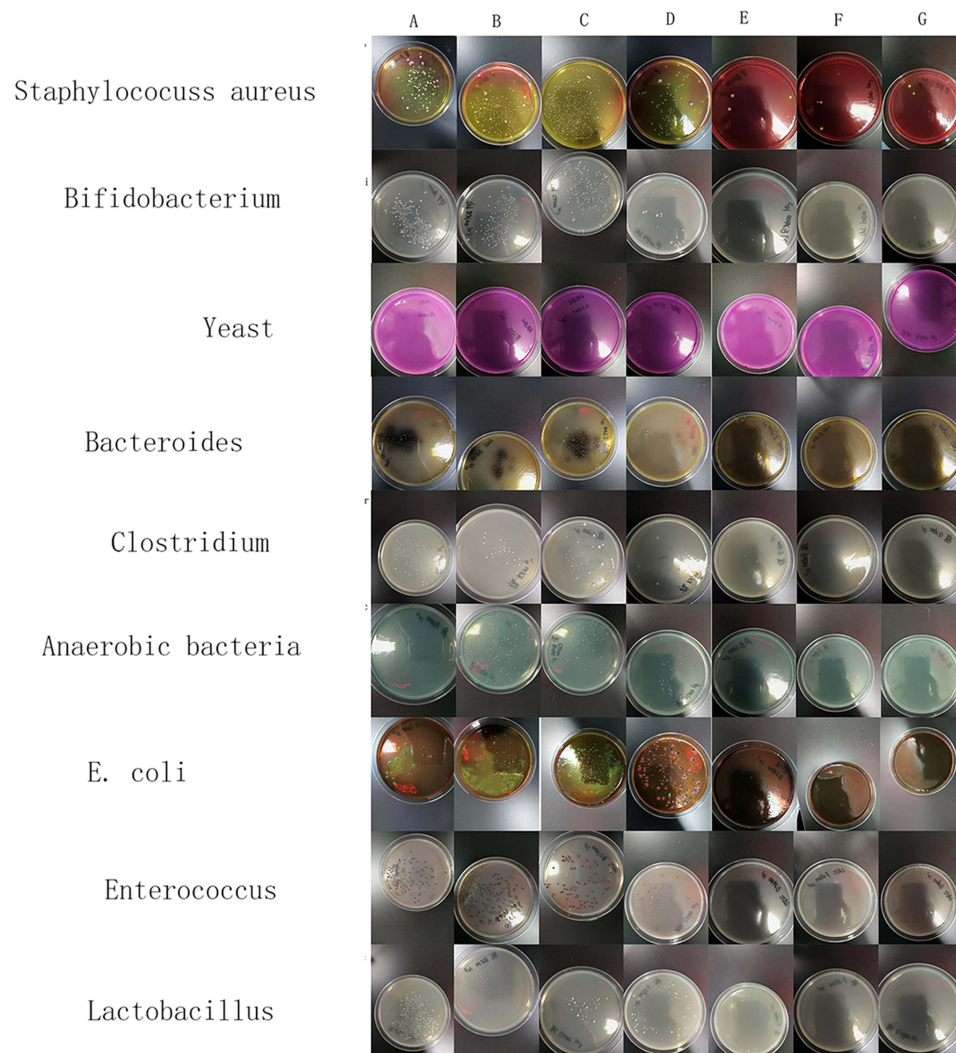| Microbiota	Culture Media	Culture Condition
Staphylococcus aureus	Mannitol sodium chloride agar medium	Aerobic
Bifidobacterium	TPY agar medium	Anaerobic
Yeast	DRBC agar	Aerobic
Bacteroides	Bacteroides-bile-escin agar (BBE)	Anaerobic
Clostridium	Reinforced cloaca culture medium	Anaerobic
Anaerobic bacteria	Anaerobic agar	Anaerobic
E. coli	Eosin blue agar medium(EMB)	Aerobic
Enterococcus	CATC agar	Aerobic
Lactobacillus	Lactic acid Bacillus selective agar	Anaerobic

Figure 2 On the fourth day, 9 kinds of microbiota culture pictures. Colony forming unit (CFU). Mannitol sodium chloride agar medium (culture of Staphylococcus aureus,48h at 37 °C); TPY agar medium (culture of Bifidobacterium, 48h at 37 °C); DRBC agar (cultured yeast, 5 days at 28°C); Bacteroides-bile-escin agar (BBE) (culture of Bacteroides, 48h at 37 °C); reinforced cloaca culture medium (culture of Clostridium, 48h at 37 °C); anaerobic agar (culture of anaerobic bacteria, 48h at 37 °C); Eosin blue agar medium (EMB) (culture of E. coli, 48h at 37 °C); CATC agar (cultured Enterococcus, 48h at 37 °C); lactic acid Bacillus selective agar (cultured Lactobacillus, 48h at 37 °C). Groups: A Control; B Low single dose; C Middle single dose; D High single dose; E Low triple dose; F Middle triple dose; G High triple dose.

no significant differences in the microbiota composition between group A and groups B–G (all $p>0.05$). On the 4th day, there were significant differences in the microbiota composition by comparing A/B ($p=0.044$), A/D ($p<0.001$), A/E ($p=0.029$), others have no significance. On the 8th day, there were significant differences in the microbiota composition between group A and groups B–G ($p= 0.003, <0.001, <0.001, <0.001, 0.002$, and <0.001, respectively). However, on the 11th, day there was only significant differences in the microbiota composition at A/F ($p<0.001$), other groups have no significance compared with A group (all $p>0.05$). Only significant difference was also observed on the 14th day at A/D (Figure 4D and E). These changes show that the intestinal flora gradually returned to normal after drugs were stopped.

Histological Changes in Intestinal Tissues

Pathological colonic images were observed under a microscope after HE staining (×100). In Figure 5, the left panel is the dissection of the rat colon tissue on day 11, and the right is the dissection of the rat colon tissue on day 15. On day 11, in group A, no obvious neutrophil infiltration and mucosal edema were observed and in groups B–G, neutrophil infiltration was severe, and mucosal edema became obvious; further, mucosal necrosis was observed, and the focal ulcer was formed; On day 15 in group A, no obvious neutrophil infiltration and mucosal edema were observed and in groups B–G,
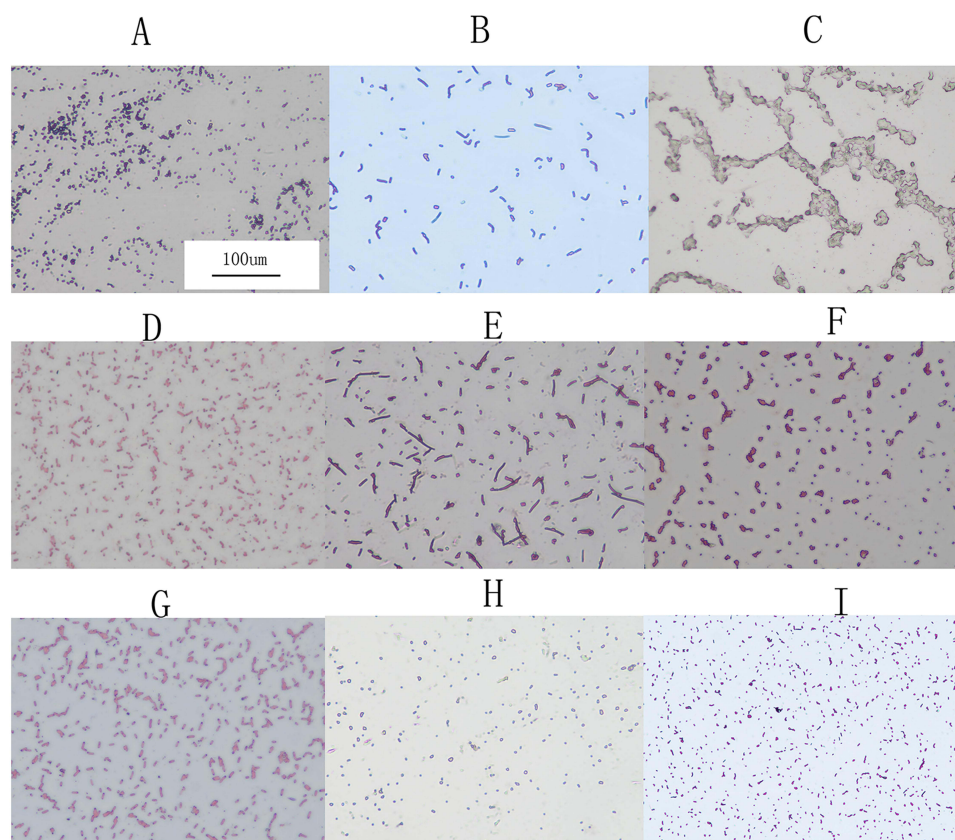
Figure 3 Pictures of counted microbiota specimen under a microscope on fourth day using Melan staining. CFU/g=number of plate colonies ×50×dilution factor. (**A**) Staphylococcus aureus, (**B**) Bifidobacterium, (**C**) yeast, (**D**) Bacteroides, (**E**) Clostridium, (**F**) anaerobic bacteria, (**G**) Escherichia coli, (**H**) Enterococcus and (**I**) Lactobacillus.

neutrophil infiltration was severe, and mucosal edema became obvious; further, mucosal necrosis was observed, and the focal ulcer was not formed. The colic inflammation was significant mild on day 15 comparing with that on day 11 (Figure 5).[22]

Comparison of Inflammation Factors Between Groups

There were significant differences in the levels of TNF-α between group A and groups B–G (p = 0.021, 0.048, 0.012, 0.046, 0.025, and 0.012, respectively) with ANOVA showing a significant difference within groups (F = 2.343, p < 0.001). Significant differences in IL1-β were observed between group A and groups B–G (p = 0.042, 0.033, 0.020, 0.047, 0.022, and 0.015, respectively) with ANOVA showing F = 1.186, p < 0.001. Significant differences existed among all groups regarding IL-6 comparison as follows: A(B/A, p = 0.012; C/A, p = 0.025; D/A, p = 0.047; E/A, p = 0.022; F/A, p = 0.015; G/A, p = 0.001); ANOVA analysis showed F = 1.337, p < 0.001. Significant differences in CRP levels were observed between group A and groups B–G (B/A, p= 0.023; C/A, p = 0.042; D/A, p < 0.001; E/A, p < 0.001; F/A, p = 0.001; G/A, p < 0.001), with ANOVA showing F = 2.807, p < 0.001 (Figure 6).

Discussion

Clinically, we have witnessed an increase in the incidence of intestinal dysfunction. Medical history has revealed various reasons for this, such as bad living habits, irregular work, and long-term use of antibiotics. Antibiotics are indispensable in modern medicine; however, proper use of these medications and standardized medical procedures are constantly demanded of most physicians. The gradual promotion of Enhanced Recovery After Surgery (ERAS)[23–26] has reduced the clinical application of antibiotics, intestinal complications, and other complications caused by double infections. This study used rats to explore the effects of antibiotics on the intestinal flora, focusing on nine common microbial organisms,
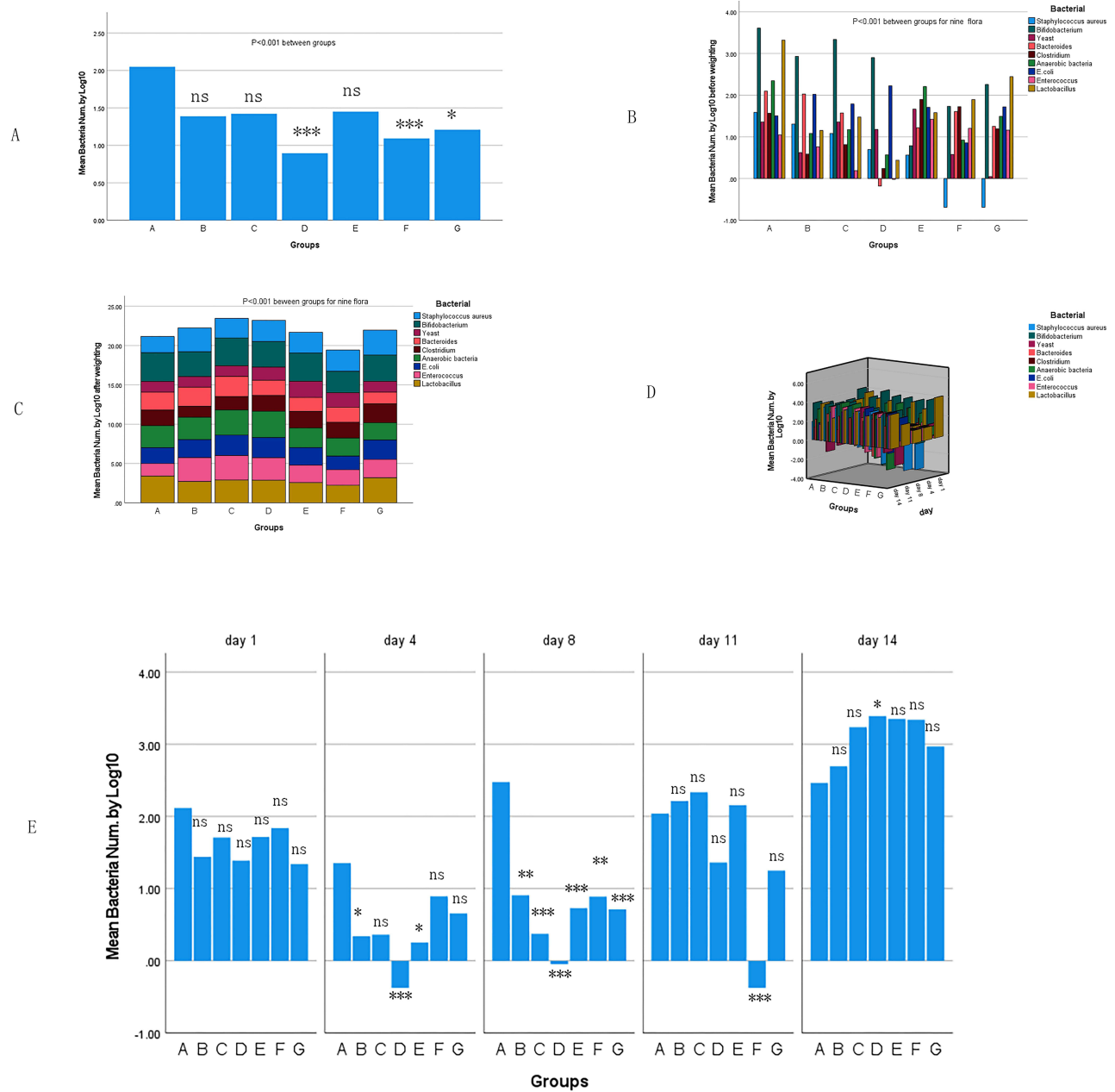
Figure 4 Nine kinds of microbiota by Log10 of all involved days were analyzed between groups. (**A**) Nine microbiota in each group were compared using ANOVA and Dunn's, showing A/B: P=0.064; A/C:P = 0.128; A/D:P < 0.001; A/E:P=0.164; A/F:P < 0.001; A/G:P = 0.013; (**B**) Nine microbiota in involved days were compared between A to G groups before weighting days (*F*-test), P < 0.001; (**C**) Nine microbiota in involved days were compared between A to G groups after weighting days (*F*-test) P < 0.001; (**D**) 3D maps of nine microbiota in different groups and days, *Bifidobacterium* and *Lactobacillus* were significantly higher in the G group on the first day than the other groups. (**E**) 9 microbiota of A group were compared to B - G group at first,4th,8th,11th, 14th days: at day 1, all P>0.05; at day 4, A/B (P = 0.044), A/D(P < 0.001), A/E(P = 0.029), others P > 0.05; at day 8 all P < 0.01, at day 11, A/F (P < 0.001), others P > 0.05; at day 14, A/D (P = 0.045), others P > 0.05. ns indicates no significance compared with control group; *Indicates P<0.05 compared with control group; **Indicates P < 0.01 compared with control group; ***Indicates P<0.001 compared with control group. Groups: A Control; B Low single dose; C Middle single dose; D High single dose; E Low triple dose; F Middle triple dose; G High triple dose.

both beneficial and pathogenic. *Campylobacter jejuni*, suggesting that both chemical and biological agents were useful to induce colitis in the rat animal model.[27] Researchers have also utilized the rat model to investigate the impact of fiber-rich diets on intestinal microbial community structure.[28,29] Based on these publications, we chose rat as a model for our research on intestinal flora following antibiotic use. This study showed that the body weight of rats changed after antibiotic molding, suggesting that antibiotics influence the nutritional status of the rat. This may be because glycolipid disorders are closely related to the intestinal flora and its metabolites.[30,31] Here, food intake decreased and water intake
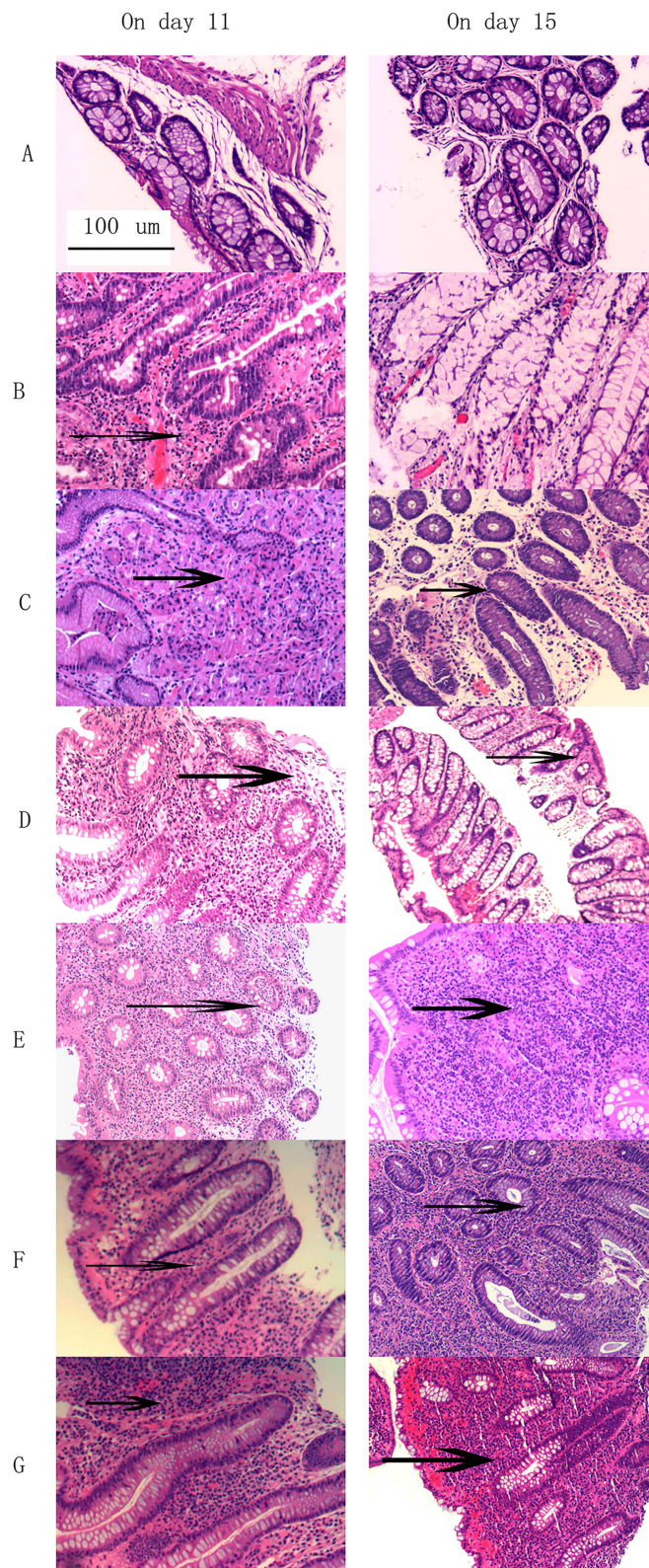
On day 11　　　On day 15

Figure 5 Pathological image of colonic HE staining (×100) of groups: (**A**) Control; (**B**) Low single dose; (**C**) Middle single dose; (**D**) High single dose; (**E**) Low triple dose; (**F**) Middle triple dose; (**G**) High triple dose. The left panel is the dissection of the rat colon tissue on day 11, and the right is the dissection of the rat colon tissue on day 15. (**A**) (left, and right): no obvious neutrophil infiltration; (**B**) (left):arrow indicates light neutrophil infiltration, (**B**) (right): no obvious neutrophil infiltration; (**C**) (left): arrow indicates moderate neutrophil infiltration, (**C**) (right):arrows indicates light neutrophil infiltration; (**D**) (left): arrows indicates severe neutrophil infiltration; (**D**) (right): arrow indicates moderate neutrophil infiltration; (**E**) (left): arrow indicates light mucosal edema with neutrophil gathering. (**E**) (right): arrow indicates neutrophil gathering without mucosal edema; (**F**) (left): arrows indicates moderate mucosal edema with amount neutrophil gathering, (**F**) (right): arrow indicates light mucosal edema with neutrophil gathering; (**G**) (Left): arrow indicate severe mucosal edema, necrosis and focal ulcer. (**G**) (right): arrow indicate severe mucosal edema, moderate necrosis, no focal ulcer. On day 15, the inflammation was significant mild comparing with that on day 11.
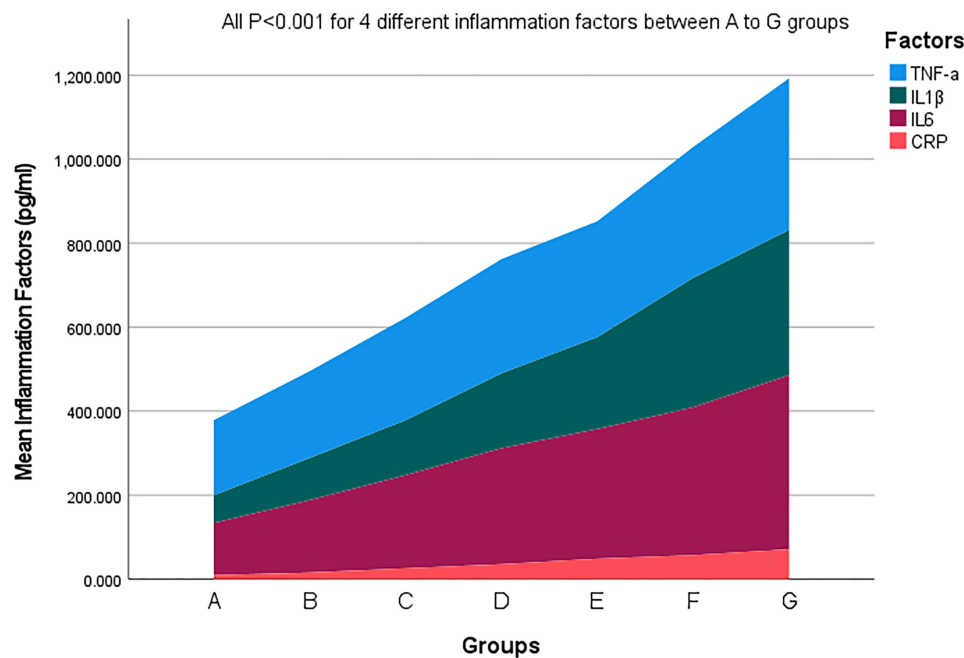
Figure 6 A regional map (pg/mL) of four inflammatory factors compared between groups: A Control; B Low single dose; C Middle single dose; D High single dose; E Low triple dose; F Middle triple dose; G High triple dose. There were a significant difference about TNF-α, IL1β, IL-6 and CRP between and within groups (all P <0.001). And the mean values from high to low are TNF-α, IL1β, IL-6, CRP.

increased significantly with an increase in antibiotic dose, and dose-dependent defecation changes were observed within 2 hours after antibiotic use. These changes indicate intestinal dysfunction, which led us to explore the changes in the intestinal micro-ecology. Previous studies[32–34] have shown that the gut microbiota is a central regulator of host metabolism, modulates body immunity, and affects the diet, such as the amount and composition of lipids. Different strains of rats have been reported to have different responses to stress,[35,36] and these differences may affect outcomes on a small scale.

The host intestinal tract contains a diverse community of bacteria, totaling 10^{13}–10^{14} bacterial cells[37], with organisms most often belonging to the *Bacteroidetes, Firmicutes, Actinobacteria, Spirochete, and Proteobacteria*phyla.[12,38,39] Here, the nine target microbial organisms studied are indicated at 10^6 (CFU/g). Homeostatic interactions between the host and the resident microbiota occur in the intestine, and microbial abundance changes might lead to intestinal inflammation.[39] Investigations have well documented that commensal microbiotas are important in maintaining a healthy intestine by preventing the overgrowth of pathogenic microorganisms and help to regulate and maintain a quiescent intestinal immune system.[40] Once the healthy flora is destroyed and the intestinal flora is disordered, damage to the intestinal mucosa can be caused by changes in the immune system through the brain-gut axis, the intestinal-bacteria axis, and abnormal flora metabolites. Reports have shown that aberrant immune responses could occur from increased exposure to the commensal microbiota.[38,41] Attack of the intestinal mucosa by immune cells can produce intestinal diseases. IBD is an intestinal inflammatory condition affecting over two million people in the United States.[42] Intestinal dysbiosis for patients with IBD has shown a characteristic pattern of decreased commensal microbial diversity, with *Firmicutes* and *Bacteroides*, the two most abundant groups in the normal flora, being decreased the most.[43–45]

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical infections. It is a leading cause of bacteremia and infective endocarditis and osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections.[46] This bacterium can reside in a wide range of host tissues, from superficial surfaces like the skin to deeper tissues like the gastrointestinal tract, heart, and bones.[47] Thus, in this study, *Staphylococcus aureus* was tested and analyzed. It decreased compared to the control group; and as the antibiotic dose increased, the decrease in bacterial load was obvious, indicating that the drug inhibited the strain. We also analyzed *S. aureus* load chronologically throughout the

tested days; we found it highest on day 14. The rebound phenomenon was obvious after stopping the drug. This might be related to the strong regeneration ability after the drug elimination, but the mechanisms are still unknown.

*Bifidobacterium*is defined as a group of living microorganism supplements, which confer health benefits on the host when administered in adequate amounts.[48] Here, the beneficial bacterium was significantly reduced in groups B, D, E, and F than in the control group; however, it was elevated in the middle-dose mono-therapy group C and the high-dose combination group G. We considered that an obvious rebound effect after stopping the drug might be observed in the middle single dose and high combined dose groups. This was also confirmed by comparison on different days.

Yeast cells are often used in industrial fermentation processes due to their ability to efficiently convert relatively high sugar concentrations into ethanol and carbon dioxide.[49] *Yeast* is not a common intestinal microbiota, and it is low in the control group. It was found increasing in the triple-agent low- and middle-dose groups (E and F). We believe this was a rare flora colonization, one manifestation of intestinal flora disorder.

Bacteroides is a genus of gram-negative, non-sporulating, obligate anaerobic *Bacillus*. This study showed a significant reduction of *Bacteroides* in the high-dose medication group, suggesting sensitivity to high-dose and combination drugs, and the rebound effect was very obvious after stopping the drug. A previous study has shown that *Bacteroides* metabolic disorders are associated with type 2 diabetes.[50]

Clostridium organisms are anaerobic, gram-positive, rod-shaped, endospore-forming bacteria belonging to the phylum *Firmicutes*, constituting both a class and genus in the phylum.[51] Infection of the colon with the gram-positive bacterium, *Clostridium difficile*, is potentially life-threatening, especially in older adults and patients who have dysbiosis of the gut microbiota following antimicrobial drug exposure. *C. difficile* is the leading cause of health care-associated infective diarrhea.[52] This study showed that compared to the control group, *Clostridium* decreased in the single-agent group but increased in the combined drug group, revealing that this organism had strong regeneration after the drug was stopped.

Anaerobic bacteria play pivotal roles in the human microbiota, and they are significant infectious agents involved in several pathological processes, especially in immunocompromised individuals. Their isolation, cultivation, and correct identification differ significantly from the workup of aerobic organisms, although using innovative technologies changes anaerobic diagnostics dramatically.[53] Here, *anaerobic bacteria* were significantly decreased in all other groups during drug feeding periods than in the control group.

Although *Escherichia coli* can be an innocuous resident of the gastrointestinal tract, it also has the pathogenic capacity to cause significant diarrheal and extraintestinal diseases. Pathogenic variants of *E. coli* have caused much morbidity and mortality worldwide.[54] Here, we found that *E. coli* increased after antibiotic drug being used, but it changed by independent drugs.

Enterococcus strains that adhere strongly to the intestinal epithelium form biofilms and possess antioxidant defense mechanisms; they seem to have the greatest influence on the inflammatory process.[55] Here, a significant decrease in this organism in groups of different doses of single and combination drugs at all tested days was observed. We found it significantly decreased on the 1st and 4th days but significantly rebounded after drugs were stopped, suggesting that both single and combined drug use affected this organism regardless of the dose.

Lactobacillus comprises 173 genera, with many genomes available to study taxonomy and evolutionary events, as reported by a previous study.[56] As a probiotic, *Lactobacillus* was significantly reduced in groups B-G than in the control group on the 4th, 8th, and 11th days. We found that *Lactobacillus* peaked on the 1st day and did not rebound significantly after the drugs were stopped (Figure 4B and C). This proves that the drug killed *Lactobacillus* by continuous administration.

Some recent literatures[57–59] showed antibiotic use and microbial imbalance in murine models which were similar with this study. Microbiota disorders associated with IBD have been reported by previous literature.[60–62] Our study showed that colon tissue inflammation became more server in a dose-dependent manner (Figure 5). Intestinal microecological disorders can also affect the immune system. This information is propagated to T-cell populations through the secretion of cytokines to facilitate cell maturation and proliferation.[63] Fitzpatrick et al[16] found Interleukin (IL)-12 and tumor necrosis factor (TNF)-alpha peaked during the earlier postnatal time points and then declined after repetitive administration of the hapten but, IL-13 and IL-17 were consistently elevated using intracolonic 2,4,6-trinitrobenzene sulfonic acid (TNBS) rat model. This study found that TNF-α and IL-6 were significantly higher in experimental groups than in the control group, and there was a significant relationship with the dose administered. Inflammation induces IL1-β

production in Kupffer cells and hepatocytes.[64] In this study, the IL1-β was significantly greater in the groups adminis-tered drug doses than in the control group. CRP is a novel topic in the study of inflammation and related diseases.[65–67] It is associated with chronic inflammation; thus, here, CRP was examined in the abdominal blood of the SD rats. The results showed that elevated CRP had a similar relationship with groups receiving antibiotics as did other inflammatory factors earlier described. The four inflammation factors increased depending on the drug dose. An increase in the single and combined dose led to an increase in intestinal flora disturbance, with the four target inflammatory factors in serum increasing significantly in the humoral and cellular immune mechanisms (Figure 6).

Based on our study, to avoid antibiotics-induced intestinal microbial disorders and pathological reactions, we recommend that prophylactic antibiotics use should not be advocated for aseptic surgery clinically. Other non-severe bacteria are moving to minimize the dose and course of antibiotic use. This is consistent with the guidelines for the clinical use of antibiotics.[68]

However, this study has some shortcomings. We did not explore the mechanisms via which intestinal flora disorder causes rat intestinal inflammation, and we did not study intestinal dysfunction after antibiotic-induced flora disorder. Further, we did not genetically analyze the gut target flora in this paper.

Conclusions

Antibiotics can cause disorder in the intestinal target flora organism of rats, without apparent law of the doses and combination of antibiotic use. Antibiotics can also cause an inflammatory response in the blood system of rats with intestinal inflammation changes of colon. The current research provides a basis for intestinal inflammatory diseases caused by intestinal microbial disorders. Antibiotic-induced flora imbalance might be applied for establishment of IBD model.

Abbreviations

BBE, Bacteroides-Bile-Enterprise; CI, Confidence interval; CRP, C-reactive protein; DRBC, Dichloran Rose Bengal Chloramphenicol; ELISA, Enzyme-linked immune sorbent assay; EMB, Eosin Methylene Blue; ERAS, Enhanced Recovery After Surgery; IACUC, Institution Animal Care and Use Committee; IBD, Inflammatory bowel disease; LSD, Least significant differences; SD, Sprague Dawley; TPY, Trypticasephytone yeast; ANOVA, Analysis of variance; CATC, Citrate Azide Tween® Carbonate; TNBS,2,4,6-trinitrobenzenesulfonic acid model; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; IL1-β, Interleukin-1β; SAP, Serum amyloid P-component.

Data Sharing Statement

All raw data were shared in Science Data Bank (https://www.scidb.cn/anonymous/bWFlWU5y), Number: 10.57760/sciencedb.09521.

Ethics Approval and Consent to Participate

This study follows the Basel Declaration of 2010. Most authors of this article have been trained in animal experiments and have obtained a certificate of competency. We used animals to a minimum following animal welfare principles and without affecting the accuracy of the experiment. Xi'an United Nations Quality Detection Technology Laboratory was commissioned to perform our experiments under his IACUC permission (No.20220722). All applicable international, national, and/or institutional guidelines for care and use of animals were followed.

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Disclosure

The contents of this manuscript have not been copyrighted or published. There are no related manuscripts or abstracts, published or unpublished, by any authors of this manuscript. The authors declare no conflicts of interests.

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