Original Article

Heat-killed *Lactobacillus sakei* HS-1 mitigates small intestinal pathophysiology on *Plasmodium berghei* ANKA infected C57BL/6 mice

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Abstract

Malaria parasites grow in erythrocytes, while the important symptoms reflecting disease severity are observed in the brain and/or the gastrointestinal (GI) tract in human and murine malaria. *Plasmodium berghei* ANKA (PbA-) is widely used as an experimental model to cause lethal murine malaria. We previously reported GI pathophysiology with weight loss on PbA-C57BL/6 mice. Accordingly, we hypothesized that mitigation of weight loss might help ameliorate GI pathophysiology on PbA-C57BL/6 mice. This study aimed to investigate whether administration of heat-killed *Lactobacillus sakei* HS-1 (HK LS HS-1), a newly identified influencing weight gain on healthy domestic animals, mitigates weight loss and/or GI pathophysiology on PbA-C57BL/6 mice.

Six C57BL/6 mice were infected with *P. berghei*-ANKA strain via infective mosquitoes. Thereafter, half of the mice were randomly assigned to receive HK LS HS-1 from day zero until the day of dissection on day 8. Disease states in mice with and without HK LS HS-1 were compared with respect to weight change, food intake, and GI pathophysiology. This experiment was performed three sets.

Overall, the weight (%) was higher in the HK LS HS-1 group than in the control group ($p = 0.001$), while the food intake (g) was comparable in both the groups. Further, pathological changes of the small intestine did not occur in the HK LS HS-1 group.

(Keywords: Heat-killed *Lactobacillus sakei* HS-1, malaria, *Plasmodium berghei* ANKA)

Introduction

Malaria is one of the most prominent mosquito-borne diseases, with an estimated 228 million cases of malaria infection and 405,000 malaria-related deaths having occurred in 2018[1]. Among the five species of malaria parasites that infect humans, the mortality associated with the *Plasmodium falciparum* infection is the highest, and this parasite reportedly causes complications such as cerebral ischemia / edema-related impaired consciousness, and convulsions[2, 3]. Furthermore, in relatively less severe cases of malaria, gastrointestinal (GI) symptoms such as epigastric pain, vomiting, and diarrhea are more frequent. Moreover, in cases of cerebral malaria, delayed excretion of gastric contents was confirmed[4]. A previous study reported the association between malaria and increased bowel obstruction in children[5]; however, the underlying reasons remain unknown.

Other than humans, malaria is species-specific including several types of rodent malaria. Among them, *P. berghei*[6] has been used as an experimental model to cause murine cerebral malaria[7, 8]. Cerebral malaria is reportedly accompanied by intestinal dysbiosis[9], and we reported GI pathophysiology with and without cerebral malaria on PbA-C57BL/6 mice[10]; however, no methods are currently available to alleviate GI pathophysiology. As an indicator of disease severity, PbA-C57BL/6 mice displayed weight loss starting 5 days post-infection[9, 10]. Since weight loss is an indicator of severity during infection[11, 12]; hence, we

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focused on the association between weight loss and GI pathophysiology post-infection on PbA-C57BL/6 mice.

In general, *Lactobacillus* exerts beneficial effects on health. In context of murine malaria, the administration of *Lactobacillus casei* ssp. is reported to strengthen the innate immunity[13], and a high proportion of *Lactobacillus* in the intestinal flora indicates resistance against malaria[14]. Thus, we hypothesized that administration of *Lactobacillus* to PbA-C57BL/6 mice might mitigate the GI pathophysiology by ameliorating weight loss. We administered heat killed *Lactobacillus sakei* HS-1 (HK LS HS-1) to PbA-C57BL/6 mice. LS HS-1 was detected in kimchi[15] (Korean pickled cabbage) and is reported to activate macrophages[16]. Moreover, LS HS-1 can reach the human digestive tract alive[17], and its heat killed form induces weight gain in healthy livestock[18, 19]. To the best of our knowledge, this is the first study to investigate the effect of HK LS HS-1on the mitigation of GI pathophysiology in PbA-C57BL/6 mice.

Methods

Mice

Animal experiments comply with laws and guidelines related to laboratory animals, including the Cartagena Act, and were approved by the Animal Experiment Committee of Jichi Medical University (18004-01). C57BL/6NCr female mice (7 weeks old) were purchased from Japan SLC. Inc. (Hamamatsu, Japan), and fed the CLEA Rodent Diet CE-2 (Japan Inc., Tokyo, Japan). The mice were housed at 23 ℃ ± 1℃ under a 12-h light-dark cycle.

In the experiment, six C57BL/6 mice were infected with *P. berghei*-ANKA strain via infective mosquitoes. Thereafter, half of the mice were randomly assigned to receive HK LS HS-1 from day zero until the day of dissection on day 8. Disease states in mice with and without HK LS HS-1 were compared with respect to weight change, food intake, and GI pathophysiology. The mice were housed in cages (3 per cage). This experiment was performed three sets.

Parasites and mice infection through infective mosquitoes

Mosquitoes (*Anopheles stephensi* SDA 500 strain) maintained in the division of Medical Zoology were used herein. *P. berghei* ANKA (PbA-) strain clone 2.34[20, 21], maintained in the laboratory were used to infect mice. When producing infective mosquitoes, approximately 200 female mosquitoes were allowed to feed on a malaria-infected mouse. After the blood meal, mosquitoes were separated and housed at 21 ℃ ± 1 ℃ with 80% humidity with a 12-hour light / dark cycle, and provided ad libitum access to 5% fructose (containing 0.05% p-aminobenzoic acid). Two weeks after a blood meal from infected mice, eight mosquitoes per cage were dissected and their midguts were screened for *P. berghei* ANKA. When seven of eight mosquitoes developed oocysts on their midguts, the mosquitoes were defined as infective mosquitoes. One week after having been confirmed as infective mosquitoes, eight of these infective mosquitoes fasted for 6 h were released in a cage (16 × 26 × 26 cm), wherein a mouse was placed by immobilizing the trunk with wire mesh. Infective mosquitoes were allowed to bite for 4 min to complete the malaria infection[10, 21].

Lactobacillus administration

Heat killed *Lactobacillus sakei* HS-1 (HK LS HS-1) was obtained from Kazami Food Science (Ashikaga, Japan). HK LS HS-1 was dissolved in tap water to a density of 2×10^6 bacteria/mL, a dose reported to increase body weight in healthy livestock[18,19]. Since live LS HS-1 is excreted in the feces[17], it is presumed that the cell wall (peptidoglycan) is not digested by gastric acid and intact HK LS HS-1 reaches the small intestine.

On the day of infection, half of the PbA-C57BL/6 mice were randomly assigned to the HK LS HS-1 group, for which drinking water containing HK LS HS-1 was freely available. HK LS HS-1was not administered to the control group.

Physical findings and anatomy

From the day of infection as day 0 until the day 8 post-infection, the changing of mice weight (%) on each day divided by weight at day zero was shown. The amount of food in each cage was weighed, and the amount eaten per cage was defined as the decrease in food weight compared with that on the previous day. The daily amount eaten per mouse was estimated by dividing the amount eaten per cage by the number of mice in the cage[10]. Peripheral parasitemia (%; the numbers of parasites per 3,000 red blood cells) was measured by blood smears each day until day 8. Briefly, the blood was collected by making a small incision in the tail with a sterile scalpel and smeared on a slide[10]. Murine cerebral malaria is a clinical condition with severe neurologic sequelae, including seizure, focal neurologic defects, and coma[22]. We defined cerebral malaria as the appearance of convulsions or paresis post infection[10]. All mice were dissected on day 8 when cerebral malaria occurs in any of PbA-C57BL/6 mouse[10]. On dissection, the GI tract was removed, the small intestinal length was measured (cm), and histopathological specimens were prepared in accordance with our previous study[10].

Microscopic observations

Pathological images were microscopically investigated, as previously reported[10]. Briefly, stereoscopic observations were conducted using an Olympus SZX7 scope (Olympus, Tokyo, Japan), and photographs were taken with a DP73 camera (Olympus, Tokyo, Japan). Histopathological observations were conducted using a BX-63 microscope (Olympus, Tokyo, Japan), and photographs were taken using a DP72 camera (Olympus, Tokyo, Japan).

Statistical analyses

In PbA-C57BL/6 mice with and without HK LS HS-1, the changing of weight (%), food intake (g), peripheral parasitemia (%), and the length of the small intestine (cm) were analyzed by Mann-Whitney U test using Eazy-R (EZR)[23]. A *p-value* less than 0.05 was considered statistically significant.

Results

On day 8 post-infection, none of the mice developed cerebral malaria in the HK LS HS-1 group. Though not significantly, cerebral malaria was confirmed in two of nine mice in the control group. Overall, the group with HK LS HS-1 showed higher weight ($p = 0.001$) with the significance on day 4 ($p = 0.02$) and day 5 ($p = 0.04$). The average weight increased in both groups until day 4 post-infection, after which the weight began to decrease. Finally, the weight drastically decreased on day 8 dropping below day zero (Figure 1a). The food intake per mouse (g) was stable by over 3.0 g until day 6 post-infection; thereafter, food intake decreased both in the HK LS HS-1 and control groups. On day 8, average food intake (g) was less than 1.0 g in both groups (Figure 1b). The peripheral blood parasites appeared in all mice on day 4 post-infection with and without HK LS HS-1. Thereafter, parasitemia in the HK LS HS-1 group was superior on day 6 ($p = 0.04$) and 7 ($p = 0.02$). While on day 8, no difference was observed in parasitemia between the HK LS HS-1 and control groups (Figure 1c). Upon dissection, the small intestinal length was found to be 29.1 ± 1.8 cm in the HK LS HS-1 group, and 26.5 ± 2.4 cm in the control group ($p = 0.02$) (Figure 1d).

On dissection, the HK LS HS-1 group presented an intact small intestine (Figure 2a), while the control group presented a change in the course of the small intestine and flattening of the edematous upper portion of the small intestine (Figure 2b). Multiple red patches were observed on the gastric mucosa both in the HK LS HS-1 (Figure 2c) and control group (Figure 2d). The small intestinal villi was intact in the HK LS HS-1 group (Figure 2e), whereas in the control group, the nuclei of the epithelial cells were edematous with reduced staining, and notable enlargement of the goblet cells was observed (Figure 2f).

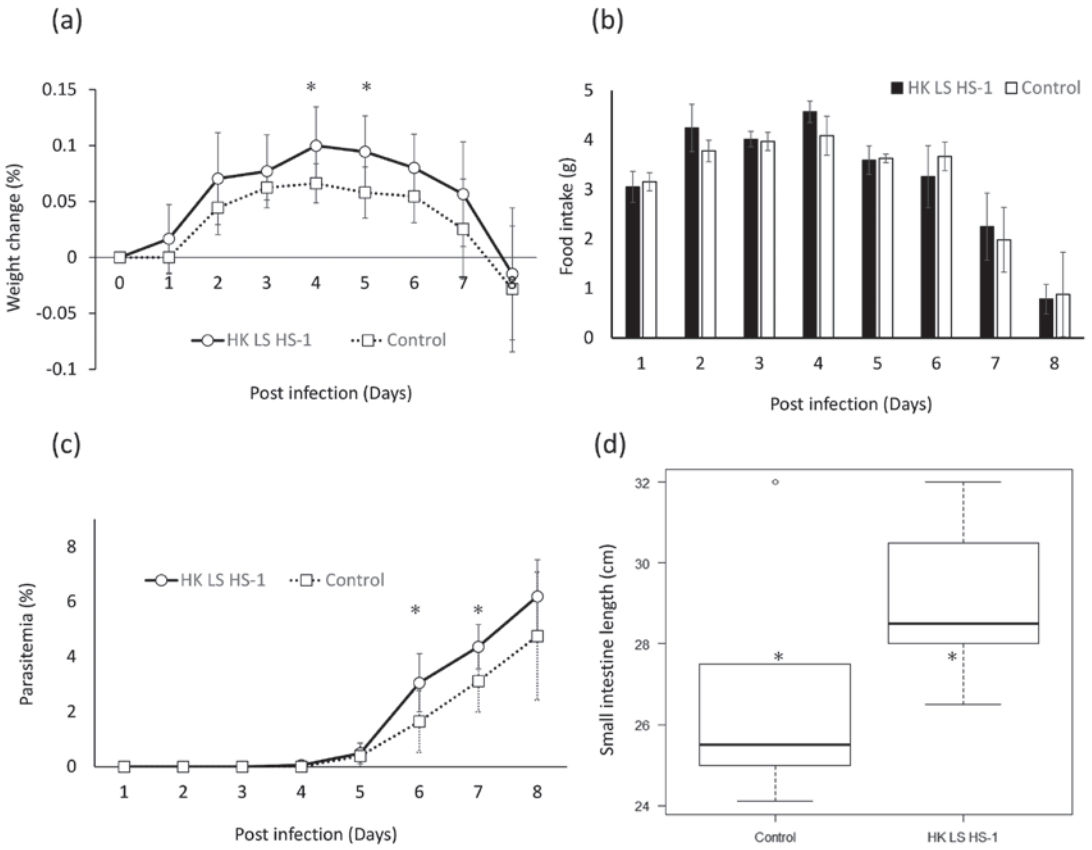

Figure 1. Changes in weight, food intake, and parasitemia on PbA-C57BL/6 mice are shown as the mean ± standard deviation (s.d.) in the HK LS HS-1 group and the control group. (a) Weight change (%) from the starting day until day 8. (b) The amount of food intake (g) per mouse from days 1 to 8. (c) The peripheral parasitemia (%) from days 1 to 8. (d) The lengths of the small intestine (cm) are shown by box plot with or without HK LS HS-1. * indicates $p < 0.05$ significance.

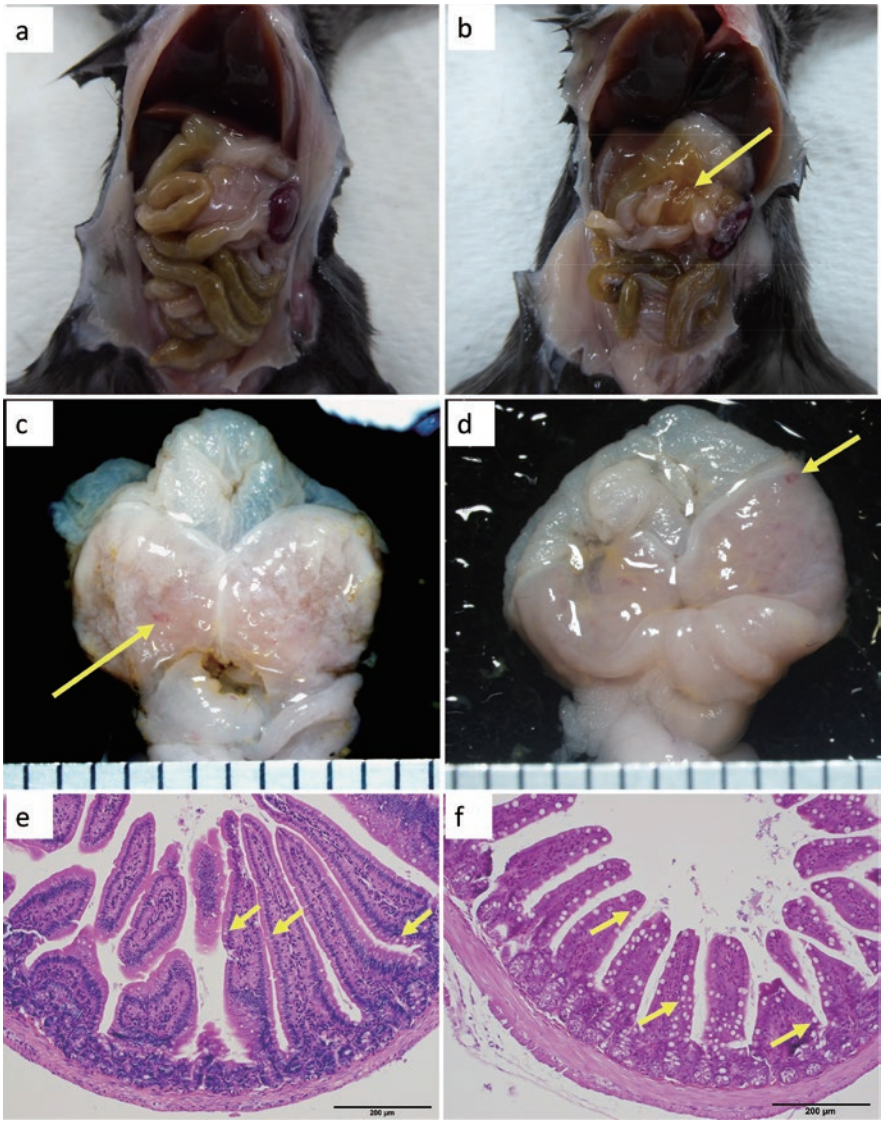

Figure 2. Pathology of PbA-C57BL/6 mice on day 8 with and without HK LS HS-1.

Representative images of (a) the abdomen in a dissected mouse administered HK LS HS-1 and (b) the abdomen in a dissected control mouse. The yellow arrow indicates the small intestine, whose course was disturbed, flattened, and edematous. Representative images of the gastric mucosa of (c) a mouse administered HK LS HS-1 and (d) a control mouse. Examples of mucosal red patches are indicated with yellow arrows. Representative histopathological images of the small intestinal villi of (e) a mouse administered HK LS HS-1 and (f) a control mouse. The yellow arrows indicate goblet cells. The scale in (e) and (f) represents 200 μm.

Discussion

When PbA-C57BL/6 mice present severity as cerebral malaria, accompanied by weight loss[9, 10] and GI pathophysiology[10]; hence, we hypothesized that mitigation of weight loss as well the GI pathophysiology might be the key to ameliorate the severity of malaria. Therefore, we investigated the effects of HK LS HS-1 on PbA-C57BL/6 mice with respect to weight changes and GI pathophysiology. Overall, HK LS HS-1 administration mitigated weight loss and pathophysiology of small intestine on PbA-C57BL/6 mice under the same amount of food intake as the control group. The mitigation of weight loss on murine malaria is a novel finding regarding HK LS HS-1 in addition to the effect of weight gain in healthy domestic animals[18, 19]. Parasitemia was superior upon weight maintenance in the HK LS HS-1 group, implying that malaria parasites multiply slightly rapidly in the HK LS HS-1 group at a more appropriate weight. These phenomena might not contradict the acceleration in the proliferation of malaria parasites in erythrocytes under favorable nutritional conditions[24]. Gastric mucosal red patches[10] were shown in both HK LS HS-1 and control groups on day 8 post-infection, and they were indicative of acute gastric mucosal lesions[25, 26]. These red patches potentially relate to the

reduction in food intake on day 8 in both groups.

In our study, the length of the small intestine in HK LS HS-1 group was similar to that of uninfected C57BL/6 mice in our previous report[10]. Results of histopathology indicated that the small intestine was intact in the HK LS HS-1 group, but showed edematous epithelial cells and goblet cell enlargement in the control group. Goblet cells are known to produce mucin[27] and their enlargement suggests functional impairment. The histopathological differences in the small intestine with or without exposure to HK LS HS-1 might explain the shortening and impairment of small intestine on PbA-C57BL/6 mice. Although the direct effects of intestinal microflora were not investigated herein, the present results nonetheless provide evidence that HK LS HS-1 administration mitigates weight loss in PbA-C57BL/6 mice and relieves pathophysiology of small intestine. Moreover, our results did not contradict the former reports of *Lactobacillus* relating resistance against murine malaria[13, 14]. In the future, it will be interesting and informative to verify whether gender or the age differences modulate the effects of HK LS HS-1 on mice.

In conclusion, weight loss was mitigated in HK LS HS-1 group compared to the control group when food intake was comparable on PbA-C57BL/6 mice. Further, HK LS HS-1 administration had beneficial effects on the small intestine in PbA-C57BL/6 mice, as it did not lead to edematous change of the epithelial cells or cause goblet cell enlargement, which in turn prevented macroscopic pathology of the small intestine.

Declaration of interest

This study was ruled by the Conflict of Interest Committee of Jichi Medical University as having no conflicts of interest.

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Plasmodium berghei 感染 C57BL/6マウスにおける heat killed *Lactobacillus sakei* HS-1投与による小腸病態の緩和

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要　約

ネズミマラリア*Plasmodium berghei* ANKA（PbA-）感染C57BL/6マウスは，脳マラリアのモデルとして広く使用されるが，我々は以前，脳マラリア出現時期に体重減少を伴う上部消化管の病態が生じていることを報告した。その知見から，体重減少緩和と消化管病態緩和の関係性を検証するため，健常家畜の体重増加に有効とされるheat killed *Lactobacillus sakei* HS-1（HK LS HS-1）をPbA-C57BL/6マウスに投与し，病態を評価した。
PbA-C57BL/6マウスを，感染当日HK LS HS-1投与群と非投与群に分け，感染後８日目までの体重，餌摂取量及び最終的な病理所見を評価した。投与群では，脳マラリア出現がなく，体重減少が軽度であった。病態緩和に関与し得る病理学的な差異として，投与群では，非投与群に認める小腸の短縮，絨毛上皮の浮腫や杯細胞の腫脹が認められなかった。
（キーワード: *Lactobacillus sakei* HS-1，マラリア，*Plasmodium berghei* ANKA）

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