RESEARCH ARTICLE

Administration *Lactobacillus casei* and *Bifidobacterium longum* improves neurological outcomes in *Plasmodium berghei* infection

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The high mortality and morbidity rates of malaria remains to be a global problem, particularly in tropical regions. Cerebral malaria, the leading cause of death, possesses a complex pathogenesis involving host immune and parasite interactions. Lately, according to the gut-brain axis concept, the gut microbiota was identified to protect the host against systemic pathogen infection through the modulation of immune signaling pathways. In this study, we aimed to explore the effect of probiotic supplementation toward neurological outcomes of cerebral malaria *in vivo*. Twenty-eight male C57BL/6 mice were randomly divided into 5 groups after 2 weeks acclimatization period. All groups except the negative control were given oral probiotic supplementation with either *Lactobacillus casei* at 10^9 colony forming unit/100 µL (CFU/100 µL), *Bifidobacterium longum* at 10^9 CFU/100 µL, or the combination of each at $5x10^8$ CFU/100 µL for five days before to six days after *Plasmodium berghei* infection. Neurological manifestations were recorded by using the SmithKline, Harwell, Imperial College, Royal Hospital, Phenotype Assessment (SHIRPA) score. The data was analyzed by using One-way ANOVA and post-hoc LSD. The results showed that there were significant differences of motor behavior ($P < 0.01$), muscle tone ($P < 0.01$), reflex and sensory ($P < 0.05$), and neuropsychiatric state ($P < 0.05$) between the control group and that receiving combination of *L. casei* and *B. longum*. The results suggested that probiotic supplementation showed promising effects in improving the neurological manifestations during cerebral malaria. Further studies are required to explicate the pathways involved in the immunological interplay. In the future, it is propitious that the mortalities by cerebral malaria could be mitigated through the optimal use of probiotics.

Keywords: *Lactobacillus casei*; *Bifidobacterium longum*; probiotics; gut microbiota; cerebral malaria; dysbiosis; immune regulation.

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Introduction

Malaria, a mosquito-borne infection by *Plasmodium spp.*, is a major cause of mortality and morbidity in endemic areas with a mortality rate of 15-25%. In 2020, there were 241 million new cases of malaria along with 627,000 deaths worldwide [1]. Among the five human-infecting *Plasmodium* species, *P. falciparum* causes the most severe complications, one of which being

cerebral malaria. It is estimated that 1 in 4 patients develops long-lasting neurological and cognitive deficits [2]. The cerebral pathogenesis of malaria is complex, involving the interplay of immune system which mediates both the defense and development of the disease [3]. The main cerebral malaria pathomechanism is hypoxia due to adhesion of parasite-infected erythrocytes to the vascular endothelial walls of internal organs [4]. Cytoadhesion of infected erythrocytes results in microcapillary sequestration and facilitates their agglutination in the microvasculature. Blockage of blood flow to the brain and various tissues results in tissue hypoxia, decreased perfusion, and parasite multiplication. Conditions that follow sequestration in cerebral malaria include neuroinflammation, oxidative stress, endothelial cell activation, apoptosis, platelet activation, and disruption of blood brain barrier. Some of the above conditions also inhibit neurogenesis, causing axonal injury and neuronal cell death, thus resulting neuroplasticity disorders and triggering long-term neurological deficits [5]. At the time of inflammation, apoptosis also occurs in endothelial cells, neurons, and glial cells which affect cognitive impairment [6]. These processes also cause T-cell modification and altered immune status. In addition, cerebral malaria is associated in high inflammatory response caused by monocyte activation and induction of pro-inflammatory mediators, such as interleukin (IL), macrophage colony growing factor (M-CSF), tumor necrosis factor alpha (TNF-α), and lymphotoxin. TNF-α production in the early cerebral phase of malaria is associated with a reduction in parasite burden, however, the excess TNF-α production in the late phase is associated with disease severity [7]. In cerebral malaria, pathogenic cells accumulating in the brain's microvasculature result in the production of TNF- α, which in turn upregulates chemokine receptors on T cells and adhesion molecules like intracellular adhesion molecule 1 (ICAM-1) on the vascular endothelium, and the accumulation of pathogenic mononuclear cells in cerebral blood vessels [8]. The central nervous system dysfunction associated with this inflammatory cytokine profile is consistent with previous studies that neurologic changes precede the migration of leukocytes into the brain. Neurologic changes that occur include motor behavior, autonomic function, muscle tone, strength, and neuropsychiatric conditions. In addition, there was also a histopathological change in the brainstem and hippocampus [9].

Plasmodium berghei-infected mice are the primary model for cerebral malaria research. Within 6–12 days of infection, *P. berghei* infection transforms into a fatal neurological condition with an incidence of 50-100% [10]. Mice C57BL/6 strain infected with the blood stages of *P. berghei* showed development of cerebral malaria symptoms between the 5th and 7th day with low parasitemia and death on the 2nd to 3rd day afterward. Symptoms suggestive of cerebral malaria are ataxia, seizures, paralysis, or coma, which are considered as signs of brain damage. Neurologic signs (ataxia, paralysis) develop within hours before death [11]. However, not all symptoms appear simultaneously and will vary in severity. A change in locomotor activity is associated with disease progression [12]. The most common neurological tests used in mice is the SmithKline, Harwell, Imperial College, Royal Hospital, Phenotype Assessment (SHIRPA) to confirm that experimental animals suffering from cerebral malaria show a variety of progressive behaviors and functionalities. SHIRPA is a standardized tool to evaluate postural control, motor activity, autonomic function, coordination, and emotional reactivity through 42 test points. The neurological signs are observed through 9 circuits, including a viewing jar, open arena, horizontal grid, tail lifting, horizontal wire, tube rotation, vertical grid, supine restraint, and a spacious and transparent inspection area. Furthermore, the signs could be observed from features of head flicking, circling, retropulsion, tripping, self-biting, seizures, myoclonic hopping, feet flexed, or grasping during tail lifting. This assessment is sensitive in detecting phenotypes of experimental animal models of neural disease and useful in estimating the neurological impact

of exposure to infectious agents and vascular anomalies [13].

Gut microbiota has been known to impact the host physiology and shape susceptibility to diseases. The effect of gut microbiota on host immunity is influenced by the composition of commensal bacteria and flora that influence the local pathogen load. In addition, to maintain gut local immunity, the gut microbiome also influences host immunity to extra-gastrointestinal tract infections [14]. Research on the gut microbiota continues to expand on the systemic implications of gut microbiota dysbiosis related to metabolic disorders, neurodevelopmental, and neuropsychiatric disorders [9]. This study aimed to explore the effect of administration of probiotics *Lactobacillus casei* and *Bifidobacterium longum* on neurological manifestations of cerebral malaria *in vivo*. In the future, it is propitious that the mortalities by cerebral malaria could be mitigated by harnessing the gut-brain axis using the power of probiotics.

Materials and Methods

Animal model for cerebral malaria

The experiment was conducted in the Animal Research Facility of Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. Experimental design and procedures were approved by the Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia with the approval number of 168/EC/KEPK/06/2021. Six-week-old male C57BL/6 mice were provided by PT. Biomedical Technology Indonesia (Bogor, West Java, Indonesia). The mice were housed at 25°C with a 12-hour light/dark cycles and free access to standard fed and water. After 2 weeks of acclimatization, 28 mice were randomly divided into 5 experimental groups, which comprised positive control, negative control, and three intervention groups. All intervention groups and the positive control group consisted of 5 mice per group, while the negative control group had 3 mice.

The mice were infected with *P. berghei* ANKA strain obtained from the Laboratory of Parasitology, Universitas Brawijaya (Malang, Indonesia) to develop cerebral malaria model. Cryopreserved *P. berghei*-infected erythrocytes were thawed and centrifuged at 2,000 rpm for 5 minutes. The pellets were washed twice using the Roswell Park Memorial Institute (RPMI) medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and injected intraperitoneally to donor mice (5×10^6 parasites in 200 µL of diluted erythrocytes per mice). Observation of the degree of parasitemia in donor mice was performed daily to determine the adequate number of parasites used to infect the mice in the experimental groups. After the parasitemia reached over 20%, the donor mice were sacrificed, cardiac blood was collected and stored in EDTA vacutainers, and the number of *Plasmodium*-infected red blood cells were counted. Then, the blood was diluted with normal saline to reach a concentration of 1×10^7 infected red blood cells per 200 µL. The mice in the positive control and intervention groups were then injected intraperitoneally with 5×10^6 parasites in 200 µL of diluted erythrocytes. The degree of parasitemia was observed on day 2 to day 6 post-infection using Giemsa-stained thin blood smears according to the World Health Organization (WHO) protocol [15].

Probiotic intervention

Isolates of probiotics *Lactobacillus casei* (*L. casei*) and *Bifidobacterium longum* (*B. longum*) were purchased from the Food and Nutritional Culture Collection (FNCC), Universitas Gajah Mada, Yogyakarta, Indonesia. The bacteria were grown in 11 mL of de Man, Rogosa and Sharpe (MRS) broth (Oxoid™, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37°C for 24 hours. The optical density (OD) was measured using SmartSpec™ Plus spectrophotometry (Bio-Rad Laboratories, Ltd., Hong Kong, China) at a wavelength (λ) of 600 nm post harvesting to estimate the number of bacteria. Then, 1 mL of the bacteria was vortexed and centrifuged at

4,000 rpm for 15 minutes. The pellets were resuspended in phosphate-buffered saline (PBS) to reach a certain concentration of bacteria. Each mouse in treatment groups received oral administration of either *L. casei* at 10^9 colony forming unit/100 µL (CFU/100 µL), *B. longum* 10^9 CFU/100 µL, or a combination of *L. casei* and *B. longum* 5 x 10^8 CFU each/100 µL 5 days before to six days after *P. berghei* inoculation. The negative and positive control groups received PBS orally.

Evaluation of clinical conditions

Neurological tests to confirm various progressive motor and functional behaviors in cerebral malaria models was conducted using SHIRPA. The evaluation included motor activity, coordination, postural control, muscle tone, autonomic function, emotional reactivity, visual, auditory, and tactile-dependent reflexes. The observation was performed on the circuits by documenting head flicking, circling, retropulsion, tripping, self-biting, seizures, myoclonic hopping, feet flexed, or grasping during tail lifting [13]. All mice from each experimental group were evaluated based on 42 measurements points of SHIRPA on day 4 to day 6 post infection.

Statistical analysis

The statistical analysis was performed by using SPSS version 24.0 (IBM, Armonk, NY, USA). The differences in behavioral task performances was analyzed by a one-way ANOVA test followed by post-hoc LSD test to evaluate the effect of different treatments on neurological manifestations. Statistical significance was determined at a *P* value of < 0.05, while very significant difference was set at a *P* value of < 0.01.

Results

The highest degree of parasitemia was observed in the *L. casei*-treated group, which was comparable to the positive control (untreated, *P. berghei*-infected) group. Until day 4 post infection, all treatment groups still maintained a parasitemia level below 0.5%, which were lower than that in the positive control group. However, on days 5 and 6, the degree of parasitemia in *L. casei*-treated group was higher than that in positive control group. The degree of parasitemia in *B. longum*-treated group was consistently lower than that in other treatment groups and positive control until day 6 post infection. While the group treated with combination of *L. casei* and *B. longum* showed significantly lower degree of parasitemia than that in positive control group on days 5 and 6 post-infection (data was not shown) [16].

Probiotic administration enhanced motor behavior in *Plasmodium berghei*-infected mice

Motor behavior in the group receiving a combination of *L. casei* and *B. longum* probiotics showed good scores comparable to those in the negative control (non-infected) group. Combined supplementation of *L. casei* and *B. longum* to *P. berghei*-infected mice very significantly improved motor behavior scores compared to the positive control group (*P* < 0.01) (Figure 1).

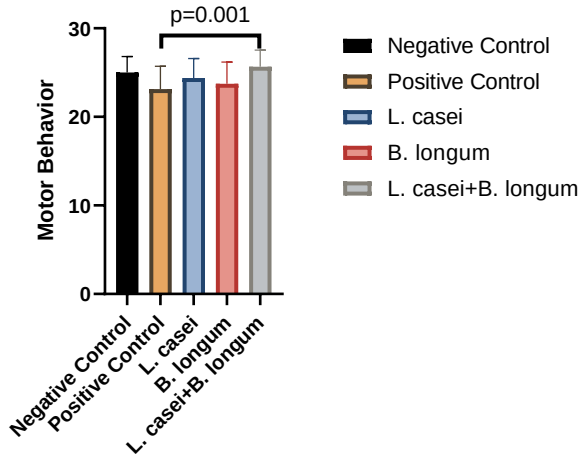

Figure 1. *Lactobacillus casei* and *Bifidobacterium longum* enhanced motor behavior in *Plasmodium berghei*-infected mice.

Improvement of muscle tone after probiotic administration in cerebral malaria mice model

To assess the muscle tone, parameters examined were body tone, extremity tone, and grip strength. The results showed that the group receiving combination of *L. casei* and *B. longum* supplementation demonstrated very significant

improvement in muscle tone compared to the positive control group ($P < 0.01$) (Figure 2).

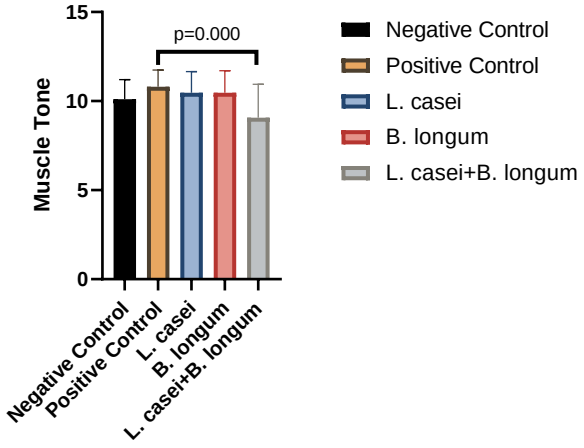

Figure 2. Improvement of muscle tone after *Lactobacillus casei* and *Bifidobacterium longum* administration in cerebral malaria mice models.

Probiotic administration did not affect autonomic outcomes of cerebral malaria *in vivo*

The examination parameters for the autonomic functions included respiratory rate, defecation, skin color, heart rate, lacrimation, and salivation. In this study, we did not find significant differences in the autonomic function among the treatment and control groups ($P > 0.05$) (Figure 3).

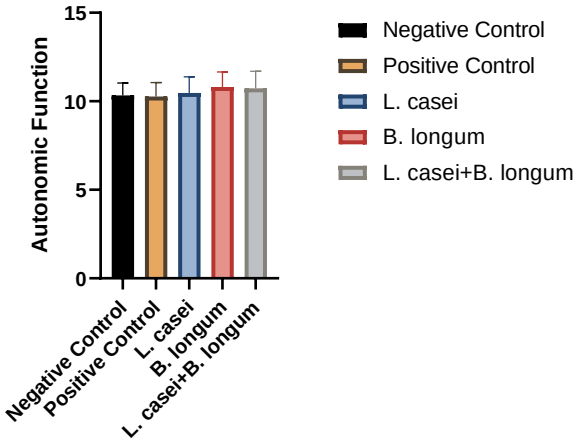

Figure 3. *Lactobacillus casei* and *Bifidobacterium longum* administration did not affect autonomic outcomes of cerebral malaria.

Reflex and sensory improvement following probiotic administration

The reflex and sensory using parameters of shock response, visual placement, pinna reflex, corneal reflex, and right reflex were further assessed. There was a significant difference between the groups receiving *L. casei* ($P < 0.05$) and combination of *L. casei*/*B. longum* ($P < 0.05$) and the positive control group (Figure 4).

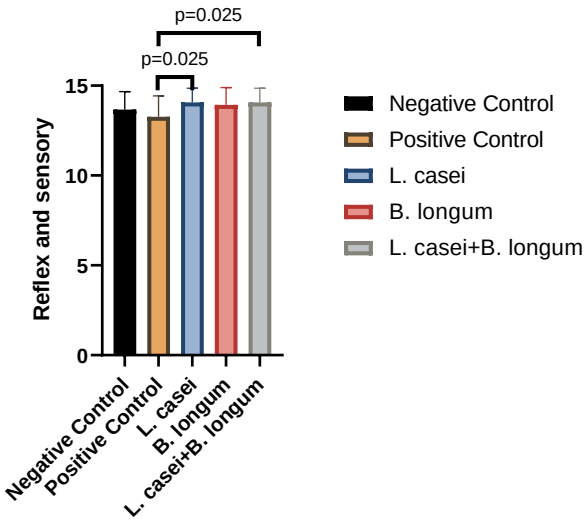

Figure 4. Reflex and sensory improvement following *Lactobacillus casei* and *Bifidobacterium longum* administration.

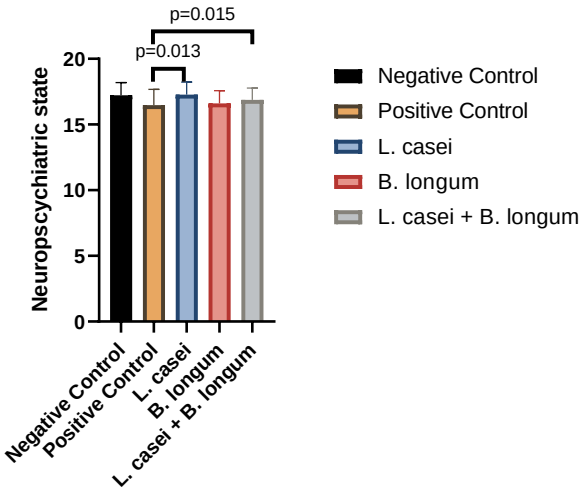

Figure 5. *Lactobacillus casei* and *Bifidobacterium longum* administration ameliorated neuropsychiatric outcomes of cerebral malaria.

Probiotic administration ameliorated neuropsychiatric outcomes of cerebral malaria

To evaluate the neuropsychiatric outcomes, the parameters of the desire to change positions, reactivity when touched, provocative behavior by biting, aggressiveness, fear, and vocalizations or screaming when touched were assessed. The results showed significant differences between the group receiving combined probiotic supplementation ($P < 0.05$) and the *L. casei*-treated group ($P < 0.05$) compared to the positive control group (Figure 5).

Discussion

Probiotics are live microorganisms that, when ingested in adequate amounts, provide health benefits due to their properties as direct pharmacological agents or drug carriers through their ability to synthesize neuroactive compounds. Probiotics also modulates the composition of the gut microbiota, strengthen the epithelial barrier, and produce intestinal antimicrobial substances, thereby regulating the host immune system [17]. They affect the brain directly by producing neurotransmitters and neuromodulators. *Lactobacillus sp*. and *Bifidobacterium sp*. have been reported to produce γ-aminobutyric acid (GABA), acetylcholine, and histamine. *Escherichia sp*. and *Bacillus sp*. produce norepinephrine, *Streptococcus sp*., *Escherichia sp*., and *Enterococcus sp*. produce serotonin, while *Bacillus sp*. produces dopamine. Probiotics also secrete or mediate the production of various biologically active compounds such as peptides and mediators related to neurotransmission [18]. They have been identified to possess immunological benefits toward neurological disorders by modulating the host immune pathways. *B. longum* strains have been shown to influence the depletion of serum and brain IFN-γ, TNF-α, and IL-6 levels, as well as the increase of plasma serotonin and brain-derived neurotropic factors (BDNF) levels in the brain [3]. In this study, the results showed significant improvement of motor neuropsychiatric state in the group receiving combination of *L. casei* and *B. longum* supplementation. *Bifidobacterium longum* has been identified to suppress cognitive decline. The gut bacteria *Bifidobacteria* and *Lactobacilli* also reduce unbalanced responses, psychiatric disorders, and cognitive disorders by regulating the expression of pro-inflammatory and/or anti-inflammatory cytokines [19]. Previous research stated that monocolonization with *B. longum* could protect against excessive stress response in the hypothalamus-pituitary-adrenal axis [20]. Treatment with the bacterial species *B. longum* eliminated the effects of anxiety due to infection [21]. Moreover, combination of the probiotics *L. helveticus* and *B. longum* have been proven to influence brain activity, especially in areas that process sensation and emotion [18].

In *P. berghei*-induced cerebral malaria, the parasite induces leukocyte migration to the brain, while the production of pro-inflammatory cytokines and chemokines causes neurological changes including the state of motor behavior, neuropsychiatry, autonomic function, muscle tone, and strength, indicating that inflammatory changes are involved in neurological disorders [6]. In this study, significant improvements in motor behavior and muscle tone were observed in the group receiving a combination of *L. casei* and *B. longum*, although autonomic functions were not affected. This might be contributed by the psychobiotic properties of *B. longum*, which was able to improve anxiety levels and sensory function, development, and processing in mice. Thus, these psychobiotics provide improvements in mental health, behavior, cognition, memory, and other general health [20].

Systemic infection and chronic stress can cause gastrointestinal dysregulation through the hypothalamus-pituitary-adrenal axis, where endocrine signals can change the balance of microflora. Gut dysbiosis is associated with gastrointestinal and psychiatric disorders [19, 22]. In this study, malaria-induced dysbiosis affected several neurological outcomes, which were mitigated by the supplementation of probiotics. This was in accordance with previous

studies reporting the therapeutic properties of probiotics in neurological disorders. Strains of *L. acidophilus*, *L. casei*, *B. bifidum*, and *L. fermentum* could improve cognitive function and metabolic rate. *Lactobacillus reuteri*, *L. rhamnosus*, and *B. longum* had been used to improve social deficits and anxiety levels [20].

Conclusion

Probiotic supplementation of *L. casei* and *B. longum* showed promising effects in improving the neurological manifestations during *P. berghei*-induced cerebral malaria. Further studies are required to elucidate the host immune components and pathways involved in the gut-brain-axis interplay. The use of optimal and standardized probiotic supplementation in combination with antimalarial treatment could potentially mitigate mortalities by cerebral malaria in the future.

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