Gut Microbiota Reconstruction Following Host Infection with Blood-stage *Plasmodium berghei* ANKA Strain in a Murine Model*

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Summary: Malaria remains a global health problem. The relationship between *Plasmodium spp*. and the gut microbiota as well as the impact of *Plasmodium spp*. on the gut microbiota in vertebrate hosts is unclear. The aim of the current study was to evaluate the effect of blood-stage *Plasmodium* parasites on the gut microbiota of mice. The gut microbiota was analyzed by 16S rRNA sequencing and bioinformatic analyses at three stages. The gut microbiota changed during the three phases: the healthy stage, the infection stage, and the cure stage (on the 9th day after malarial elimination). Moreover, the gut microbiota of these infected animals did not recover after malaria infection. There were 254 operational taxonomic units (OTUs) across all three stages, and there were unique strains or OTUs at each stage of the experiment. The percentages of community abundance of 8 OTUs changed significantly (P<0.05). The dominant OTU in both the healthy mice and the mice with malaria was OTU265, while that in the cured mice was OTU234. In addition, the changes in OTU147 were the most noteworthy. Its percentage of community abundance varied greatly, with higher values during malaria than before malaria infection and after malaria elimination. These results indicated that the external environment influenced the gut microbiota after host C57BL/6 mice were infected with blood-stage *P. berghei* ANKA and that the same was true during and after elimination of blood-stage *P. berghei* ANKA. In addition, we could not isolate OTU147 for further study. This study identified gut microbiota components that were reconstructed after infection by and elimination of blood-stage *P. berghei* ANKA in host C57BL/6 mice, and this process was affected by *P. berghei* ANKA and the external environment of the host.
Key words: malaria; gut microbiota; operational taxonomic units; *Plasmodium berghei*; C57BL/6 mice; reconstruction of the microbiota

Malaria, which is one of the top ten tropical diseases and is caused by *Plasmodium spp.* (*P. spp.*), remains a global health problem. The World Malaria Report 2017 from the World Health Organization (WHO) reported that there were an estimated 216 million cases of malaria and 445 thousand deaths from malaria globally in 2016. Insecticide resistance and multidrug resistance, including artemisinin resistance and partner drug resistance, have become obstacles to control and elimination strategies. Therefore, further studies in the field of malaria are needed.

Many studies have examined the links between *Plasmodium spp.* (*P. spp.*) and the gut microbiota in mosquitoes. However, little is known about the relationship between *P. spp.* and the gut microbiota in vertebrate hosts[1–3]. Studies have demonstrated that the gut microbiota may participate in long-distance communication during extragastrointestinal tract infections by *P. spp.*[1–4]. *Escherichia coli* O86:B7 may promote host production of anti-α-gal antibodies that target *P. berghei* sporozoites to block malaria transmission[1]. The composition of the human stool microbiota may be associated with the prospective risk of *P. falciparum* infection, according to Cox regression analysis[2]. Ectogenic *Lactobacillus* and *Bifidobacterium* may decrease the *Plasmodium* burden in C57BL/6 mice infected with *P. yoelii* and modulate the severity of malaria following *P. yoelii* infection[3]. However, there are few studies on the impact of *P. spp.* on the gut microbiota in vertebrate hosts, although a study showed that *P. berghei* ANKA caused intestinal pathological changes in C57BL/6 mice and affected intestinal microbiota changes[5].

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C57BL/6 mice are common experimental animals, and *P. berghei*, which is a rodent-infecting parasite, is often used in malaria research. In the present study, we examined the effect of infection with blood-stage *P. berghei* ANKA on the gut microbiota of host C57BL/6 mice in an attempt to understand whether the host gut microbiota may recover after malaria treatment and whether gut microbiota changes result in disorder or dysbiosis after malaria infection.

1 MATERIALS AND METHODS

1.1 Ethics Statement

All experiments that used mice in this study were approved by the Medical Ethics Committee of Hainan Medical College and were performed in a way that minimized suffering.

1.2 Mice, Parasites, Experimental Infection and Specimen Collection

Five-week-old specific pathogen-free female C57BL/6 mice were obtained from Hunan SLAC Jingda Experimental Animal Co., Ltd. (Changsha, China). Blood-stage *P. berghei* ANKA parasites were provided by the Hainan Provincial Key Laboratory of Tropical Medicine. Four weeks after C57BL/6 mice were raised in an ordinary laboratory, they were infected with 1×10^5 erythrocytes parasitized with blood-stage *P. berghei* ANKA by intraperitoneal injection. Eight days after *P. berghei* was inoculated, the mice were intraperitoneally injected with artemether (Ar). Tail blood was collected for thin smears and stained with Giemsa dye, and parasitemia was quantified by microscopic examination. Parasitemia was used to determine the degree to which *P. berghei* infected erythrocytes. Mouse feces were collected at three time points, namely, before inoculation of *P. berghei*, 8 days after *P. berghei* inoculation, and 9 days after elimination of malaria parasites. The requirements for feces collection were as follows: the procedure was performed next to an alcohol lamp, the anus was wiped with alcohol-soaked cotton balls, mouse feces were clipped with sterile tweezers, and feces were placed in a sterile centrifuge tube. These samples were stored in a freezer at –80°C for inspection.

1.3 DNA Extraction, PCR Amplification and MiSeq Library Construction and Sequencing

Mouse feces were subjected to DNA extraction, PCR amplification and MiSeq library construction and sequencing. The bacterial 16S rRNA primers 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 533R: 5′-TTACCGCGGCTGCTGGCAC-3′ were used. To ensure the accuracy of the data, we entrusted the company Shanghai Majorbio Biopharm Technology Co., Ltd. to complete this part of the work.

1.4 Bioinformatic Analyses and Statistical Analyses

Operational taxonomic unit (OTU) clustering, species composition analysis, species difference analysis, evolutionary analysis and statistical analyses of the data were performed on the online platform from Shanghai Majorbio Biopharm Technology Co., Ltd. The classification level was selected as OTU or species, and OTUs or species with an abundance greater than 1% were analyzed.

2 RESULTS

2.1 Effect of Blood-stage *P. berghei* Infection on Host C57BL/6 Mice

To determine the time of stool collection, we first examined the effect of blood-stage *P. berghei* on host C57BL/6 mice. C57BL/6 mice began to die on day 9 after infection with blood-stage *P. berghei*. When the medication was stopped, the baseline status of the mice had been basically restored. To better facilitate recovery from intestinal injury in mice and further promote the stability of the gut microbiota, we decided to conduct an experimental program (fig. 1A). Mice infected with blood-stage *P. berghei* were treated with Ar on the 8th day after *P. berghei* infection. On the 13th day after *P. berghei* infection, the malaria parasites had been eliminated (fig. 1B), and the behavior of the mice returned to normal. On the 22nd day after *P. berghei* inoculation, which was the 9th day after malarial elimination, the mice were in good health. Mouse feces were collected at three points [the healthy stage, the infection stage, and the cure stage (on the 9th day after malarial elimination)].

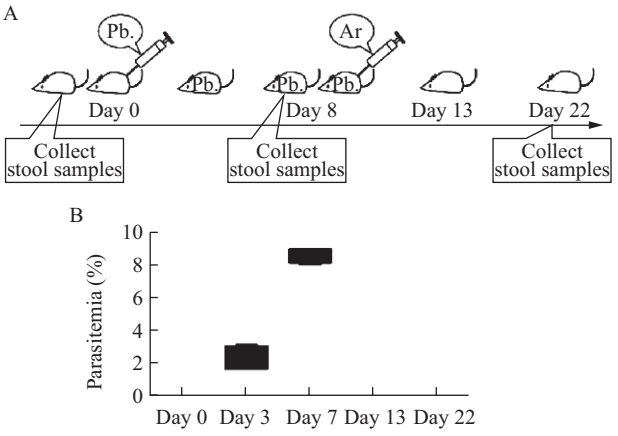

Fig. 1 Experimental program and parasitemia

A: experimental and sample collection program. Four weeks after C57BL/6 mice were raised in an ordinary laboratory, they were infected with blood-stage *P. berghei* (Pb.) by intraperitoneal injection. Eight days after *P. berghei* was inoculated, the mice were injected with artemether (Ar) by intraperitoneal injection. Mouse feces were collected at three time points, namely, before *P. berghei* was inoculated, 8 days after *P. berghei* was inoculated, and 9 days after *P. berghei* was eliminated. B: Parasitemia was used to signify the degree to which *P. berghei* infected erythrocytes.

2.2 Number of OTUs in the Gut Microbiota after Infection with Blood-stage *P. berghei*

To determine whether the host gut microbiota may recover after malaria treatment, we first analyzed the number of OTUs in the gut microbiota. There were 254 OTUs in all three stages. Twelve OTUs were unique to healthy mice (healthy mice), 6 OTUs were unique to mice infected with *Plasmodium* parasites (mice with malaria), and 15 OTUs were unique to mice after treatment (cured mice). The mice with malaria had three more OTUs than the healthy mice and had 274 OTUs in common with the healthy mice; however, 33 OTUs from the healthy mice were different from those from the mice with malaria, and 36 OTUs from the mice with malaria were different from those from the healthy mice (fig. 2). The cured mice had 10 more OTUs than the mice with malaria, and they had 284 OTUs in common with the mice with malaria. Thirty-six OTUs from the cured mice were different from those from the mice with malaria, and 26 OTUs from the mice with malaria were different from those from the cured mice. While the cured mice had 13 more OTUs than the healthy mice, they had 275 OTUs in common with the healthy mice; in addition, 45 OTUs from the cured mice were different from those from the healthy mice, and 32 OTUs from the healthy mice were different from those from the cured mice (fig. 2). Therefore, blood-stage *P. berghei* caused the gut microbiota of host C57BL/6 mice to change, but the host gut microbiota did not recover after malaria treatment.

2.3 Changes in Percentages of Community Abundance for OTUs and Dominant OTU

To understand the differences in OTUs throughout the process of health, malaria infection and treatment, we analyzed the percentage of OTU community abundance and the dominant OTU (fig. 3). The percentages of community abundance for 8 OTUs, namely, OTU265, OTU234, OTU191, OTU147, OTU199, OTU4, OTU74 and OTU282, changed significantly (fig. 3A, 3B) (P<0.05). The dominant OTU in both the healthy mice and the mice with malaria was OTU265, while that in the cured mice was OTU234 (fig. 3). In addition, the changes in OTU147 were the most noteworthy.

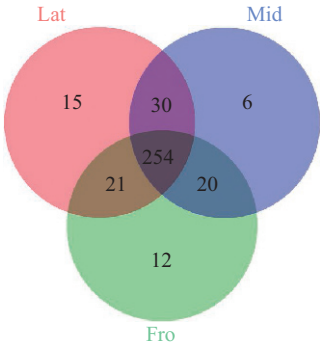

Fig. 2 The quantitative differences in gut microbiota OTUs at three stages
Green represents the number of gut microbiota OTUs before *P. berghei* was inoculated (Fro). Red represents the number of gut microbiota OTUs at 9th day after malaria was cured (Lat). Blue represents the number of gut microbiota OTUs at 8th day after *P. berghei* was inoculated (Mid). The overlapping areas represent the species common to multiple groups (or samples), the non-overlapping areas represent the species unique to each group (or sample), and the numbers represent the corresponding numbers of species.

Before and after mice were infected with *Plasmodium* parasites, the percentages of community abundance at the OTU level showed large changes for OTU265, OTU234, OTU147, OTU4, OTU74, OTU191 and OTU199. After *P. berghei* infection, the percentage of community abundance for OTU265 decreased significantly, while those for OTU4 and OTU74 decreased significantly and those for OTU234, OTU191, OTU147 and OTU199 increased significantly.

Before and after treating the mice with malaria, the percentage of community abundance at the OTU level showed large changes for OTU265, OTU234, OTU147, OTU293, OTU273 and OTU261. After malaria treatment, the percentage of community abundance for OTU261 decreased significantly, while those for OTU265 and OTU147 decreased significantly and those for OTU234, OTU293, and OTU273 increased significantly. Together, these results indicated that the percentages of community abundance for OTU265, OTU234 and OTU147 varied greatly throughout the process. The *Firmicutes/Bacteroidetes* ratio of the gut microbiota may change in the physiological state[13]. Therefore, we considered the gut microbiota to be remodeled after infection by and elimination of blood-stage *P. berghei* ANKA in host C57BL/6 mice.

2.4 No Direct Species Relationship among OTU265, OTU234 and OTU147

To clarify the relationship among OTU265, OTU234 and OTU147, we carried out an evolutionary tree analysis. These OTUs did not belong to the same genus of bacteria. Analysis at the evolutionary level indicated that OTU147 was the oldest, OTU234 was the most recent, and OTU265 was in the middle (fig. 4). OTU147 belongs to *Enterobacteriaceae*, OTU234 belongs to *Bacteroidales*_S24, and OTU265 belongs to *Lactobacillaceae* (fig. 4). Therefore, there was no direct species relationship among these OTUs.

2.5 Exclusion of OTU147 as a Pathogenic Bacterium

To analyze the possible relationships between the three OTUs and malaria, we compared their percentages of community abundance during the three stages, namely, before infection, during malaria infection, and after being cured. The percentage of community abundance of OTU265 during malaria was between that obtained before *Plasmodium* infection and that obtained after malaria cure. The same was true

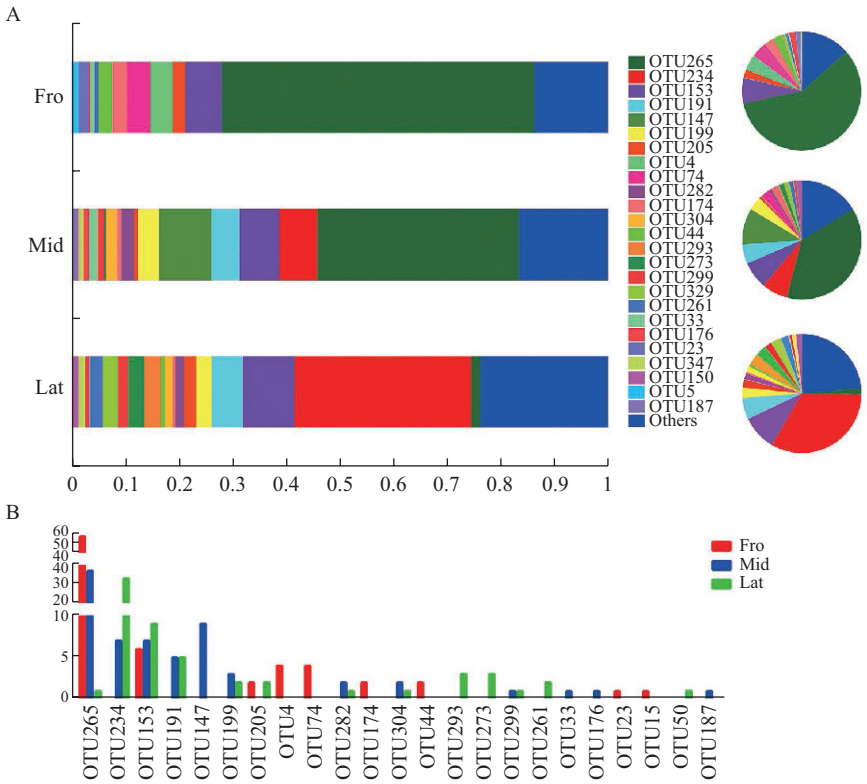

Fig. 3 The percentage differences of gut microbiota OTUs at three stages

A: community histogram and pie chart. Different colors represent different species, and the length of the bars on the histogram and the area of the pie chart represent the percentage of the number of species among the total number of species. B: comparison of the percentages of each species in the three stages.

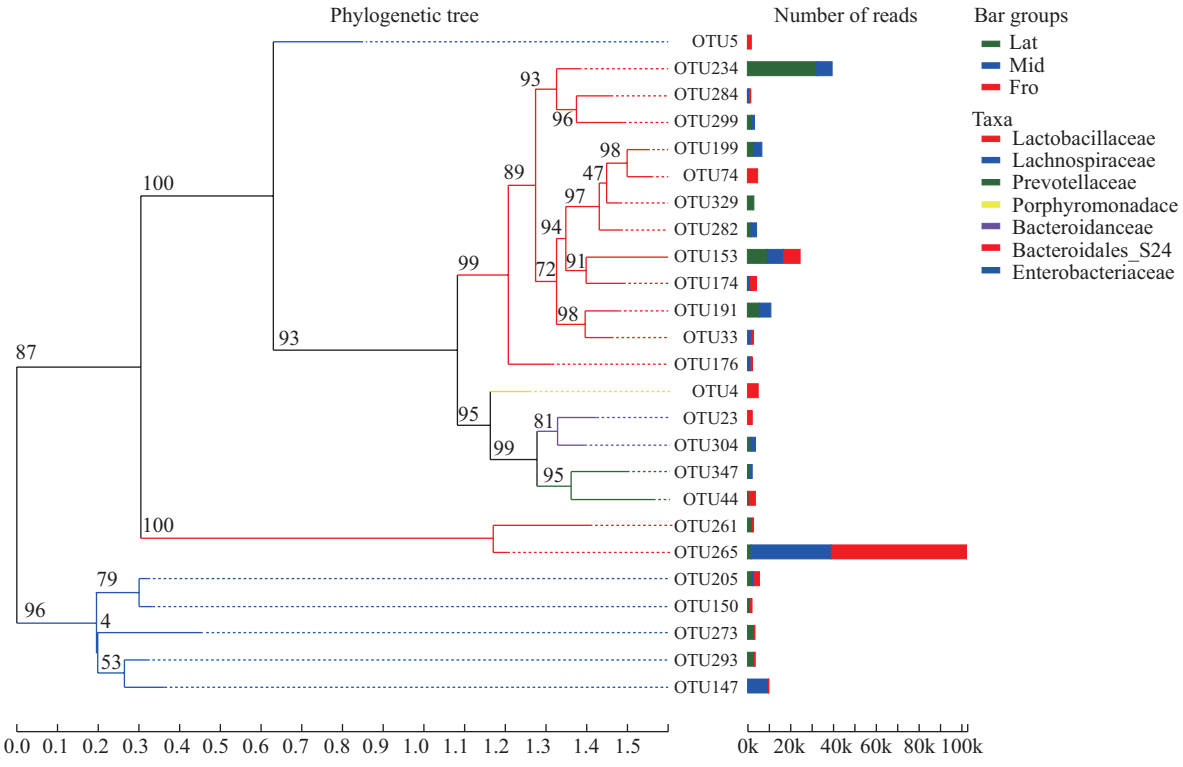

Fig. 4 Evolutionary tree

Each branch of the evolutionary tree represents a species, and the length of the branch is the evolutionary distance between two species, that is, the degree of difference between species. No direct species relationships among OTU265, OTU234 and OTU147 were observed.

for OTU234. However, the percentage of community abundance of OTU147 during malaria infection was higher than that obtained before *Plasmodium* infection and that obtained after malaria cure (fig. 5). These results suggested that OTU265 and OTU234 may not be pathogenic bacteria and that OTU147 may be a pathogenic bacterium. Therefore, these three bacteria, especially OTU147, are worth studying.

2.6 Community Barplot Analysis Result

To identify these gut bacteria that varied greatly before and after malaria infection and to conduct further studies, we performed a "community barplot analysis" at the level of species selection. As shown in fig. 6, OTU265 belonged to the genus *Lactobacillus*. Many bacteria belonged to uncultured or unclassified bacteria; the red part represents OTU234 (OTU234-bacterium), and the yellow part represents OTU147 (OTU147-bacterium). Unfortunately, both were uncultured bacteria. Therefore, we were unable to conduct further studies involving isolation of the two bacteria and investigate their impact on malaria and pathological changes. However, these data supported the view that OTU234-bacterium and OTU147-bacterium may play a role in malaria.

3 DISCUSSION

In tropical developing regions, malaria and diarrhea are common risk factors for some diseases[6, 7]. Malaria may cause enteritis[8] or diarrhea[9, 10], but research on the impact of *P. spp.* on the gut microbiota in vertebrate hosts is limited. Although a study showed that *P. berghei* ANKA affected intestinal microbiota changes[8], it remains unclear whether the host gut microbiota may recover after malaria treatment. In line with a previous report[8], in the present study, we showed that the gut microbiota was remodeled after infection by and elimination of blood-stage *P. berghei* ANKA in host C57BL/6 mice; that is, the host gut microbiota after malaria treatment did not recover to the state that was present before the experiment. These findings emphasize host gut microbiota reconstruction and provide evidence for such studies of blood-stage malaria, which may need to consider changes in the

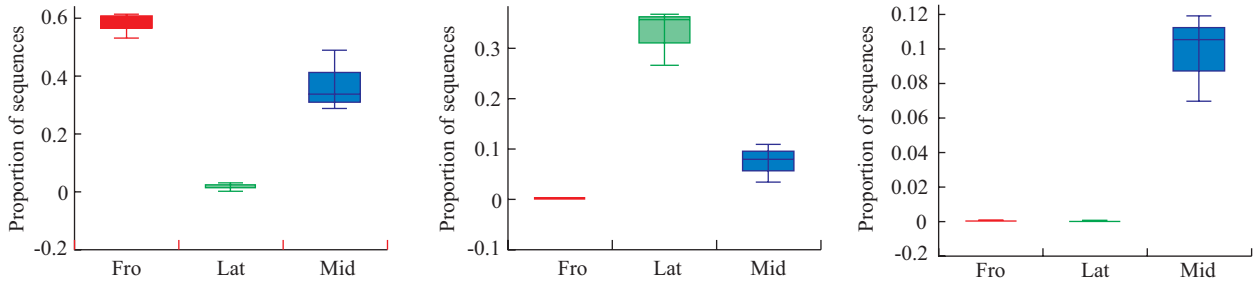

Fig. 5 The differences in abundance percentage changes among OTU147, OTU265 and OTU234 across three stages
A: abundance percentage change in OTU265 in the three stages; B: abundance percentage change in OTU234 in the three stages; C: abundance percentage change in OTU147 in the three stages. The variation in OTU147 is different from that in OTU265 and OTU234.

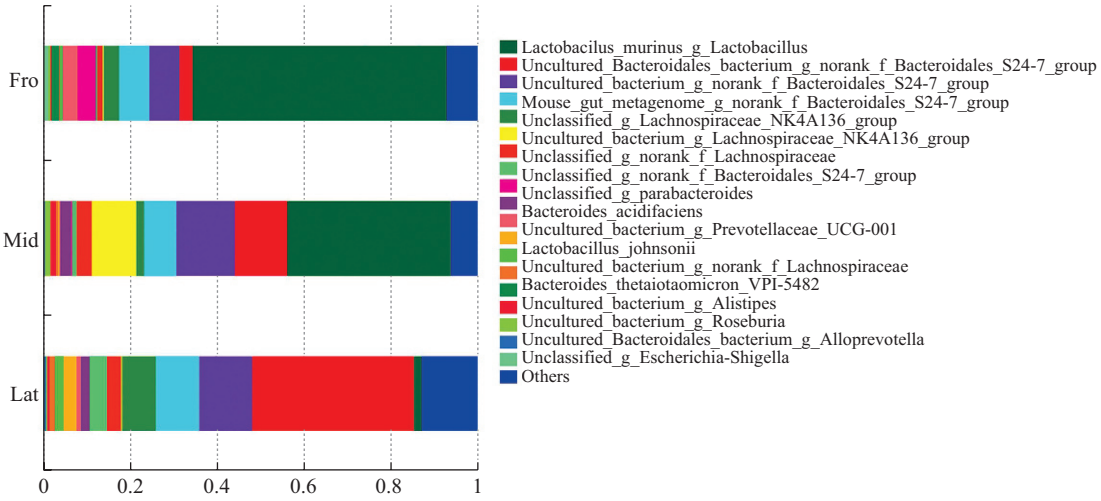

Fig. 6 Community barplot analysis at the level of species selection
Different colors represent different species, and the length represents the percentage of the number of species among the total number of species.

host intestinal microbiota.

The gut microbiota may affect malaria transmission[1], decrease the *Plasmodium* burden and modulate the severity of malaria[3]. In this report, we showed that the dominant bacterium in healthy mice was a *Lactobacillus sp.*, while that in the cured mice was OTU234-bacterium. A previous study showed that an ectogenic *Lactobacillus sp.* decreased the *Plasmodium* burden and modulated the severity of malaria[3]. These results confirmed our previous speculation that OTU256 and OTU234 may not be pathogens. In addition, *Lactobacillus sp.* tended to decrease and OTU234-bacterium tended to increase throughout the process, which suggests that *Lactobacillus sp.* and OTU234-bacterium may compete with each other; that is, they may inhibit each other. In the hosts with malaria and the hosts that had been cured of malaria, OTU234-bacterium had an opportunity to be the dominant bacterium. Previous studies showed that the gut microbiota from C57BL/6 mice maintained by different vectors were different[11,12], and the *Firmicutes/Bacteroidetes* ratio of the gut microbiota may change in the physiological state[13], which suggests that the *Firmicutes/Bacteroidetes* ratio of the gut microbiota does not necessarily indicate bacterial dysbiosis. Therefore, we believe that the change in the gut microflora is not necessarily a disorder or dysbiosis of the microbiota but may reflect the reconstruction of the microbiota.

Among the changing intestinal species in this study, we must mention OTU147-bacterium, for which the percentage of community abundance varied dramatically and was higher during malaria than before malaria infection or after malaria was cured. The percentage of community abundance of OTU265 during malaria was between that obtained before *Plasmodium* infection and that obtained after malaria was cured. The same was true for OTU234. These data suggested that a possible link between OTU147-bacterium and malaria could not be excluded. The evolutionary tree showed that OTU147-bacterium belongs to the *Enterobacteriaceae*. However, it is regrettable that OTU147-bacterium could not be isolated in this study. Isolation of OTU147-bacterium is needed in further studies.

To date, there are few studies on the mechanism by which malaria influences the gut microbiota. The changes caused by malaria in the host are complex; for example, malaria can cause changes in the host immune system[14], intestinal pathological changes[5] and other effects, and it is difficult to isolate a single influencing factor for mechanistic research. Different diets or sources can also affect the gut microbiota[11, 12]. Psychological and physical stressors also change the gut microbiota[15]. The gut microbiota also plays a role in pathogen colonization, immune responses and inflammatory disease[16]. Furthermore, it is also difficult to rule out an interaction between the gut microbiota and malaria. These characteristics make relevant studies more difficult and even less repeatable than they might be otherwise. In this report, the results showed that there were 254 OTUs in all three stages and that there were unique strains or OTUs at each stage of the experiment. Twelve OTUs were unique to the healthy mice, 6 OTUs were unique to the mice infected with *Plasmodium* parasites, and 15 OTUs were unique to the mice after treatment. These results indicated that the external environment also influenced the host gut microbiota after host C57BL/6 mice were infected with blood-stage *P. berghei* ANKA and after blood-stage *P. berghei* ANKA were eliminated. Taniguchi *et al* used two kinds of mouse malaria models to clarify that changes in the gut microbiota were related to intestinal pathological changes[5]. However, the gut microbiota differed among mice, leading to some deficiencies in the experimental results. Although their study implies a possible relationship, the absence of direct evidence adds uncertainty to the validity of the relationship. These results suggested again that the mechanisms underlying changes in the gut microbiota are extremely difficult to study. In this report, the changing microflora is in a single group of mice at different stages, which is another aspect that makes it difficult to further study the underlying mechanism. Changes in the microflora varied with changes in malaria status, histopathological changes and immune status, and the microflora did not return to the healthy state as predicted, also showing the complexity of the mechanism, which is most likely the result of a combination of factors. Therefore, it will be a long process to study the mechanism by which malaria influences the gut microbiota.

Overall, we provided evidence that when malaria occurs, changes in the gut microbiota, in addition to malaria, are affected by the external environment of the host. This finding reminds us that to avoid severe intestinal symptoms or complications during malaria, it is necessary to place the patient in a pathogen-free environment and to address the surroundings of patients in a timely manner.

Acknowledgments

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Conflict of Interest Statement

The authors declare that they have no competing interests.

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