

Changes in the Gut Microbiota Diversity of Brown Frogs (*Rana Dybowskii*) After Local Antimicrobial Therapy

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Abstract

Background: Amphibians frequently receive an antibiotic bath after feedlot placement to control bacterial diseases. The potential collateral effect of these antibiotics on the frog microbiota is largely unknown. Antibiotics are frequently employed to examine the role of the gut microbiota. Existing research relies mainly on oral antibiotics, but knowledge regarding the effects of antibiotics on the gut microbiota through a bath or local antimicrobial therapies is limited.

Results: The gut microbiota of gentamicin, recovered, and control *Rana dybowskii* groups were compared by Illumina high-throughput sequencing, and the functional profiles were analysed using a phylogenetic investigation of communities by the reconstruction of unobserved states (PICRUSt). Furthermore, the relationship between gut microbiota structures and forecast function compositions was determined. The results showed that the alpha diversity indices were significantly reduced by the gentamicin bath, which significantly changed the composition of the gut microbiota. After 7 days, the gut microbiota was still similar to that during the gentamicin bath. Forty-four indicator species were selected at the genus level, namely, 42 species indicating the control group and 2 species indicating the gentamicin and recovery groups. Potential pathogenic bacteria belonging to *Aeromonas*, *Citrobacter*, and *Chryseobacterium* significantly decreased after the gentamicin bath. The community similarity assays did not show an obvious discrepancy in the functional composition between the gentamicin and control frogs, indicating that the functions of the gut bacterial community were highly redundant.

Conclusions: The gentamicin bath significantly reduced the alpha diversity of the gut microbiota of *R. dybowskii*. Gentamicin significantly changed the structure of the gut microbiota, and the gut microbiotas exhibited weak resilience and did not totally recover after seven days. The gentamicin bath did not change the functional composition of the gut microbiota of *R. dybowskii*, and there was no significant correlation between the composition of the gut microbiota and the functional composition, illustrating the high intestinal functional redundancy of the frog gut bacterial community. This work offers basic data for upcoming research, including the establishment of the amphibian gut microbiota and local antibiotic administration, and has important implications for aquaculture management and amphibian conservation.

1 Background

The diversity of bacterial communities is closely related to animal health, especially the gut diversity of the animal microbiota [1]. The gut bacterial community is dynamic, and its composition is continually changing to cope with changes in the internal and external environment [2]. The gut bacterial community can form a barrier against pathogens, producing many essential products and playing a role in the digestion, gut morphology, immunity, and regulation of host gene expression [3]. With the development of high-throughput sequencing technology, microbial diversity has gradually attracted attention. Currently, research investigating the gut bacterial community of farmed animals is rapidly increasing, reflecting the

increasing importance of the role of the intestinal bacterial community in animal health [4]; however, research investigating aquatic products, especially amphibians, has not attracted much attention.

The use of antibiotics is essential in aquaculture but is associated with several adverse side effects related to the perturbation of the microbiota [5]. Various antibiotics have been used to prevent such outbreaks in aquaculture sectors. Antibiotics may be used to treat gastrointestinal infections in amphibians; however, other diseases, including bacterial diseases (such as red leg syndrome), diseases related to improper husbandry (such as sunburn), and protozoan parasites (such as sporozoans), may also be treated with antibiotics [6]. Many studies have shown that the use of antibiotics has a significant impact on the animal intestinal bacterial community, such as a reduction in bacterial diversity, changes in the species composition, the introduction of new species, and the total eradication of existing species [7, 8]. The widespread use of antibiotics has led to the rapid emergence of drug-resistant bacteria and severely damaged the ecological stability of animals' healthy microbiome and species diversity [9]. However, studies investigating the effects of antibiotics on the intestinal bacterial community of amphibians have not been reported.

Local antimicrobial therapy is a common treatment for diseases that may affect the microbiome in a local part of the body [3]. For microbial infections of the skin, local antibiotic treatment has many benefits as follows: the local concentration of drugs is high, adverse reactions are infrequent, and the risk of bacterial resistance and cross-resistance is reduced [10]. Transdermal absorption is a complex process that includes applying chemicals to the skin that are metabolized by enzymes in the skin and bind skin components. However, transdermal absorption also involves the passive diffusion of a chemical through the epidermis into the capillaries of the dermis [11]. The gills and skin of amphibians are highly permeable, resulting in their introduction as a remarkable indicator for assessing the harmful effects of pollution [12]. In research, antibiotics are frequently employed to examine the role of the microbiota. However, these studies mainly use oral antibiotics, and very limited research attempting to understand the effects of antibiotics on the bacterial community in other parts of the body, such as the intestines, through a bath or local antimicrobial therapies has been performed.

Functional predictions can connect gut microbiota function with the gut microbiome structure and probably further explain dysbiosis/perturbation [13, 14]. The functional redundancy of gut microbes may differ depending on the species [15]. Each species of frogs has a unique gut microbiota structure, but bacterial biofunctions are similar among species [16]. Functional redundancy heavily affects the consequences of orbits caused by differences in age, diets, and diseases [17]. Gut microbiotas significantly differ between normal and diseased animals (e.g., frogs and shrimps), and the community is significantly and positively correlated with the functional composition [14, 18]. Disease may also be accompanied by the disappearance of functional excess, which is likely related to the intensity and advancement of dysbiosis [17]. Thus far, studies investigating the properties of the gut microbiota in amphibians are scarce; specifically, few functional studies investigated how community variations impact microbially modulated functions.

Rana dybowskii (brown frog) is a major aquaculture species with medical and nutritional values in China [18]. The current stagnation of the frog culture industry is mainly caused by diseases, primarily bacterial diseases [12]. If a disease is found during the breeding process of *Rana dybowskii*, it is difficult to carry out effective treatment [12]. Mainly, frogs are ingested quickly after the onset of disease, and drugs are difficult to administer orally; furthermore, due to the number of farmed frogs, small individuals and susceptibility to stress, the intramuscular injection and intravenous injection methods are difficult to achieve [12]. Therefore, frog disease prevention and appropriate methods of administration are particularly important. To better understand the effects of local antimicrobial therapy on the intestinal bacterial community of the brown frog, some brown frogs were subjected to a gentamicin bath treatment (G group), and the gut microbiota was examined and compared with that in the control (C group) and recovery (R group) groups. In this study, we hypothesized that (1) the gentamicin bath affected the microbial diversity of the frog gut bacterial community, (2) the gut microbiota would return to the pre-treatment state within 7 days after the bath, and (3) the changes in the gut microbiota result in changes in function.

2 Results

2.1 Alpha diversity and the core microbiota

The ACE, Chao1, Shannon, and Sob indices showed a tendency to first decrease and then increase. The ACE, Chao1, and Shannon indices differed among the control, gentamicin, and recovery groups (Kruskal-Wallis H test; $P < 0.05$). The ACE, Chao1, and Shannon indices differed between the antibiotic bath group and control group (Wilcoxon rank-sum test; $P < 0.05$, Fig. 1). However, there were no differences in the ACE, Chao1, Shannon, and Sob indices between the antibiotic bath group and recovery group (Wilcoxon rank-sum test; $P < 0.05$, Fig. 1). The rarefaction curves tended to plateau, indicating that the amount of sampling is reasonable and that more sampling produces only a small number of new OTUs (Fig. S1).

The Venn diagrams showed that the number of unique OTUs in the gentamicin bath group (21) was lower than that in the recovery group (60) and control group (119) (Fig. S2). As the number of samples increased, the number of core OTUs in the control (C) group decreased to a lesser extent, while that in the gentamicin group decreased to a more significant extent (Fig. S3a). The core OTU numbers in the control, gentamicin, and recovery groups and all 21 frogs were 47, 25, 39, and 16, respectively (Fig. S3b). The 16 core OTUs were from four phyla, including 8 from Bacteroidetes, 3 from Actinobacteria, 3 from Proteobacteria, and 2 from Firmicutes (Table S1). The most abundant core OTUs were OTU198 (*Vagococcus*), OTU179 (*Citrobacter*), OTU417 (*Bacteroides*), OTU38 (*Bacteroides*), OTU333 (*Faecalitalea*), and OTU753 (*Arthrobacter*) (Table S1).

2.2 Beta diversity

The gut bacterial community composition differed between the G and C groups and between the R and C groups based on the Bray-Curtis dissimilarity matrix (Adonis: $P < 0.05$; ANOSIM: $P < 0.05$; Table 1 and Fig. 2) and the unweighted UniFrac distances (Adonis: $P < 0.05$; ANOSIM: $P < 0.05$; Table 1 and Fig. 2).

However, the gut bacterial community composition did not significantly differ between the G and R groups based on the Bray-Curtis dissimilarity matrix (Adonis: $P > 0.05$; ANOSIM: $P > 0.05$; Table 1 and Fig. 2) and unweighted UniFrac distances (Adonis: $P > 0.05$; ANOSIM: $P > 0.05$; Table 1 and Fig. 2). The gut bacterial community remarkably split into two major groups on the NMDS plot that corresponded to the C group and the G + R group (Fig. 2).

Table 1
Pairwise comparisons showing differences in the gut bacterial community in different groups.

	Bray-Curtis				Unweight UniFrac			
	ANOSIM		Adonis		ANOSIM		Adonis	
	R	P	R^2	P	R	P	R^2	P
C vs. G	0.489	0.002	0.214	0.006	0.249	0.023	0.146	0.019
G vs. R	0.022	0.329	0.081	0.362	0.095	0.174	0.094	0.192
C vs. R	0.338	0.010	0.164	0.018	0.270	0.037	0.154	0.033
All	0.284	0.005	0.204	0.005	0.207	0.018	0.170	0.015

C indicates the control group, G indicates the gentamicin group, and R indicates the recovery group.

2.3 Composition of and differences in the gut microbiota

The taxonomic assignment analysis showed that the most abundant phyla in the control, gentamicin, and recovery groups were Firmicutes (C: 38.19%, G: 15.90%, R: 30.09%), Bacteroidetes (C: 35.15%, G: 31.00%, R: 40.94%), Proteobacteria (C: 21.96%, G: 44.74%, R: 23.44%), and Actinobacteria (C: 3.23%, G: 5.37%, R: 3.38%) (Figs. 3a and S4). In total, 10 phyla were shared among all groups, and no bacterial phylum was found to be significantly different in abundance in the control, gentamicin, and recovery groups (Kruskal–Wallis H test, FDR correction, CI: Scheffer, $P > 0.05$).

At the genus level, the most abundant microbial genera were *Bacteroides*, *Morganella*, *Vagococcus*, *Faecalitalea*, *Parabacteroides*, *Arthrobacter*, *Alistipes*, *Pseudomonas*, and *Myroides* (Fig. 3b and S5). Of all 290 genera, the 5 bacterial genera *Crenobacter*, *Morganella*, *unclassified_f_Eggerthellaceae*, *unclassified_f_Veillonellaceae*, and *Weissella* exhibited significant differences among the control, gentamicin, and recovery groups (Kruskal–Wallis H test, FDR correction, CI: Scheffer, $P < 0.05$; Fig. S5).

The LEfSe showed that Fusobacteria were significantly enriched in the control group (LDA > 2 , $P < 0.05$, Fig. 4a). This bacterial taxon with significant differences accounted for most of the control group, while the gentamicin and recovery groups had fewer bacterial taxon (Fig. 4a). The LEfSe analysis at the genus level revealed that *Morganella*, *CL500_29_marine_group*, *Paenarthrobacter*, and *Plesiomonas* were significantly enriched in the gentamicin group and that *Butyricicoccus*, *Corynebacterium_1*, *Enterococcus*, *Phascolarctobacterium*, *Providencia*, *Vagococcus*, and *Weissella* were significantly enriched in the recovery group (LDA > 2 , $P < 0.05$, Fig. 4a). When LDA > 4 , the LEfSe analysis showed that at the genus

level, *Citrobacter* (C group), *Morganella* (G group), and *Vagococcus* (R group) were significantly enriched (LDA > 4, $P < 0.05$, Fig. 4b).

2.4 Indicator taxa of frog gut dysbiosis and potentially pathogenic genera

At the genus level, forty-four indicator species were selected, including 42 species indicating the C group and 2 species (*Butyricoccus* and *Morganella*) indicating the G + R group (Fig. 5). A heat map depicting the normalized abundances of the 44 indicator taxa across the samples was generated and showed their abilities to discriminate among the samples according to the sample grouping process (Fig. 5).

The distribution and comparison of potentially pathogenic genera in the gut of the control, gentamicin, and recovery groups are shown in Table 2 and Fig. S7. *Aeromonas*, *Acinetobacter*, and *Chryseobacterium* differed among the control, gentamicin, and recovery groups (Kruskal-Wallis H test; $P < 0.05$). The relative abundance of the bacterial genera belonging to *Aeromonas*, *Citrobacter*, and *Chryseobacterium* was significantly decreased in the G group (Wilcoxon rank-sum test; $P < 0.01$, Table 2). After 7 days of recovery, *Aeromonas*, *Citrobacter*, and *Chryseobacterium* still significantly differed between the C and R groups (Wilcoxon rank-sum test; $P < 0.01$, Table 2).

Table 2

Changes in the relative abundance of potentially pathogenic genera after antibiotic baths (Wilcoxon rank-sum test).

Genus	OTU	C group (mean \pm SD)	G group (mean \pm SD)	R group (mean \pm SD)
<i>Acinetobacter</i>	OTU666, OTU528, OTU332, OTU41, OTU175, OTU173 OTU160	1.51 \pm 3.06 ^a	0.14 \pm 0.18 ^a	0.09 \pm 0.12 ^a
<i>Aeromonas</i>	OTU2	1.25 \pm 1.98 ^a	0 ^b	0 ^b
<i>Citrobacter</i>	OTU179, OTU74	12.70 \pm 11.14 ^a	0.85 \pm 1.71 ^b	3.16 \pm 3.82 ^b
<i>Chryseobacterium</i>	OTU413	0.28 \pm 0.41 ^a	0.01 \pm 0.01 ^b	0 ^b
<i>Proteus</i>	OTU405	0.01 \pm 0.01 ^a	0.01 \pm 0.02 ^a	0 ^a
<i>Pseudomonas</i>	OTU192, OTU552, OTU185	3.06 \pm 3.51 ^a	1.98 \pm 3.81 ^a	2.71 \pm 5.04 ^a
<i>Staphylococcus</i>	OTU222, OTU733	0.01 \pm 0.01 ^a	0.02 \pm 0.04 ^a	0.03 \pm 0.03 ^a
<i>Streptococcus</i>	OTU346, OTU322	0.01 \pm 0.02 ^a	0.01 \pm 0.0 ^a	0 ^a

2.5 Relationship between the bacterial community structure and function

Three hundred functional pathways were obtained in the C and G + R groups. The principal coordinate analysis did not show significant differences in the functional composition between the C and G + R groups (Fig. 6a). Similarly, the bacterial community similarity test did not show a significant difference in the functional composition between the C and G + R groups (C:G + R, ANOSIM statistic $R = -0.041$, $P = 0.637$, Fig. 6a).

The linear regression analysis showed that the gut microbiota composition and functional composition of the C and G + R groups were not significantly and positively correlated (C and G + R: $r = 0.125$, $P = 0.079$, Fig. 6b), indicating that changes in the gut microbiota of *R. dybowskii* did not alter bacteria-mediated physiological functions. Significant differences were observed in six KEGG pathways (cancers: overview, circulatory system, environmental adaptation, excretory system, infectious diseases: bacterial, and substance dependence) among the control, gentamicin, and recovery groups (Kruskal-Wallis H test, $P < 0.05$; Fig. S8).

3 Discussion

3.1 Variation in gut microbiota diversity

Antibiotics are often used in research to examine the role of the microbiota. However, these studies mainly use oral antibiotics, and few studies have examined the effects of antibiotics on the microbiota of other parts of the body (such as the intestine) through a bath or local antimicrobial therapies. The present study showed that the ACE, Chao1, and Shannon indices of the gut microbiota in the gentamicin group were lower than those in the control group, and the Venn diagrams showed that the number of unique OTUs in the gentamicin bath group was lower than that in the recovery and control groups. These results are consistent with the microbiota of weaned piglets treated with chlortetracycline [19]. Antibiotic treatment caused changes in the alpha diversity in individual hosts of honeybees [9]. Similarly, an investigation of mosquito-eating fish exposed to antibiotics in water for 7 days showed that the antibiotics significantly reduced the diversity of the gut and skin bacterial community in the mosquito-eating fish [20]. High microbiota diversity is favourable for the fitness and overall health of animals [1]. Many studies have shown that the use of antibiotics has a critical impact on human and animal gut bacterial communities, such as a reduction in bacterial diversity, the introduction of new species, and the total eradication of existing species [7]. The use of antibiotics significantly reduces the alpha diversity of the gut bacterial community, which may be a manifestation of either the adverse side effects of antibiotics or dysbiosis of the gut bacterial community [21].

The ability of the gut microbiota to recover to the baseline level after stopping antibiotics may vary depending on differences in the antibiotic administration, the host species, the community context, and environmental reservoirs [2, 22]. In the present study, the gentamicin bath significantly changed the composition of the gut bacterial community; however, after 7 days, the gut microbiota was still similar to that during the gentamicin bath. After mice were treated with antibiotics, it was found that the structure of the microbiome in the intestines of the mice was significantly changed and that the structure of the microbiome could be quickly restored to its previous configuration after the treatment [2]. However, previous studies examined antibiotic treatment in aquatic animals, and within a few days of antibiotic consumption, the intestinal microbiota biodiversity was reduced, while the initial bacterial community composition rarely fully recovered [23]. In an animal model of mosquito-eating fish exposed to water containing antibiotics, the diversity of the fish gut microbiota rapidly decreased, and the composition of the gut microbiota changed [20]. The effect of antibiotics on the gut bacterial community persists after the withdrawal of antibiotic treatment. Severe antibiotic pressure resulted in non-reversible, long-lasting alterations in the gut microbiota [2].

3.2 Variation in the gut microbiota composition

Some studies involving humans or animals have shown that the use of antibiotics has a significant effect on the relative abundances of some bacteria in the gut bacterial community [5]; usually, the less-abundant phylum Proteobacteria is increased at the expense of the usually dominant Bacteroidetes and Firmicutes [2, 19, 24]. However, in this study, no bacterial phylum was found to significantly differ in

abundance among the control, gentamicin, and recovery groups. The reason may be the use of antibiotic baths such that the drugs did not directly enter the intestinal tract; thus, the effect on the intestinal flora is not direct, and the effect on the gut microbiota is limited.

In the present study, the LEfSe analysis indicated that *Morganella* was significantly enriched in the gentamicin group (LDA > 4, $P < 0.05$). The composition of the gut bacterial community of *R. dybowskii* before and after the antibiotic treatment and the different microbial profiles between these groups may be related to the selection pressure of gentamicin. In addition to pathogens that may have a strong tolerance to gentamicin, the concentration of the remaining bacteria in the treatment group may be related to the severe stress of gentamicin. Bacteria, such as *Morganella*, are functional bacteria associated with the formation of bacterial biofilms, and the bacteria of bacterial biofilms are strongly resistant to antibiotics; the formation of biofilms is a fundamental cause of resistance in bacteria [25]. When bacteria are in an environment that is not conducive to their growth, they form a mutually adhered bacterial community to resist the action of antibiotics. These reasons may explain the change in the composition of the gut bacterial community of *R. dybowskii* under the pressure of gentamicin.

3.3 Frogs in an antimicrobial bath

Oral and local antimicrobial therapies are preferred for animal use because they are safe, rapid, and reliable [26]. In this study, the antibiotic gentamicin may penetrate blood and fluids through the skin, which may lead to changes in the gut microbiota, e.g., disrupting the steady state of the gut bacterial community. Providing medicine in a bath is also a good method for animals. In this study, gentamicin was used at a dose of 20 mg/L, which is close to the dose used in fish seedlings in aquaculture [12]. The drug bath method is non-invasive with less stress to animals than other methods, possibly reducing animal pain and damage to the skin [11]. Although many reports discuss the treatment of frog diseases with medicine baths, most medicines are recommended for oral use or through injection [12]. This lack of information indicates that dermal treatments for frog use are often chosen based on the treatment efficacy in other species and that the dose is inferred from its use in these species. Riviere et al. found that gentamicin can enter the bloodstream after a gentamicin bath, but when the concentration of gentamicin was increased (from 10 µg/mL to 50 µg/mL), the concentration of gentamicin in the blood only slightly increased (from 1.4 µg/mL to 1.5 µg/mL) [11]. This finding indicates that the relationship between the concentration of the drug in the blood and the concentration of the drug in liquid may be complicated. However, in this study, we did not detect the concentration of the drug in the blood or set a gradient of different drug concentrations. More work needs to be carried out in drug and intestinal microbiota research involving amphibians.

The capacity to absorb a drug may vary across animals and skin types. In the same species, the ability to absorb through the skin may vary across different areas of the skin [27]. The drug absorption capacity of the same skin area can widely vary across different host species and is often associated with the habitat of the host species [27]. For example, aquatic frogs tend to have a relatively uniform skin thickness, with lower vascularization in all skin areas, while the abdominal skin of terrestrial or tree-dwelling species tends to be much thinner and more vascularized than the back skin. In this study, the bathing depth was

1.8 cm, which ensured that the liquid could soak one-third of the body of each brown frog. The frogs' abdomen could be mostly immersed in the liquid, which may promote the percutaneous absorption of the drug [11]. The medicinal bath could affect the diversity of the skin bacterial community, and it is well known that the skin bacterial community plays a vital and particularly important role in amphibians. The bacterial community of amphibian skin can vary with the external environment, which may lead to changes in the species and abundance of opportunistic pathogens and endanger the fitness and health of the host [12]. Compared with other animal species, the skin microbiota is particularly important for maintaining the health of amphibians. Antibiotic baths may disturb the stability of skin microbiota; thus, we need to consider this issue when treating diseases through amphibian antibiotic baths.

The gut microbiota is easily disturbed by internal and external factors [28]. Among humans and livestock, antibiotic exposure is a vital source of interference that can seriously alter the gut microbiota composition [29]. Few studies examined the effects of local drugs (e.g., antibiotics) on the gut microbiota. Antibiotics may be used to treat humans or animals when they develop a disease; however, antibiotic abuse can cause much harm [9]. The steady state of the gut bacterial community is critical; if the steady state is destroyed, the pathogens or potential pathogens in the intestine have the opportunity to multiply in large numbers and break through the intestinal mucosa into tissue, eventually leading to systemic infection [24]. In the present study, the relative abundances of some potential pathogenic bacteria were significantly reduced, and the functional prediction of infectious diseases, such as those caused by bacteria and environmental adaptation, was increased in the drug bath group, which may represent a positive effect of the antibiotics [30].

3.4 The effect of an antimicrobial bath on the function of the gut microbiota

Functions are sometimes conserved among diverse microorganisms, and functional redundancy may occur in the microbiota [17]. The present study demonstrates that the gut bacterial community changes in brown frogs are not accompanied by severe variations in predicted community functions, suggesting that *R. dybowskii* has relatively high community redundancy under gentamicin treatment (Fig. 6). However, studies have shown remarkable differences in the gut microbiota between healthy and ill animals (e.g., *R. dybowskii*), and a significant positive correlation exists between the community and functional composition, indicating that the composition of the gut bacterial community in diseased animals has lower functional redundancy [14, 18]. The degree of dysbiosis caused by antibiotics and diarrhoea in the gut microbiota may differ. Diarrhoea is also probably followed by the disappearance of functional redundancy, which may be related to the severity and progression of dysbiosis [17]. The functional redundancy of the same species may differ among developing statuses [18]. For example, gut redundancy in infants may largely surpass that in adults [17].

4 Conclusions

In this study, we dissected the impact of antibiotics on potential pathogenic bacteria and the gut microbiota diversity of *R. dybowskii* and examined the correlation between the structure of the intestinal bacterial community and predicted functional components. The gentamicin bath significantly reduced the alpha diversity of the gut microbiota of *R. dybowskii*. Gentamicin significantly changed the structure of the gut microbiota, and the gut microbiotas exhibited weak resilience and did not totally recover after seven days. A few potentially pathogenic bacteria associated with red leg syndrome were significantly decreased after the gentamicin bath. The gentamicin bath did not change the functional composition of the gut microbiota of *R. dybowskii*, and there was no significant correlation between the composition of the gut microbiota and the functional composition, illustrating the high intestinal functional redundancy of the frog gut bacterial community. These findings offer a window into the role of the safe use of antibiotics in amphibians and alleviation of the effects of antibiotic treatment on the gut microbiota.

5 Materials And Methods

5.1 Sample collection

The captive *R. dybowskii* were caught in August 2017 from a farm in Huanan County (N 46°44'54", E 130°69'32", 80 m alt.), Heilongjiang, China. *Rana dybowskii* are completely terrestrial frogs [2]. *Rana dybowskii* were cultivated in a greenhouse, where sparse plants were planted, water spray equipment and shade nets were installed, and the ground humidity was 25%-35%. The feed was *Tenebrio molitor* (fed twice a day), and the feeding amount was 2%-4% of body mass. The culture density was approximately 40/m². The frogs on the farm were not diagnosed with disease and were not treated with antibiotics. During the experiment, the frogs were collected from the farm, transported to the laboratory, and kept in the laboratory terrarium for 5 days. The frogs used in the experiment were robust and lively. During the experiment, *Tenebrio molitor* were fed daily once a day, and the feeding amount was 2%-4% of their body mass.

Twenty-one *R. dybowskii* (18.07 ± 0.39 g) were separated into the gentamicin group, recovery group, and control group (n = 7 each, female:male ratio of 4:3). The gentamicin group was treated with a 20 mg/L gentamicin (E003632; Sigma, US) solution bath for 7 days. Simultaneously, the control group was treated with a distilled water bath for 7 days. The recovery group was fed for another week after receiving the same treatment as the first group. The bath depth was 1.8 cm, which ensured that the liquid could soak one-third of the body of each brown frog. The depth of the treatment bath was decided in accordance with the frog size to promote the percutaneous absorption of the drug [11].

Approximately 2 L of the solution (submerged 1/3 of the frog's body) was used in the treatment bath, which lasted for 1 hour per day. The frogs in the three groups were raised in separate plastic boxes (43.0 × 32.0 × 27.7 cm³) in the laboratory. Each plastic box was covered at the bottom with a watery pad, which guaranteed that the frog skin was wet [12]. The frogs were raised at 16°C and fed *Tenebrio* daily and freely (food was always available). All frogs were housed similarly, except for during the 1-hour treatment bath.

All frogs were sacrificed to facilitate the collection of the gut samples. The brown frogs were euthanized and intestinally dissected, and the gut contents were sampled within 20 min after euthanasia. The digestive tract of each frog was cautiously acquired, and a part of the gut starting from (but excluding) the stomach to the anus was separated. A fresh pair of sterile tweezers was used in each frog to avoid cross-contamination. The contents of each intestine were poured into a sterile vial and quickly placed and maintained at -80°C.

All animal protocols were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (IACUC#2015-035). All experiments were performed in accordance with the approved guidelines and regulations. All experiments involving animals followed the principles of the 3 Rs (replacement, reduction, and refinement) to prevent unnecessary killing.

5.2 DNA extraction and PCR amplification

Genomic DNA was extracted with a FastDNA® spin kit for soil (MP Biomedicals, US) according to the manufacturer's instructions. Using a GeneAmp 9700 PCR thermocycler (ABI, US), the hypervariable V3–V4 region of the bacterial 16S rRNA gene in each specimen was amplified with the primers 5'-ACTCCTACGGGAGGCAGCAG-3' and 5'-GGACTACHVGGGTWTCTAAT-3'. The PCR program was as follows: denaturing at 95°C for 3 min; 27 cycles of denaturing at 95°C for 0.5 min, annealing at 55°C for 0.5 min, and elongating at 72°C for 0.75 min; and extension at 72°C for 10 min. The PCRs were repeated three times using a 20-µL system as follows: 5× buffer (4 µL), polymerase (0.4 µL) (both FastPfu), 5 µM primers (each 0.8 µL), template DNA (10 ng), 2.5 mM dNTPs (2 µL), and BSA (0.2 µL). The PCR products were isolated by 2% agarose gel, purified with an AxyPrep DNA gel extraction kit (Axygen Biosciences, US) and quantified using QuantiFluor™-ST (Promega, US) as instructed by the manufacturers.

5.3 Illumina MiSeq sequencing

The pure amplicons were pooled in equal molar concentrations and paired-end (2 × 300) sequenced on a MiSeq system (Illumina, US) as specified. Raw FASTQ files were subjected to demultiplexing, quality control via Trimmomatic and integration via FLASH under the following three criteria: 1) the primers were exact matches with no more than two nucleotide mismatches, and reads with ambiguous bases were discarded; 2) sequences with more than 10 bp of overlap were integrated per overlap; and 3) reads with a mean quality score < 20 over a 50-bp sliding window at any site were deleted.

The operational taxonomic units (OTUs) were gathered using UPARSE (<http://drive5.com/uparse/>) at a 97% similarity limit, and chimeric sequences were recognized and removed using UCHIME. The taxonomic assignments of each 16S rRNA gene sequence were performed by RDP Classifier 2.2 (<http://sourceforge.net/projects/rdp-classifier/>) relative to the relevant SILVA database (Release119, www.arb-silva.de) at a confidence limit of 70%.

5.4 Ecological and statistical analyses

The software mothur 1.30.2 (https://www.mothur.org/wiki/Download_mothur) was used to generate the rarefaction curves and calculate the alpha diversity indices. Differences in the alpha diversity indices

(abundance coverage-based estimator (ACE), Chao1, observed richness (Sobs), and Shannon) were analysed via the Wilcoxon rank-sum test. The core OTUs should exist in all samples from each group and represent $\geq 0.1\%$ of the reads. To compare the differences in the gut microbiota among the different groups, we calculated the Bray-Curtis dissimilarities and unweighted UniFrac similarity values on an OTU-level table using an analysis of similarities (ANOSIM) and a multivariate ANOVA (Adonis) [31]. The data were visualized through non-metric multidimensional scaling (NMDS). The unique and shared OTUs [32] were represented in a Venn diagram generated using R software 3.0.0 (R Core Team, New Zealand).

The relative abundances of the phyla and genera in the control, gentamicin, and recovery groups were statistically compared by the Kruskal–Wallis H test. Given the multiple tests performed, the *P*-values were corrected for the false discovery rate (FDR), and the confidence interval (CI) was computed by Scheffer software. The relative abundances of the phyla in the microbiota in the two groups were determined via Welch's t-test. Significant connections between bacterial taxa and host groups were identified by the linear discriminant analysis (LDA) effect size (LEfSe) [33], which considers both consistency and statistical significance and can recognize differentially abundant taxa among groups.

A Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthologue (KO) functional profile analysis was performed with a phylogenetic investigation of the communities by the reconstruction of unobserved states (PICRUSt) based on the 16S rRNA sequencing data [34]. Significant differences in the relative abundances of the predicted functions among the control, gentamicin, and recovery groups were evaluated by the Kruskal–Wallis H test. Only differences with *P*-values < 0.05 are presented.

General discrepancies in the phylogenetic structures and forecast functional compositions were assessed by a principal coordinate analysis (PCoA) and similarity analysis using the Bray-Curtis distance, and the connections among the composition changes were examined using an ANOSIM analysis. The indicator taxa connected to each group were identified by indicator values (IndVal) [35]. The analyses were completed using the "labdsv" package in R v3.0.0. Rare taxa that could incorrectly reflect special taxa were rejected [36]. Only taxa with IndVal > 0.90 (*P* < 0.05) and a relative abundance > 0.1% were retained [35, 37].

5.5 Potential pathogenic genera

Several bacteria were chosen for a comparison to clarify the potential pathogenic bacteria of red leg syndrome in the gut of *R. dybowskii*. *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Proteus*, *Edwardsiella*, *Chryseobacterium*, *Citrobacter*, and *Aeromonas* are all potential pathogenic genera related to red leg syndrome in amphibians [38–42]. Differences in the potential pathogenic genera were evaluated by a Wilcoxon rank-sum test.

Declarations

Ethics approval and consent to participate

All animal protocols were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (IACUC#2015-035). All experiments were performed in accordance with the approved guidelines and regulations. All experiments involving animals followed the principles of the 3 Rs (replacement, reduction, and refinement) to prevent unnecessary killing.

Consent for publication

Not applicable

Statement of ARRIVE guidelines

The study was carried out in compliance with the ARRIVE guidelines.

Availability of data and materials

All microbial sequences have been uploaded to the NCBI SRA under accession numbers PRJNA635022, [PRJNA691728](#), and [PRJNA691728](#).

Competing interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

QT, ZFH, JB, and LYC: data collection, data analysis and interpretation, and drafting of the article. JTZ, HBW, and QT: conception or design of the work. QT, LYC, and JTZ: sample collection. QT, JTZ, and ZFH: writing and critical revision of the article. JTZ: final approval of the version submitted.

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Figures

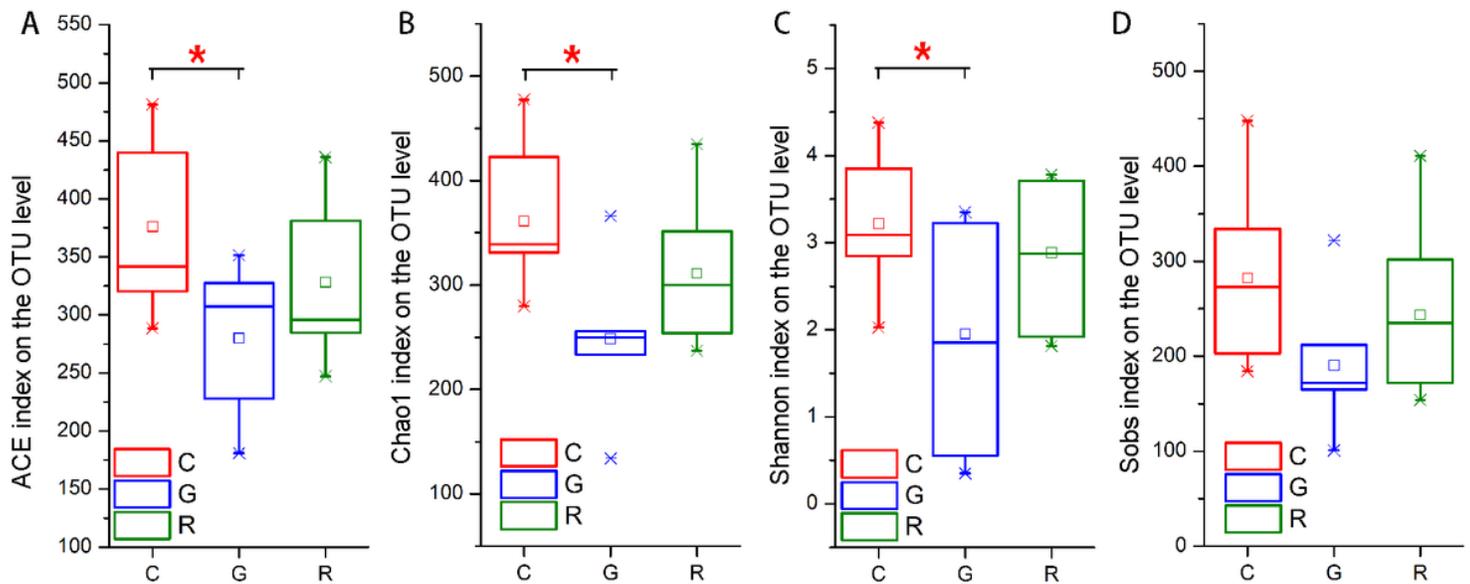


Figure 1

The alpha diversity of the intestinal bacterial community of *R. dybowskii*. Comparison of the alpha diversity (ACE, Chao1, Shannon and Sobs indices) of the intestinal bacterial community of *R. dybowskii* among the control (C), gentamicin (G), and recovery (R) groups (Wilcoxon rank-sum test, $0.01 < P \leq 0.05$ marked as *).

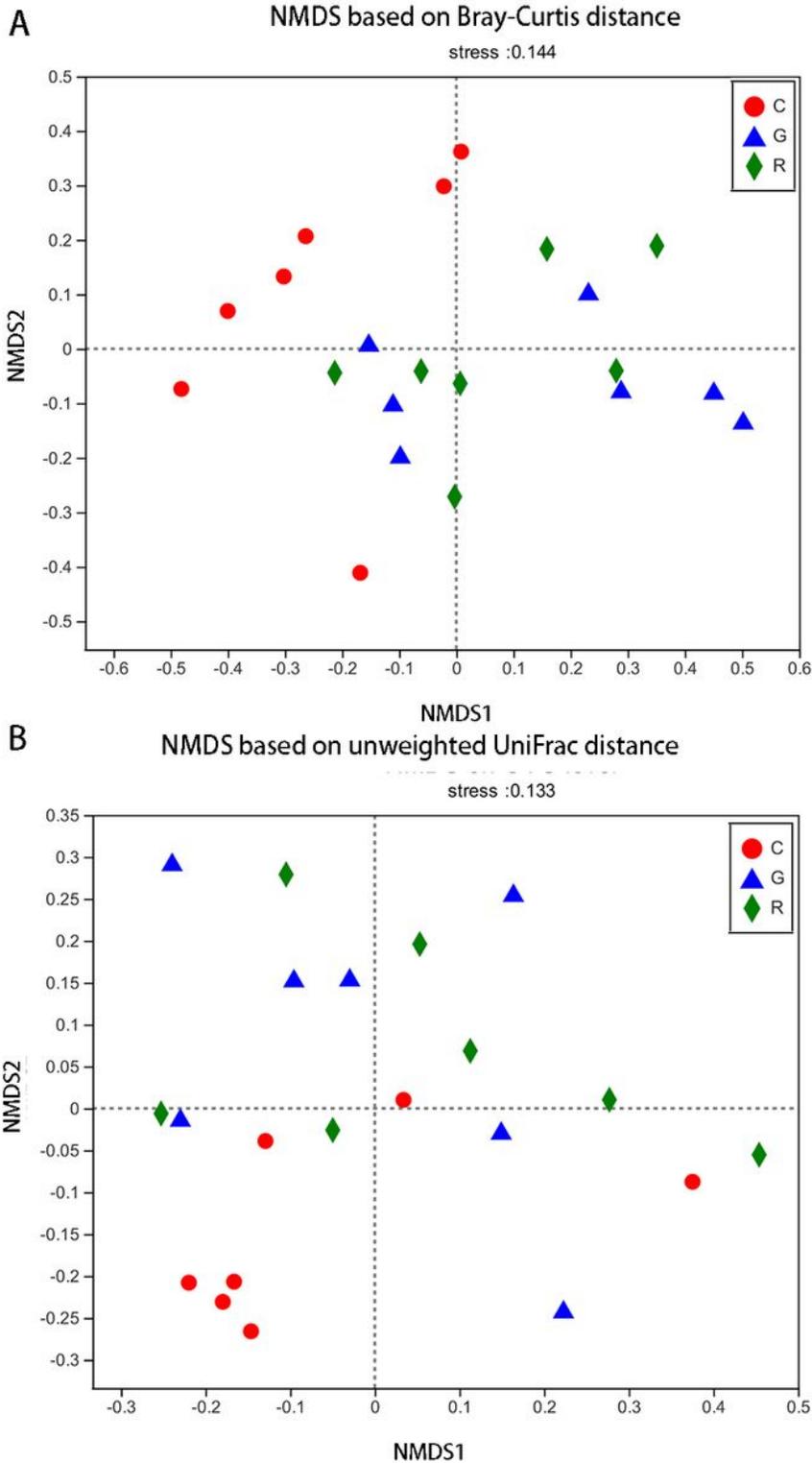


Figure 2

Microbial community shifts associated with the antibiotic bath in captive *R. dybowskii*. Non-metric multidimensional scaling (NMDS) shows patterns of separation in the control (red indicates C), gentamicin (blue, G), and recovery (green, R) groups based on Bray-Curtis (a) and unweighted UniFrac (b) distances. NMDS was based on all OTUs. Each point represents the gut microbiota of an *R. dybowskii* individual.

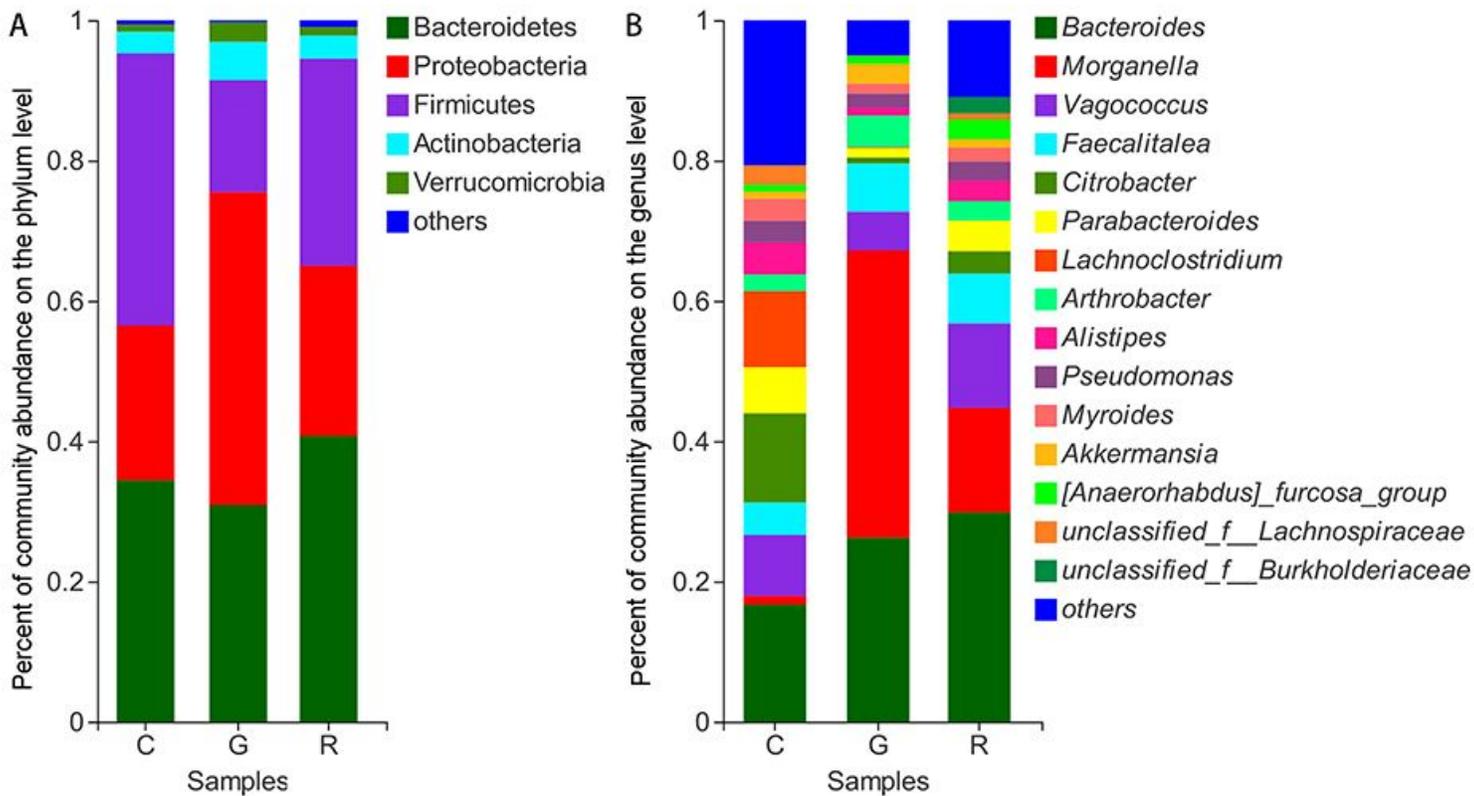


Figure 3

Gut microbiota composition across the groups. Gut microbiota composition across the groups at the phylum (a) and genus (b) levels. Only genera with relative abundances over 2% in at least one sample are shown.

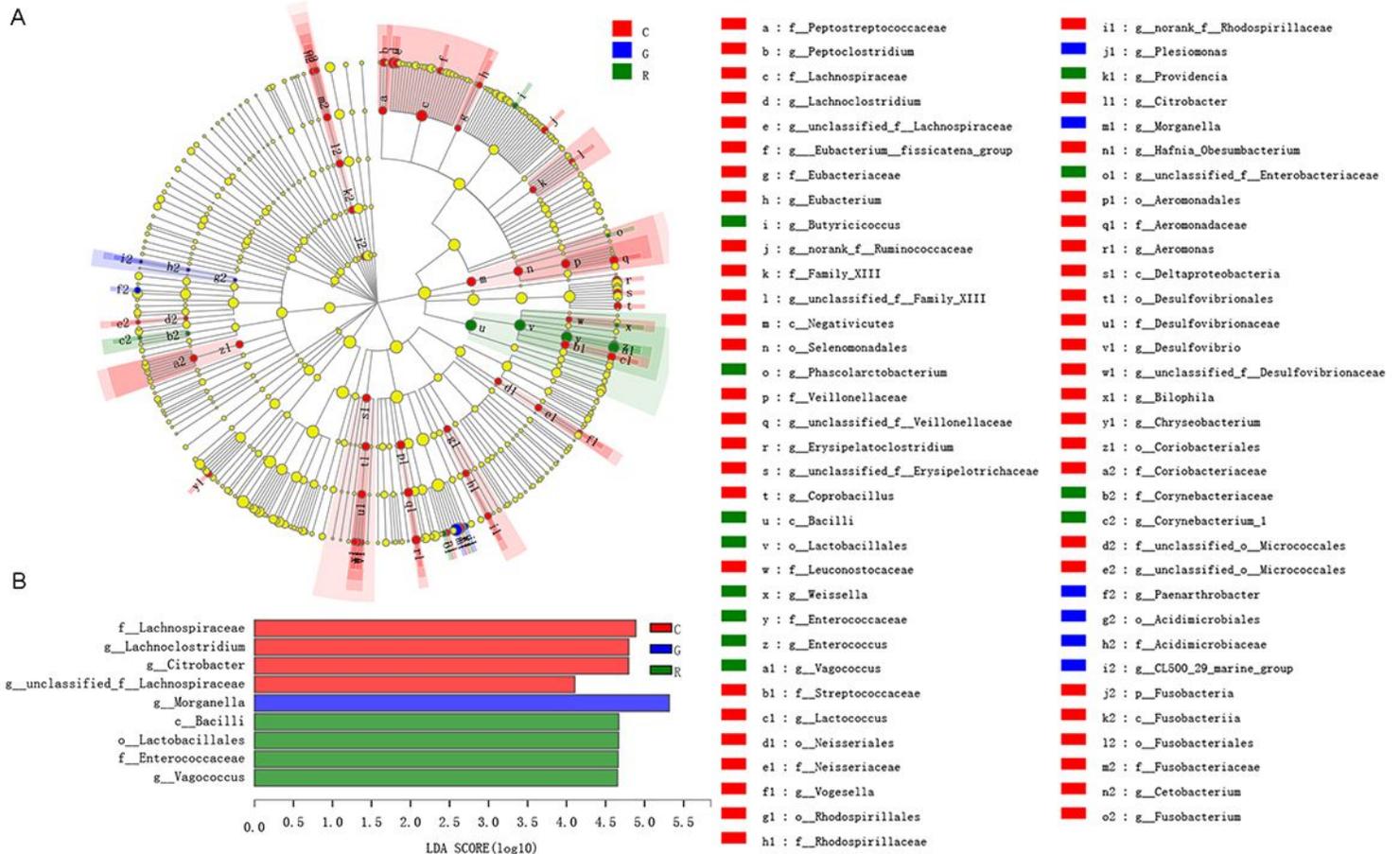


Figure 4

The results of LefSe (LDA Effect Size) analysis. Cladogram showing the phylogenetic distribution of microbial lineages connected with the differences among the C, G, and R groups using LefSe with LDA > 2 (a) or 4 (b). Differences among the control, gentamicin, and recovery groups are represented by the treatment colour (red indicates the control group (c), blue indicates the gentamicin group (G), and green indicates the recovery group (R)). Each circle's diameter is proportional to the taxon's abundance. The strategy of the multiclass analysis is all-against-all. Circles represent taxonomic ranks from domain to genus from the inner circle to the outer circle. Labels are shown at the class, order, and family levels.

Community heatmap analysis on Genus level

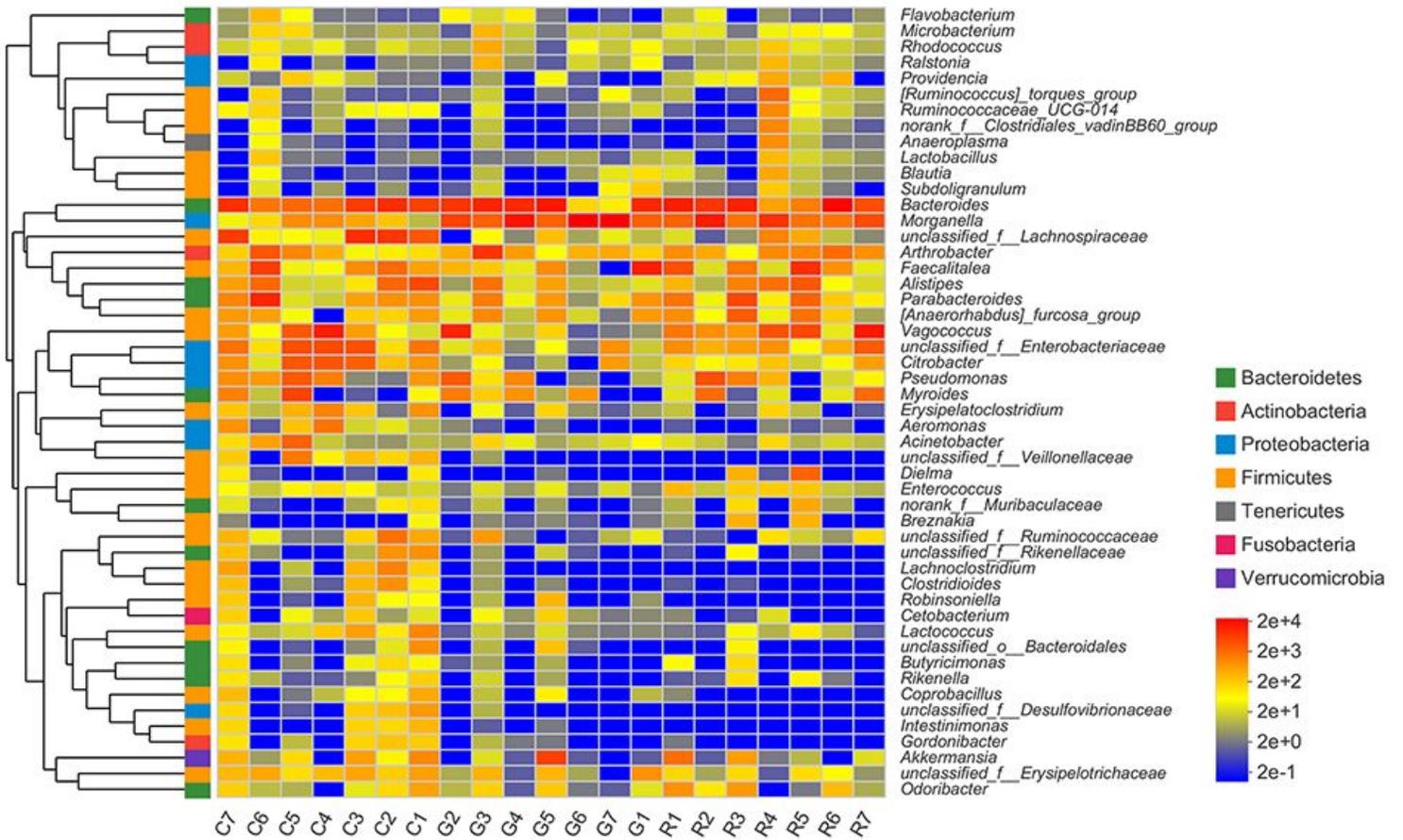


Figure 5

Heatmap showing the relative abundances of the 44 recognized indicator taxa between the C and G+R groups. The text near the ordinate and under the horizon present the names of the species and samples, respectively. The colour patch gradient shows the abundance changes among the different species in the sample, and the relevant data are listed on the right side.

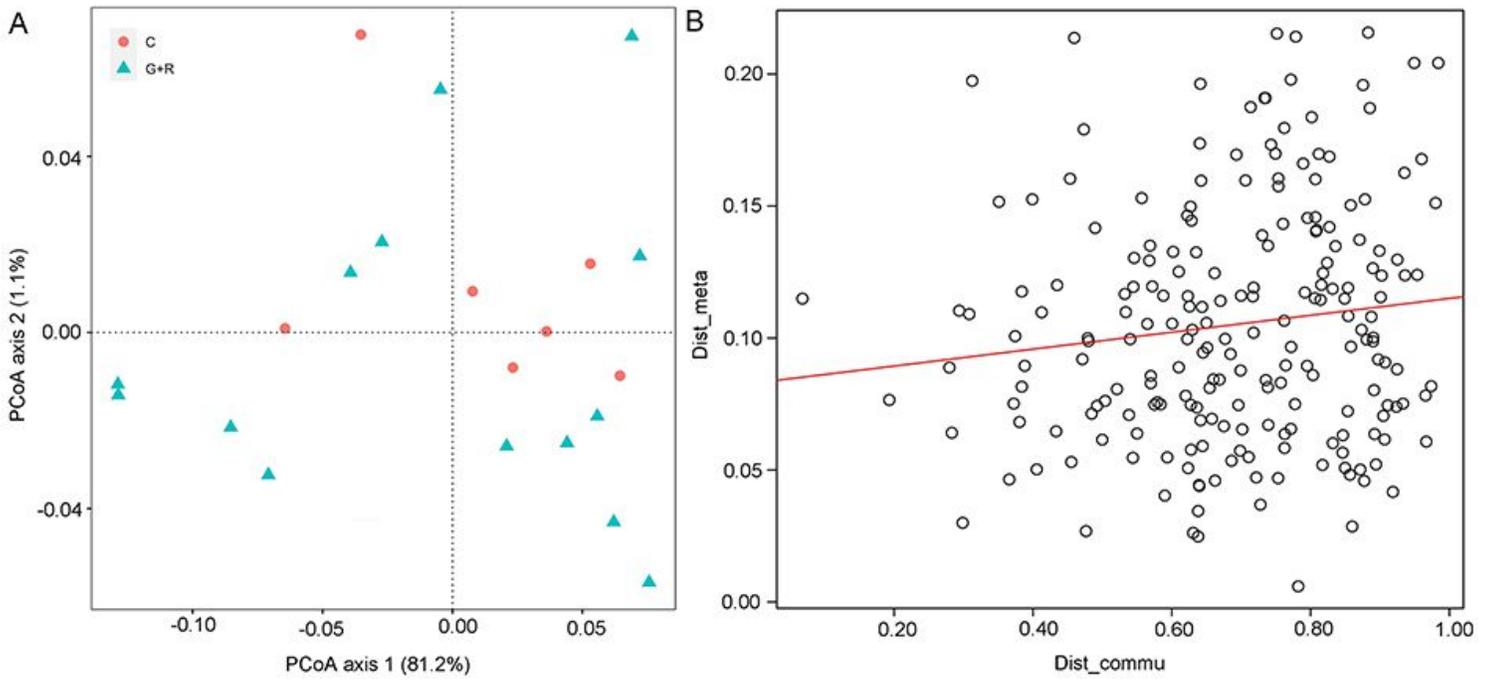


Figure 6

Functional differences in the gut bacterial community between the C and G+R groups. PCoA of the functional structures of the gut microbiota using the Bray-Curtis distance (a) and correlation between functional and compositional similarities (b).

Supplementary Files

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