Evaluation of Effect of Probiotics as an Add-on Therapy With Conventional Therapy and Alone in Malaria Induced Mice

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Research note

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Abstract

Objective: Chloroquine is used as conventional drug therapy for the treatment of malaria. The existence of resistance for chloroquine shown among various species of *Plasmodium* leads to the search for more efficacious therapy to treat malaria. Probiotics (*Lactobacillus casei*) have been tried as add-on therapy with chloroquine. Probiotics are ingested microorganisms associated with a beneficial effect on humans and other species. The study was done to check the efficacy of probiotics as an add-on therapy along with conventional drug therapy (chloroquine) to treat malaria.

Results: Probiotics in combination with chloroquine showed complete suppression in parasitemia count. Representation of parasitemia count was done using mean ± SD. $P<0.05$ is considered significant. The results showed a reduction in parasitemia with probiotics treatment, which was further confirmed through histological observation of two major organs liver and spleen. Interestingly, further suppression of parasitemia and hemosiderosis was observed when probiotics were given along with chloroquine.

Introduction

Malaria is among one of the deadliest threats for the human species which is caused by various strains of malaria parasite e.g. *P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi*. The risk for developing malaria is about 3.3 billion all over the world [1]. The immune response plays a major role in the pathophysiology of malaria. Different immune protein which is released during the parasite attack can prohibit the growth of the same. Moreover, some signaling pathways act against the parasite e.g. Tolls like receptor, signal transducers and activators of transcription pathway (STAT), Janus kinase pathway (JNK) [2]. Chloroquine was the choice of drug for the treatment of *P. falciparum* malaria but these days there is an emergence of resistance among *P. falciparum* species [3]. Microorganisms that are believed to provide health benefits to the consumer are known as probiotics. Probiotics are generally gram-positive in nature and are isolated from gut microflora known to provide an enhancement in the immune response to the consumer. Probiotics provide strain-specific effectiveness as it is having immune stimulatory protection among pathogens of various types. It modulates intestinal microorganisms. Probiotics have shown their effects on various epithelial cells, Payer's patches cells, and immune cells [4]. The result of this interaction is an increase in the number of IgA along with IgM [5]. Past studies had shown that gut microbiota correlates with the severity of malaria parasite infection. Probiotics have beneficial effects against malaria parasite infection [6]. Many studies which have specific protocol towards understanding the molecular mechanism of probiotics needed to be done which involves clinical application also. The present study evaluates the effects of probiotics on parasitemia count, histopathological effects in malaria-infected mice and demonstrate the synergistic effect of probiotics along with chloroquine (conventional drug therapy) which can further lead to the development of fixed-dose probiotic combination for the treatment of malaria.

Materials And Methods

Study area

The study was designed as a single experimental, observational study which was conducted in the Department of Parasitology and Department of Pharmacology of the PGIMER, Chandigarh, India, for a duration of 1 year after taking approval from Institutional Animal Ethics Committee (IAEC), PGIMER, Chandigarh, vide Ref No. 81/IAEC/521. The total of 32 mice were obtained from Institutional Central Small Animal Facility, PGIMER, Chandigarh and were divided into four groups with 8 mice in each group. The mice were infected with 0.2 mL suspension of 10^6 parasitized erythrocytes intraperitoneally. Eight Balb/c mice were present in each of the four groups (i) GroupI (non-infected) (ii) GroupII (Infected group) (iii) GroupIII (*P. berghei*+ *Lactobacillus casei*) (iv) GroupIV (*P. berghei*+ *Lactobacillus casei*+ chloroquine). Parasitized RBCs were checked using the Giemsa staining technique under the light microscope. Laboratory standard cages were used for the housing of mice and acclimatized for 7 days. Standard livestock feed and clean drinking water were provided to them. This study was conducted according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

Treatment procedure

The mice were infected via injection with 0.2 mL suspension of 10^6 parasitized erythrocyte intraperitoneally. Samples of blood were taken from the tail of mice. Parasites were determined after preparation of thin blood smear and stained with Giemsa. PBS was administered to the mice in the first group (positive control) for four consecutive days. *P. berghei* infection was given to the second group. *L.casei* along with *P. berghei* was given to the third group. Chloroquine was administered to the mice at the dose of 15mg/kg once a day for four consecutive days of the fourth group [7]. After completion of all the experimental procedures, animals were sacrificed by giving the anesthesia followed by cervical dislocation. This euthanasia procedure is done according to CPCSEA guidelines.

Determination of Parasitemia

Administration of extract was done after the collection of blood from the tail of each mouse which was inoculated with the parasite for about four days. For the determination of the baseline parasitemia, a thick and thin smear was made using the blood sample [8]. By using the random field microscope, we have checked the parasitemia percentage by counting infected erythrocytes (parasitized) out of the 200 normal (non-parasitized) erythrocytes. The following formula used for the calculation of percentage parasitemia and average percentage parasitemia:-

$$\text{PP (percentage parasitemia)} = \frac{\text{Total number of PRBC(parasitized RBC)}}{\text{Total number of RBC (non-parasitized RBC)}} \times 100$$

Histopathology

For histopathological studies, the animals were sacrificed by cervical dislocation 3 days after the last dose on the fourth day of respective treatment and the organs were harvested. Pathological changes were observed in two organs i.e. liver, spleen. Tissue from each group was fixed in 10% formalin, embedded with paraffin. After routine processing paraffin sections from each tissue were cut into 5µm thickness and stained with hematoxylin and eosin. The photomicrographs of the relevant stained sections were taken with the aid of a light microscope [9]. The following scores were used to grade the degree of histopathological changes or lesions observed in the organs: not observed (−), mild (+), moderate (++), and severe (+++).

Data analysis

Data analysis was done using statistical software SPPS Version 21. Representation of parasitemia count was done as mean± SD. Post hoc analysis by using ANOVA and Bonferroni multiple comparison test was used for the comparison of means. $P<0.05$ is considered statistically significant.

Results

Mainly 4 groups were taken for the study each containing 8 animals (Balb/c mice). (i) GroupI (non-infected) (ii) Group II (Infected group) (iii) Group III (*P.berghei* + *Lactobacillus casei*),(iv) Group IV (*P.berghei* + *Lactobacillus casei* + chloroquine). Parasitized RBCs were seen using light microscopy on the 4th day of inoculation by using the Giemsa staining technique on the microscopic slides. On the 1st day as compared to Group II (*P.berghei* treated*)*, GroupIV (*P.berghei* + *Lactobacilluscasei* + chloroquine) has shown a statistically significant decrease ($p < 0.01$) in % parasitemia, On 2nd day, Group III (*P.berghei* + Probiotic) and Group IV (*P.berghei* + *Lactobacillus casei* + chloroquine) has also shown a statistically significant decrease in % parasitemia ($p < 0.05$ and $p < 0.001$) as compared to group II (*P.berghei*) while on day 3rd, Group IV has shown statistical significant ($p < 0.01$) decrease in % parasitemia as compare to group II. Finally, on the last 4th-day group IV has shown a statistically significant decrease in % parasitemia ($p < 0.05$) as compared to the *P. berghei* treated group as shown in Fig. 1. Overall, it has been shown that there is a reduction in parasitemia count when probiotics alone and probiotics along with chloroquine were given as compare infected group (*P. berghei* treated), shown in (Additional file 1: Figure S1).

There were no changes found in the case of a control group as it has shown normal pathology (Fig. 2a & 3a). Hemosiderrhosis and Periportal inflammation in the liver section was found more in an infected group (2b) but there is a reduction of Hemosiderrhosis and Periportal inflammation when treatment of probiotic (2c) was given and further suppression was seen in the group treated with chloroquine and probiotics (2d). In the case of the spleen, megakaryocytic hyperplasia, lymphoid hypoplasia along Hemosiderrhosis have been seen in the infected group (3b). It has become mild and traces of Hemosiderrhosis pigments were seen when probiotic treatment was given (3c) but these were further reduced in the chloroquine and probiotics treated group (3d).

Discussion

This study is planned to show the synergistic effect of *L. casei* (Probiotics) along with chloroquine in malaria-induced mice. Probiotics were given 0.1ml for 3 days. A similar study was conducted by Oyetayo VO *et al.*, [10] in which they showed *Lactobacillus acidophilus* and *l.casei* protective effects. Liver functions are improved by *L.casei* which is confirmed by Toxicological data of rat serum. The rat has been dosed with *Lactobacillus* were found to have a lowering of serum alanine aminotransferase activities which have value 15.50 and 18.27 IU/L as compare to that of the control. In the present study, probiotics treatment along with conventional drug therapy showed a statistically significant reduction in parasitemia count along with conventional drug therapy. There is an improvement in histopathological damages that are caused by *the plasmodium* parasite in organs like the liver and spleen. Hence, the present study confirmed that the use of probiotics as an add-on therapy along with conventional drug therapy has beneficial effects [10]. Probiotics treatment causes a reduction in the parasitic count and it also leads to suppression in parasitemia count. Chloroquine was given along with probiotics causes a further decrease in the parasite and thus complete suppression of the parasite takes place. Blood film microscopic examination lead to the production of a mean parasitemia count which is found to be lesser than 2% within the period of 4 days before starting treatment while chloroquine + Probiotics (*L. casei*) cleared parasitemia on the third day of treatment [11]. A previous study done by Khalifa EA, 2016, has shown that as compared to the non-treated group, *the L. casei* treated group has decreased the parasite load in the infected mice. So, probiotics can be considered as a promising and hopeful alternative for the treatment of various parasitic diseases. Different standard drug therapies in combination with probiotics can provide a definite treatment to eradicate the various parasitic infections [12]. Moreover, probiotics treatment has also been effective in treating bacterial infections as well e.g. *Salmonella*. Probiotics have several mechanisms of action for example, by increasing the production of acid which kills the acid-sensitive bacteria's or by releasing the bacteriocins [13]. Another study which has been conducted in mice shown a reduction in the number of worms (about 33%) and egg output specifically which was infected by *S. venezuelensis* by probiotics activity by improving the immune response but the factors responsible for these effects are still not clear [11].On the other hand, the study which was done by Juliette Guitard *et al.*, has shown that administration of *L.casei* mixture (daily) was unable to eradicate the complete parasite load (*Cryptosporidium parvum*) in the neonatal rat model. The reason for this may be due to lack of production of INF-γ which could show the protective effect [14].

This study has shown that when *L. casei* is given along with the standard drug therapy (chloroquine) showed a synergistic effect in the mice model of malaria as it has reduced the parasitemia count and improved the pathological changes that appeared after getting the infection.

Limitations

The study includes the only preliminary finding that shows the only effect of probiotics on malaria parasite in *vivo* environment. However, lacks to depict host response while taking probiotics in case of malaria infection. A more elaborated protocol is required for further deeper investigations such as

studying of involvement of innate and adaptive immunity through estimation of antibodies, T subset regulation, and cytokines estimation. Additionally, experiments such as a survival plot would better explain the usefulness of these prophylactic measures.

Abbreviations

ANOVA Analysis of variance

ATCC American type culture collection

IgA Immunoglobulin A

IL-10 Interleukin-10

INF-γ Interferon-gamma

IU/L International units per liter

L.casei Lactobacillus casei

LS Lateral section

P .falciparum Plasmodium falciparum

PBS Phosphate Buffer Saline

RBCs Red blood corpuscles

SD Standard deviation

SPSS Statistical Package for the Social Sciences

TNF-α Tumor necrosis factor-α

Declarations

Acknowledgments

Not Applicable

Authors' contributions

BM, RS designed the study and contributed reagents and materials. EM performed experiments and AB, EM and SS did the result's analysis. EM, SS and BM helped in the writing, reviewing, and editing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethical Approval and consent to participate

The ethical approval was taken from Institute Animal Ethics Committee, Project Ref No. 81/IAEC/521, at Postgraduate Institute of Medical Education and Research, Chandigarh, India for maintenance of *P. berghei NK-65* strains in Balb/c strain of mice.

Consent for publication

Not applicable

Competing Interests

The author declares that they have no competing interests.

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Figures

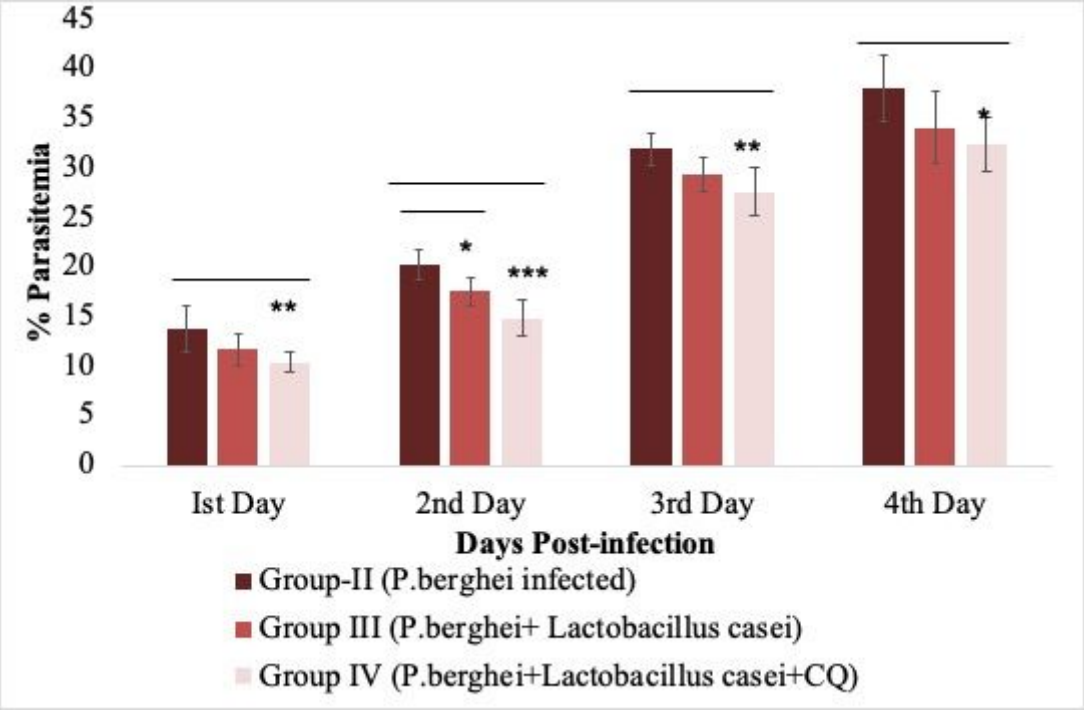
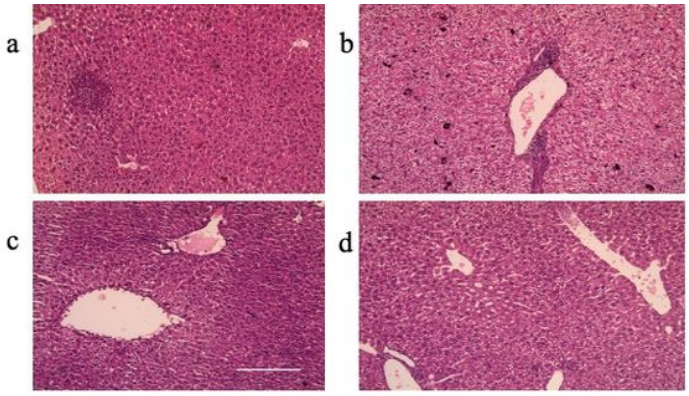


Figure 1

Graph showing percentage Parasitemia. The data are represented as mean ± SD. Statistical Significance of data are given as *p < 0.05; **p < 0.01;***p < 0.001

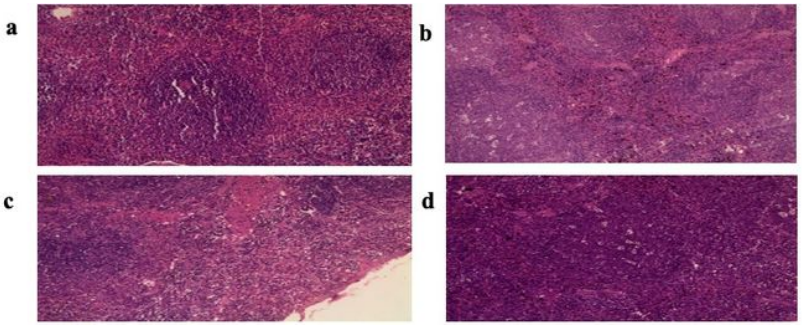


e

Animal Groups	Kuffer cell hyperplasia	Hemosiderosis	Periportal inflammation
Non infected group	–	–	–
P. berghei infected	++	++	++
P.berghei+ lactobacillus casei	+	–	–
P.berghei+ lactobacillus casei+ Chloroquine	–	–	–

Figure 2

LS of control liver and treated liver under high magnification (20x). (a) Group I (Non-infected group): In this liver showing no periportal inflammation, Hemosiderrhosis and Kuffer cell hyperplasia (b) Group II (P.berghei infected): Showing severe Periportal inflammation with Kuffer cell hyperplasia and Hemosiderrhosis (c) Group III (P.berghei+ Lactobacillus casei) liver: showing mild kuffer cell hyperplasia, periportal inflammation and traces of Hemosiderrhosis (d) Group IV (P. berghei+ Lactobacillus casei+ Chloroquine) liver: showing Kuffer cell hyperplasia, Hemosiderrhosis and traces of Periportal inflammation. (e)The scoring chart to show the effect of treatment on histopathological changes in the liver section.



e

Animal Groups	Megakaryocytic hyperplasia	Hemosiderosis	Lymphoid hypoplaisa
Non-infected	–	–	–
P. berghei infected	+++	++	+
P.berghei+ lactobacillus casei	+++	–	–
P.berghei+ lactobacillus casei+ Chloroquine	–	+	–

Figure 3

LS of Spleen under high magnification (20x). (a) Group I (Non-infected group) spleen (b) Group II (P.berghei infected) spleen: showing severe hemosiderosis, lymphoid hypoplasia and Megakaryocytic hyperplasia (c) Group III (P. berghei+ Lactobacillus casei) spleen: showing mild Hemosiderrhosis and Megakaryocytic hyperplasia (d) Group IV (P. berghei+ Lactobacillus casei+ Chloroquine) spleen: showing mild Hemosiderrhosis, Megakaryocytic hyperplasia, and no lymphoid hypoplasia. (e) The scoring chart to show the effect of treatment on histopathological changes in the spleen section.

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