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The probiotic bacterium *Pediococcus ethanolidurans* modulate malaria infection in *Plasmodium berghei* ANKA infected mice

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ABSTRACT: *Background:* Malaria still remains one of the most leading causes of morbidity and mortality in the world and particularly in sub-Saharan Africa. Until date, Resistance to the known fast acting antimalarial drugs, lack of license effective malaria vaccine and resistance of vector to insecticides remain a key challenge in eradicating the scourge of this disease. Therefore, there is a dire need for alternative antimalarial therapeutic agents or approaches that will help in preventing further increase of this disease. One alternative antimalarial possibility is the use of probiotic bacteria such as *Lactobacillus* spp. as dietary supplements.
Aim: This study aimed at assessing the effects of a probiotic bacterium *Pediococcus ethanolidurans* on Bal/c mice infected with chloroquine sensitive *Plasmodium berghei ANKA.*
Methods: The probiotic bacterium used in this study was isolated from the Cameroonian Mborro Fulani's traditionally fermented milk and was identified by sequencing its 16S r RNA gene. The repository activity of *Pediococcus ethanoliduran on* malaria infection in *Plasmodium berghei* infected mice was evaluated using the method described by Peters (1965) with slight modification. Forty-two healthy young adult balb/c were randomly divided into 7 group of 6 mice each and were orally given 9.10^8 cfu/ mL, $1.8x10^9$cfu/ mL and 2.7. $x10^9$ cfu/ mL of *Pediococcus ethanolidurans,* 0.1mL of Chloroquine (10 $mgkg^{-1}$), sulfadoxine pyrimethamine (30 mg/kg^{-1}) and 0.1mL of vehicle (PBS) for seven and for fourteen days before infection with 0.1 mL of 10^7 *Plasmodium berghei* parasite. Parasitemia, parasitemia percentage suppression, body weight loss, body temperature, survival time and some inflammatory cytokines level were evaluated. Data were presented as Mean ± SEM (standard deviation error of the mean) and analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0 statistical software (IBM, SPSS, Inc., Chicago, IL, USA).
Results: The probiotic bacterium significantly increase the parasite suppression as the dose of the probiotic bacterium increased, with maximum suppression being 100% at dose 3 on day 20. Also, they significantly prevented body weight loss and body temperature reduction and significantly ($p<0.05$) increase Interleukin 10 (Il-10) and reduce some proinflammatory cytokines (TNF-α, INF-γ, IL-1β and IL-6) in treated mice as compared to untreated mice. This bacterium was also capable to significantly increase the level of red blood cells, hemoglobin, white blood cells, lymphocytes monocytes, eosinophils and neutrophils of treated mice when compared with that of untreated mice.
Conclusion: Based on these results we therefore concluded that *Pediococcus ethanolidurans* is a probiotic bacterium with protective effects on malaria infection in the chloroquine sensitive *Plasmodium berghei* infected mice.

KEYWORDS: Malaria, Probiotics, Parasitemia, *Plasmodium berghei*, Balb/c mice, cytokines.

1 INTRODUCTION

Malaria is a parasitic disease cause by protozoan parasite of the genus plasmodium. It is transmitted by the bite of female anopheles' mosquitoes. Five species of *Plasmodium* infect humans (*Plamodium. falciparum*, *Plasmodium vivax*, *Plasmodium ovale* spp., *Plasmodium malariae* and *Plasmodium. knowlesi*) (Sinka et al., 2010). Several species infect animal among these are: *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium vinckei* and *Plasmodium yoelii* that infect rodents and plasmodium knowlesi that infect monkeys (Déchamps et al., 2010). Rodent malaria has long been used in the screening and development of new antimalarial compounds. Malaria is endemic in sub-Saharan Africa, Asia, Latin America and to a lesser extent the Middle East and parts of Europe and the intensity of transmission depends on factors related to the parasite, the vector, the human host, and environment (WHO, 2018). Although the global malaria burden has considerably decreased over the last 15 years, malaria still remains one of the most leading causes of morbidity and mortality of human populations in the world and particularly in sub Saharan Africa (WHO, 2018). Malaria disease burden was estimated to be 228 million cases and 405,000 deaths worldwide in 2018 with approximately 93% of the cases and 94% of deaths occurred in Africa (WHO, 2019). Between 2010 and 2017 the incidence rate of malaria decreases from 72 to 59 cases per 1000 population at risk. (WHO, 2018). Malaria poses serious problems to under five years children and pregnant women. The disease threatens an estimated of 25 to 125 million pregnant women world-wide every year and of all the deaths caused by malaria, 219 000 to 421 000 are children under five years living mostly in Africa (Bahizire et al., 2017; Goshu and Yitayew, 2019). Until date, resistance to the known fast acting antimalarial drugs remains a key challenge in eliminating the disease. Even the latest artemisinin-based combination therapy is already facing problem of resistance in some part of the world (Coelho et al., 2017). Also, there is no effective license vaccine against malaria, the promising vaccine RTS, S is now ongoing the phase 4 clinical trials (Coelho et al., 2017). Vector controls measures relaying on insecticides treated bed nets, indoor residual spray for the protection of human against mosquitoes' bites, insecticides and biological control measures for the reduction of mosquito vectors used in malaria endemic areas are also facing problem of resistance (WHO, 2018). Therefore, the control of this disease is among the most challenging tasks for public health. Drug overuse and misuse are recognized as the main drivers for drug resistance (Cheeseman et al., 2015). Hence, there is an urgent need for alternative antimalarial therapeutic agents or approaches that will help in preventing further increase in malaria associated morbidity and mortality.

One alternative antimalarial strategy is the use of probiotics (defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit to the host') bacteria. (Vijayakumar et al., 2015). This approach has the advantage in that, probiotic-based products are generally safe, easy to distribute and well received by the public (Holzapfel et al., 2001). In addition, probiotic-based vaccines may have short-term and long-term beneficial effects to mucosal and systemic immunity (Maidens et al., 2013). Many studies carried out on probiotic bacteria have shown their immunomodulatory properties resulting in the production of cytokines (Rigo-Adrover et al., 2016). Cytokines are known for their role in the antiparasitic immune response and in the immunopathology of malaria. However, no detailed study has yet been conducted on the relationship between immunomodulation caused by probiotics on malaria. (Farrington et al., 2017; Mandala et al., 2017). The work done by Martínez-Gómez et al. (2006) has shown that the administration of the probiotic strain Lactobacillus casei spp rhamnosus in NIH mice causes considerable resistance of these animals to infection with Plasmodium chabaudi. chabaudi AS. Lactobacillus is one of the common probiotic strain in some fermented foods or beverages, including traditionally fermented milk. Given the persistence of the occurrence of severe malaria in young children in sub-Saharan Africa, it is important to conduct research on new approaches to protection. Locally fermented drinks such as Mbororo Fulani traditionally fermented milk have been reported for their richness in probiotic lactic acid bacteria (Tatsinkou et al., 2015). These strains have never been evaluated for their ability to confer protection against malaria. Hence the interest of conducting a study on the protective effects of the probiotic bacterium *Pediococcus ethanolidurans* isolated from Cameroonian traditionally fermented milk on *Plasmodium berghei* ANKA infected mice.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 MEDIA, DRUGS AND CHEMICALS

The main media used in this study were de Man Rogosa and Sharpe (MRS) agar, MRS broth purchased from Sigma Aldrich.

The main chemicals that were used in this study include: Distilled water, Methanol, Normal saline, Trisodium citrate (BDH), Phosphate buffer saline (PBS), Gemsia stain, Chloroquine (Sigma Aldrich, Germany).

2.1.2 Source Of Probiotic

Probiotic bacterium was isolated from traditionally fermented milk randomly purchased from Bamenda Mbororo (Fulani ethnic group in Cameroon) and identify by the sequencing of its 16 S rRNA gene.

2.1.3 Malaria Parasites (Plasmodium Berghei ANKA)

Malaria parasite (*Plasmodium berghei* ANKA) were donated by *«Institut de Recherche pour le Développement, unité MIVEGEC (UM1-UM2-CNRS 5290-IRD 224), 911 avenue Agropolis, Montpellier Cedex 5 34394, France»*. The parasites were stored at -80 °C until used.

2.1.4 Experimental Animals

Young adults healthy Swiss albino mice (balb/c), 6–8 weeks old weighting 20 to 25 g were purchased from National Veterinary Laboratory (LANAVET), Cameroon. The animals were housed in standard cages at 25°C, 12/12 h light/dark cycle, with free access to clean drinking water and food. All animals were housed and cared for according to international guidelines for the caring and use of laboratory animals (Couto, 2011). Mice were acclimated into the experimental environment for five days before starting the experiment. Ethical clearance for this study was obtained from the University of Buea Institutional Animal Care and use Committee (UB-IACUC).

2.2 Methods

2.2.1 Isolation And Molecular Identification Of Lactic Acid Bacteria From Traditionally Fermented Milk By The Sequencing Of 16 S Rrna Gene

Pour plate technique was used to isolate the microorganism. One millilitre of each milk sample was serially diluted up to the ten logarithmic fold in sterile test tube containing 9 ml of 0.85% saline solution (Nacl). De Man Rogosa and Sharpe (MRS, Sigma-Aldrich, Germany) agar, a selective medium for lactic acid bacteria isolation was weighed, prepared, sterilised and allowed to cool at room temperature according to the manufacturer's instructions. One millilitre aliquot of three different dilution factors (10^{-5}, 10^{-6} and 10^{-7}) of the sample were poured into plates and about 15 ml of the selective medium (MRS agar) was added and allow to solidify at room temperature. Parafilm was then used to seal the plates to prevent contaminations and allow preferential growth of lactic acid bacteria since they are anaerobe facultative. The plates were incubated up-side-down at 37ºC for 24h to 48 h. After incubation, isolated colonies were subjected to catalase test. The catalase negative isolates were selected and sub cultured on fresh MRS agar. The purpose of this step was to purify the selected colonies with streak plate technique. A code was given to each isolate and they were examined for to their cell morphology, catalase and Gram reactions. Gram positive and catalase negative isolates were taken as presumptive lactic acid bacteria and were kept at 4°C in 1.5 ml Eppendorf tubes containing MRS agar for further investigation. At the same time duplicates were cultured in MRS broth containing 20% glycerol and stored at -80 °C for long term preservation.

2.2.2 Functional Characterisation And Safety Evaluation

The functional properties study were acid and bile tolerance. Probiotic ingested orally should resist gastrointestinal tract stress environment, such as resistance to acid and to bile salt. These two properties were evaluated as described by Ding *et al*., (2017). For this purpose, active cultures (incubated for 24 h in MRS broth) were used. Cells were harvested from the suspension by centrifugation (4000 rpm, for 10 min at 4 °C) and washed once in phosphate saline buffer (1X PBS at pH 7.2). Cell pellets were resuspended in PBS and used for *in vitro* acid tolerance test. Cell suspension (0.5 mL) in PBS was diluted to 5 mL with sterile PBS and adjusted to a series of pH between 1.0 and 3.0. using 1N HCL or NaOH. The suspensions were maintained at 37 °C and viable organisms were enumerated after 0, 1, 2 and 3 h on MRS agar plates. reflecting the minimum and maximum time which food spends in the stomach (Bengmark, 1998). Isolates that exhibited final counts ≥ 10^3 cfu/ml or ≥ 10^6 cfu/ml at low pH for 3 h, were considered to have moderate or good resistance, respectively**.** To perform this enumeration, 1 mL of each of the suspensions was serially diluted up to the ten logarithmic fold and the viable microorganisms were counted in triplicates on MRS agar.

Resistance to bile salt was evaluated based on viable colony counts on MRS agar in triplicates after incubation at 37 °C for 0 and 4 h, reflecting the time spent by food in the small intestine. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2), then suspend into 1 ml MRS broth containing 0.2 % (w/v) and 0.4 % (w/v) oxgall-bile respectively. Broth without oxgall-bile serve as control.

Safety of the isolated lactic acid bacteria was evaluated by testing their haemolytic activity. A bacterium having probiotic properties should not have haemolytic activity. This test was performed in isolated LAB with tryptic soy agar (TSA) (Sigma-Aldrich, Germany), containing 5% (w/v) sheep blood according the method described by Schmitt et al. (2012). The plate was incubated at 37°C for 48 h, and blood agar plates were examined for evidence of β -haemolysis (clear zones around colonies), α -haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies). *Staphylococcus aureus* strains were used as positive control. The assay was repeated in triplicates. The isolated LAB were selected and considered as candidate probiotic based on their functional properties: resistance to acid, resistance to bile salt, absence of haemolytic activity.

2.2.3 Identification And Phylogenetic Analysis Of The Probiotic Bacterium

Direct colony PCR was carried out to amplify the 16S rRNA gene of the probiotic. Lactic acid bacterium isolate was taken directly from pure and fresh cultures that were 24 h old. Using heat sterilised inoculating loop, two colonies of the lactobacillus isolate was directly transfer into 20 µL of the PCR reaction mixture consisting of 10 µL of master mix, 0.5 µL of forward primer, 0.5 µL of reverse primer and 9 µL of sterile distilled water. The PCR conditions for the 30 cycles were as follows: 95°C 5 min (initial denaturation), 94°C for min 30 s (denaturation), 42°C for 1 min 30 s (annealing), 72°C for 1 min 30 s (extension) 72°C for 10 min (and final extension). The PCR amplicons were examined by agarose gel electrophoresis (1%w/v). Two μl of each amplification mixture were subjected to electrophoresis in 1.5% (w/v) agarose gels in 0.5 x TEA buffer for 1 h at 100 V. DNA molecular mass marker (250 to 10000 bp) molecular ladders from INQUABA, South Africa was used as standard. After electrophoresis, the gels were stained in ethidium bromide, washed and photographed with UV transilluminator (Bio-Rad, Hercules, CA, USA). The partial 16S rRNA sequence analysis of the PCR products was determined by INQUABA (South Africa). The sequences obtained were compared using BLAST (basic local alignment search tool) and submitted to the GenBank sequence database for accession numbers

2.3 Prophylactic Activity Of Pediococcuc Ethanolidurans On Plasmodium Berghei Infected Mice

2.3.1 Animal Grouping And Dosing

Mice were randomly divided into seven groups of six mice each. One Normal group (NoG), one negative control group (NeG), two positive control groups, and three test groups. The normal group was not infected and received PBS only, the negative control group (NeG) were infected and received PBS only while those in the positive control groups were treated with 10 mg/kg of the standard drug chloroquine (CHL) and 30 mg/kg of sulfadoxine pyrimethamine (SPT) for 7 and for 14 days before infection. On the other hand, the three test groups were infected and received *pediococcus ethanolidurans* at three different concentrations (Doses) (dose 1= 9.10^8 cfu/ mL, dose 2=1.8. 10^9 cfu/ mL and dose 3= $2.7.10^9$ cfu/ mL for 7 and for 14 before infection**.**

2.3.2 Parasite Inoculation

The chloroquine sensitive strain of *Plasmodium berghei* (strain ANKA) was prepared through monthly passage of the 1×10^7 pRBCs in naive mice. Donors with parasitaemia level of 20-30% were sacrificed and blood collected by cardiac puncture. The blood was then diluted with phosphate buffered saline (PBS) based on parasitaemia level of each donor and the RBC count of normal mice, such that 1 mL blood contained 5×10^7 parasites. The experimental animals were each infected with 1×10^7 pRBCs by intraperitoneal (ip) injection during antimalarial test (Basir et al., 2012).

2.3.3 Preparation Of Different Doses Of Probiotic Inocula

Bacteria cells for *in-vivo* studies were grown on MRS broth overnight at 37 °C, then separated from the culture supernatant by centrifugation (4 min at 4000×g) at 4 ^{0}C, washed three times with ice cold phosphate buffer saline (PBS) (pH=7.2) and resuspended in PBS. The final concentration of the mixture was adjusted to McFaland standard 3 (9×10^8 cfu/ mL), 6 (1.8×10^9cfu/ mL) and 9 (2.7×10^9cfu/ mL).

2.3.4 Prophylactic Activity Test

With the aim of verifying if the probiotic bacterium could be useful in protecting or preventing malaria infection in *Plasmodium berghei* infected mice, prophylactic activity test was carried out. This was done using the method described by Peters (1965) with slight modification. The modification was based on the treatment duration. Mice were treated for 7 and for 14 days consecutively with different doses of probiotic strain before infection rather than 4 days as described by this author. Mice were randomly divided into seven groups of six animals each. The first group was the normal control group and was administered an equivalent volume

of PBS only while the second group was the negative control and was administered PBS before infection. The third, the fourth and the fifth group were test groups and received orally (through an intragastric feeding tube) 0.1mL of the probiotic strain at three different concentrations (9.10^8, $1.8.\ 10^9$ and $2.7.10^9$ cfu/ mL**)** for seven and for fourteen days before infection with 0.1 mL of 10^7 *Plasmodium berghei* parasite. The sixth and the seventh groups were positive control and were administered respectively 0.1mL of Chloroquine (10 $mgkg^{-1}$) and Sulfadoxine pyrimethamine (30 mg/kg^{-1}) orally for seven and for fourteen days before infection. After infection with *Plasmodium berghei,* body weight and rectal temperature were recorded for each experimental mouse just before infection and weekly during the experiment. Parasitaemia density and percentage inhibition were evaluated daily for 21 days through preparation of Giemsia thin blood film smear of blood collected from the tail of mice. Mice were then sacrificed on the day 22, blood was collected through cardiac puncture to evaluate haematological parameters using an Auto haematological analyser, ADVIA 60 (Bayer Bayer, Germany) and to assess cytokines profile using sandwhich ELISA. The mean survival time (MST) of each animal was evaluated during the follow-up.

2.3.5 EVALUATION OF PARASITEMIA

Parasitemia level was evaluated daily using Giemsa stain technique as described by De Oca et al. (2013). Briefly, a drop of blood was milked from the tail of each mice using sterile lancet and thin film smear prepared on to clean microscopic slide. These films were fixed with absolute methanol for 30 seconds and stained with 10% Giemsia stain for 15 minutes. Parasitemia was then examined under a standard light microscope using a 100× oil immersion objective. Using a microscopy counter, the number of infected red blood cells and the number of total red blood cells per field were counted in at least 8 fields per slide. Percentage parasitemia and percentage suppression were calculated using the formula below:

$$\%\ Parasitemia\ = \frac{Number\ of\ parasitized\ RBC}{Total\ Number\ of\ RBC\ count} \times 100$$

$$\%suppression = \frac{(\%Parasitemia\ of negative\ control - \%Parasitaemia\ of treated\ group)}{\%Parasitaemia\ of\ negative\ control} \times 100$$

2.3.6 EVALUATION OF BODY WEIGHT, BODY TEMPERATURE AND HEAMATOLOGICAL PARAMETERS

Body weight and body temperature values were evaluated to assess the effectiveness of the of the lactobacillus strain in preventing the reduction of these parameters by the *plasmodium* parasite. For the determination of weight, each experimental mouse in a group was weighed using sensitive digital weighing balance (ADAM equipment germany). Body temperature of each mice was measured using a digital rectal thermometer and their means were used to compare the change, that occurred before and after infection, between treatment groups and the untreated group.

For the evaluation of hematological parameters, Blood samples with EDTA collected from mice were used for this assay. Haematological profile of mice was evaluated using the method described by Cheesbrough (2006). Briefly, an Auto haematological analyser, ADVIA 60 (Bayer Bayer, Germany) was used to analyse the following blood parameters: full blood count (white blood cell, red blood cell, haematocrit, haemoglobin, MCV, MCH, MCHC) and differential leucocytes count (neutrophil, eosinophil, basophil, monocytes and lymphocytes).

2.3.7 EVALUATION OF CYTOKINE PROFILE

Blood without EDTA collected from mice was used to prepare sera and sera was used to evaluate the cytokine profile of the *Plasmodium berghei* infected mice using Sandwhich ELISA according to the manufactory instructions.

2.4 STATISTICAL ANALYSIS

All data presented as Mean ± SEM (standard error of the mean) were analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0 statistical software (IBM, SPSS, Inc., Chicago, IL, USA). For the evaluation of antimalarial efficacy tests, statistical analysis by one-way analysis of variance (ANOVA) was used to compare the level of cytokines of the *Plasmodium berghei* infected mice between the control and *lactobacillus* treated group. Throughout the analysis, Post hoc tukey test was used and a statistical significance was set at ($p < 0.05$).

3 RESULTS

3.1 IDENTIFICATION OF THE SELECTED PROBIOTICS

The isolate used for antimalarial activity was isolated from the Mbororo Fulani traditionally fermented milk and identified by sequencing of its 16 S rRNA gene as a strain of *Pediococus ethanolidurans* 97% similarity. The sequence was deposited in the gene bank for accession

3.2 EFFECTS OF PEDIOCOCCUS ETHANOLIDURANS ON MALARIA INFECTION IN PLASMODIUM BERGHEI INFECTED MICE

3.2.1 EFFECT OF PEDIOCOCCUS ETHANOLIDURANS ON PARASITEMIA, PARASITEMIA PERCENTAGE SUPPRESSION AND SURVIVAL TIME DURING PROPHYLACTIC ACTIVITY

Figure 1, 2 and 3 show the malaria parasitaemia, parasitaemia percentage suppression and mean survival time of *Plasmodium berghei* infected mice untreated (NeG) and *Plasmodium berghei* infected mice previously treated with Chloroquine (CHL), Sulfadoxine pyrimethamine (SPT), and different doses of *Pediococcus ethanolidurans* for seven days and for fourteen days beforer infection. These results showed that mice receiving oral administration of this probiotic bacterium for 7 and for 14 days befor infection presented a gradual significant ($p<0.05$) dose and duration of treatment dependent reduction of the level of malaria parasitaemia compared to untreated group. There was also a gradual significant ($p<0.05$) increase of the parasitemia suppression as the dose of the probiotic bacterium increased as from day 5 till the end of the experiment, with maximum suppression being 100% on day 20. When Comparing the effect among the doses of bacterium, parasitemia suppression was significantly greater ($p < 0.05$) with dose 3 than dose 2 and dose 1. In addition, all the mice survived (100%) in treated groups as compared to the untreated groups (MST 53.6%).

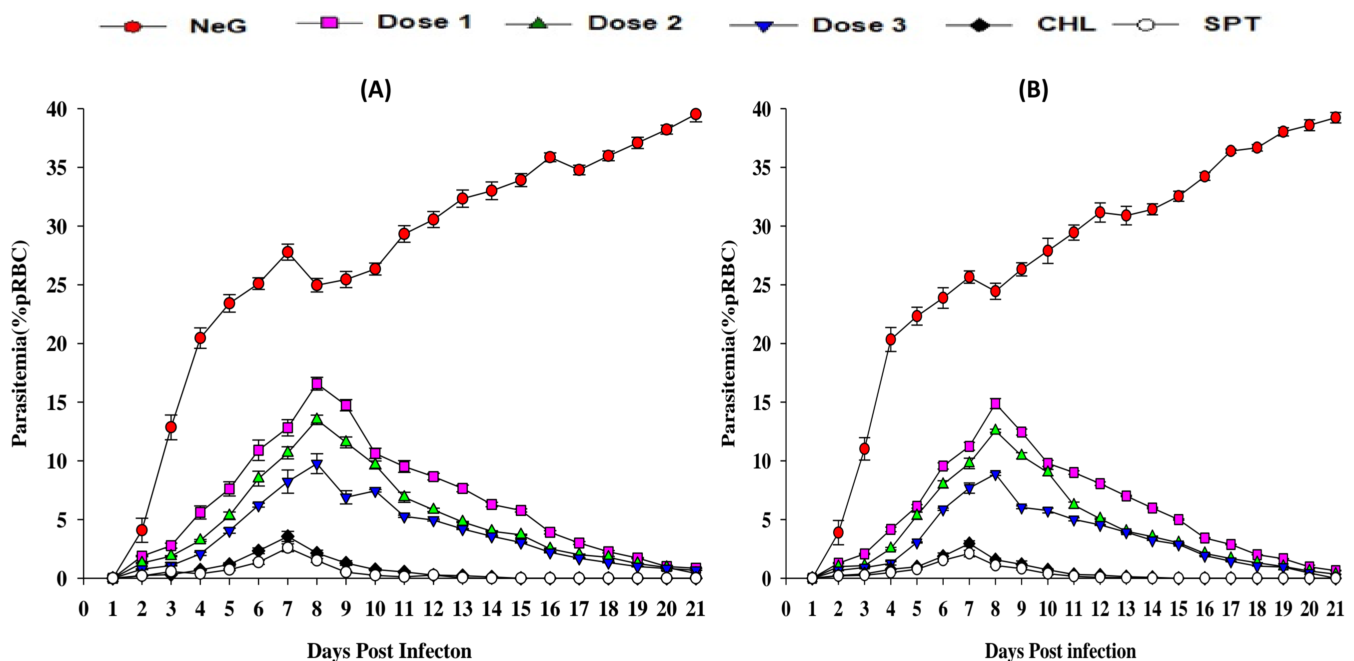

Fig. 1. Effect of Pediococcus ethanolidurans on the malaria parasitemia of Plasmodium berghei ANKA infected mice during prophylactic activity

(A): 7 days oral administration; (B) 14 days oral administration; NeG. is the negative control, CHL= Chloroquine (10mg/Kg), SPT= Sulfadoxine pyrimethamine (30 mg/Kg). Dose1 = 9 x 10^8 CFU/mL; Dose 2= 1.8. x 10^9 CFU/mL; Dose 3=2.7. x 10^9 CFU/ mL of Pediococcus ethanolidurans). All the results are expressed in term of mean ± standard error, for n = 6 mice per group.

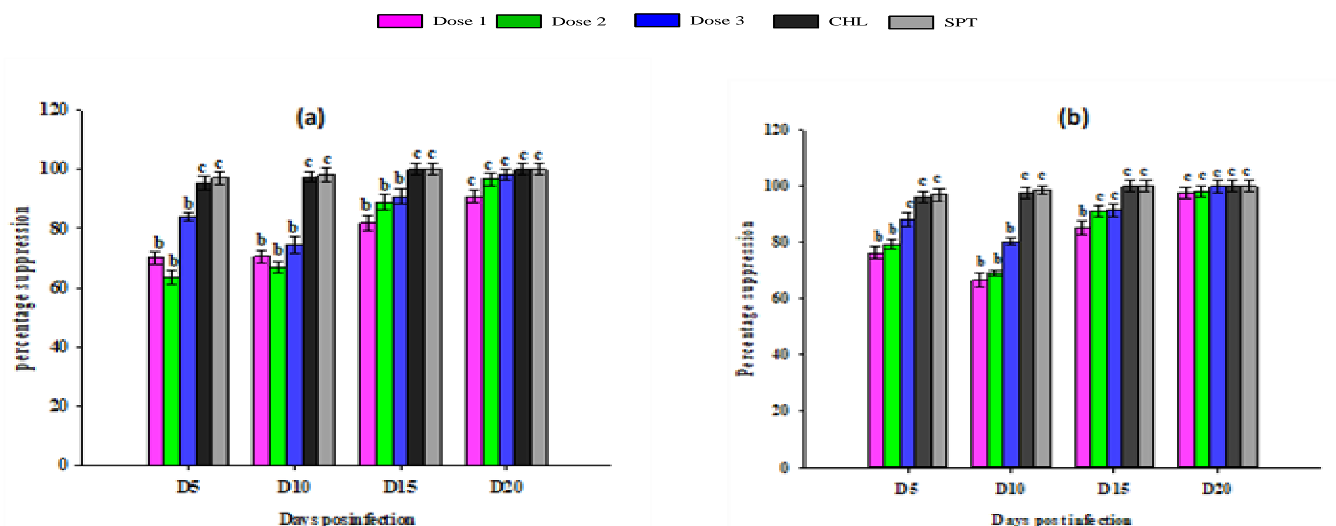

Fig. 2. ***Effect of Pediococcus ethanolidurans on the malaria parasitemia percentage suppression oPlasmodium berghei infected mice during prophylactic activity***

(a): 7 days oral administration; (b): 14 days oral administration; NeG. is the negative control, CHL= chloroquine (10mg/Kg), SPT= Sulfadoxine pyrimethamine (30mg/Kg). Dose1 = 9. x 10^8 CFU/mL; Dose 2= 1.8. x 10^9 CFU/mL; Dose 3=2.7. x 10^9 CFU/ mL of Pediococcus ethanolidurans). All the results are expressed in term of mean ± standard deviation.

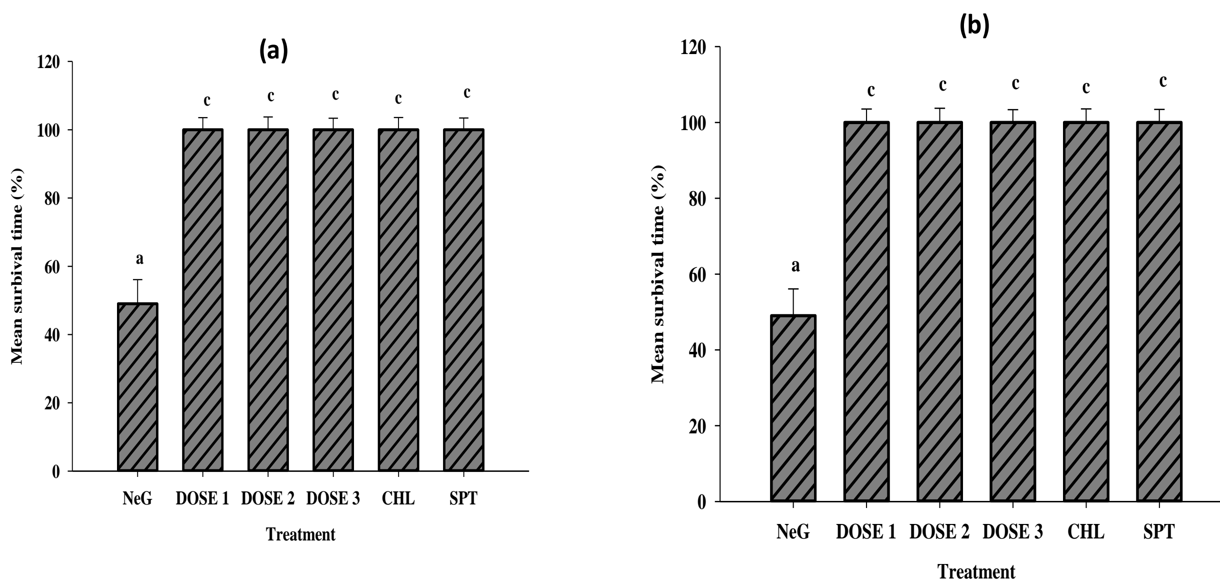

Fig. 3. ***Effect of on the mean survival time Plasmodium berghei mice during prophylactic activity***

(a): 7 days oral administration; (b): 14 days oral administration; NeG. is the negative control, CHL= chloroquine (10mg/Kg), SPT= sulfadoxine pyrimethamine (30mg/Kg) Dose1 = 9 x 10^8 CFU/ml; Dose 2= 1.8.x 10^9 CFU/mL; Dose 3=2.7 x 10^9 CFU/ mL of pediococcus pentosaceus). All the results are expressed in term of mean ± standard deviation). All the results are expressed in term of mean ± standard deviation with letters a, b and c been the level of significance where a = b = ** and c=****

3.2.2 Effect Of Pediococcus Ethanolidurans On Bodys Weight And Body Temperature Of Plasmodium Berghei ANKA Infected Mice

One feature of malaria infection is body weight loss and hypothermia. This study showed that *Lactobacillus sakei* significantly ($p<0.05$) prevented decrease of body temperature and body weight of *Plasmodium berghei* infected treated as comparedwith that untreated mice. Remarkably, there was significant increase in the body weight of the positive control groups (groups treated with chloroquine and sulfadoxine pyrimethamine) as compare to the negative control.

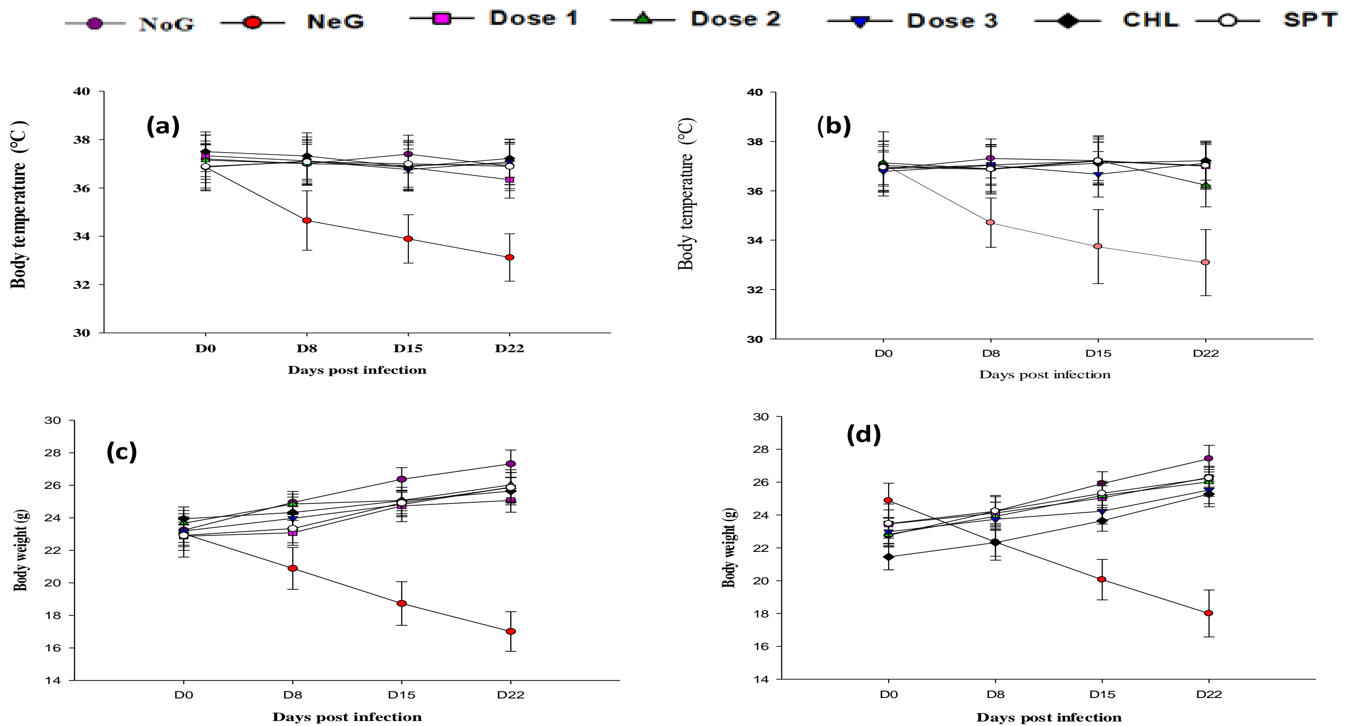

Fig. 4. Effect of pedioccocus ethanolidurans on the body weight and body temperature of Plasmodium berghei infected mice during prophylactic activity

(a): body temperature after 7 days oral administration; (b): body temperature after14 days oral administration; (c): body weight after 7 days oral administration; (d): body weight after14 days oral administration NeG. is the negative control, CHL= Chloroquine (10mg/Kg), SPT= Sulfadoxine pyrimethamine (30mg/Kg). Dose1 = 9 x 10^8 CFU/ml; Dose 2= 1.8. x 10^9 CFU/ml; Dose 3=2.7. x 10^9 CFU/ ml of pediococcus ethanolidurans). All the results are expressed in term of mean ± standard deviation.

3.2.3 Effect Of Pediococcus Ethanolidurans On Haematological Parameters Of Plasmodium Berghei ANKA Infected Mice

Table 1 showed the haematological parameters of plasmodium berghei infected treated with different doses of Pediococcus etthanolidurans, chloroquine and sulfadoxine pyrimethamine respectively before infection. These parameters include red blood cell count (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and White Blood Cell (WBC). The level of RBC, haemoglobin, haematocrit, MCV, MCH, MCHC, platelets, WBC, neutrophils, eosinophils, lymphocytes and monocytes counts were found to be significantly ($p<0.05$) raised in treated groups when compared with the untreated group. However, the significantly raised levels of the haematological parameters observed was only evident in mice treated that had received probiotic bacterium for a duration of 14 days and not in those who did for 7 days. The results of this study also showed a significant decrease of the numbers of lymphocytes, monocytes, platelets, neutrophils platelets, neutrophils and eosinophils in untreated group of mice.

Table 1. Effects of isolate UB20 (Pediococus Ethanolidurans) on the haematological parameters of Plasmodium berghei infected mice. Giving treatment orally 1 per Day for 7 and for 14 Days before infection

Parameters	Normal group	Negative control group	Treatments (Mac Farland Standard)			Treatments (mg/kg)	
			(9.10^8 cfu/ mL)	($1.8.10^9$cfu/ mL)	($2.7.10^9$cfu/ mL)	Chloroquine	Sulfa. Pyrim.
Group treated 7Days							
RBC ($\times 10^6$/µl)	6.84± 0.08^c	3.36± 0.13	4.53± 0.23^a	5.42 ± 0.40^a	5.89 ± 0.09^b	6.44 ± 0.26^b	6.53 ± 0.20^b
Haemoglobin (g/dl)	11.77 ± 0.02^c	7.08 ± 0.38	9.09± 0.5^a	9.62 ± 0.61^a	10.71 ± 0.03^a	11.30 ± 0.10^b	11.47 ± 0.10^b
Haematocrit (vol.%)	38.04 ± 0.43^c	28.42 ± 0.92	31.06 ± 0.67^a	33.05 ± 0.47^a	35.30 ± 0.25^a	37.75± 0.36^b	37.87 ± 0.40^b
MCV (fL)	58.49 ± 0.46^c	38.31 ± 0.32	42.18 ± 0.49^b	50.79 ± 0.08^c	54.68 ± 0.42^c	58.08 ± 0.48^c	58.30 ± 0.04^c
MCH (pg)	17.97± 0.39^c	12.76 ± 0.29	14.50 ± 0.49^a	15.06 ± 0.31^a	15.89 ± 0.40a	17.75 ± 0.31^b	17.81 ± 0.20^b
MCHC (g/dL)	30.10 ± 0.13^c	23.51 ± 0.45	24.33± 0.32	26.13 ± 0.246^a	28.69± 0.67^c	29.68 ± 0.56^c	29.81± 0.47^c
Platelets ($\times 10^3$/µl)	472.17 ± 2.67^c	401.00 ± 9.11	435.33 ± 2.44^b	446.00 ± 2.33^c	457.68 ± 0.3.15^c	466.63 ± 0.67^c	468.17±0.67^c
WBC ($\times 10^3$/µl)	17.66 ± 0.14	17.75± 0.16	17.83 ± 0.46	18.85 ± 0.39^a	19.79 ± 0.10^a	17.47 ± 0.05	17.50 ± 0.06
Neutrophils (%)	23.49± 0.31	19.75 ± 0.44	24.43 ± 0.38	25.40 ± 0.29^a	26.43 ± 0.60^b	24.28 ± 0.40	24.68 ± 0.17
Eosinophils (%)	3.90 ± 0.04	1.98 ± 0.11	3.22± 0.41	4.34 ± 0.47^a	4.97 ± 0.20^a	4.55 ± 0.20^a	4.71 ± 0.38^a
Basophils (%)	00 ± 0.00	00 ± 0.00	00 ± 0.00	00 ± 0.00	00 ± 0.00	00 ± 0.00	00 ± 0.00
Lymphocytes (%)	69.50 ± 0.20	44.78 ± 0.43	70.96 ± 0.58^a	75.60± 0.47^b	77.40 ± 0.70^b	71.78± 0.54	71.98 ± 0.61
Monocytes (%)	6.86± 0.06	3.96 ± 0.18	7.03± 0.10^b	8.08 ± 0.22^a	9.18± 0.53^b	7.09 ± 0.48	7.18 ± 0.44
Group treated 14 Days							
RBC (×106/µl)	6.89± 0.04^c	3.38± 0. 25	4.80 ± 0.11	5.48 ± 0.07^a	6.15 ± 0.30^b	6.78 ± 0.08^b	6.79 ± 0.07^b
Haemoglobin (g/dl)	11.86± 0.13^c	7.86 ± 0.46	9.51± 0.2^a	10.07 ± 0.46^b	11.00 ± 0.03^b	11.79 ± 0.20^b	11.82 ± 0.19^b
Haematocrit (vol.%)	38.85 ± 0.13^c	28.51 ± 0.20	33.60 ± 0.21^a	35.66 ± 0.12^b	37.06 ± 0.23^b	38.16± 0.43^b	38.46 ± 0.35^b
MCV (fL)	59.32 ± 0.32^c	39.64 ± 0.72	48.39 ± 1.17^b	53.21 ± 0.82^b	56.34 ± 0.50^c	58.78 ± 0.23^c	58.62 ± 0.39^c
MCH (pg)	18.62± 0.25^c	13.22 ± 0.34	15.75 ± 0.28^a	16.29 ± 0.24^a	16.83 ± 0.25^a	18.23 ± 0.33^b	18.43 ± 0.41^c
MCHC (g/dL)	30.52 ± 0.34^c	23.78 ± 0.67	27.67± 0.26^b	28.86 ± 0.21^b	29.86± 0.49^b	30.19 ± 0.46^c	30.34± 0.16^c
Platelets (×103/µl)	483.33 ± 3.11^c	396 ± 4.67	459.5 ± 4.17^c	469.83 ± 3.83^c	476.33 ± 2.00^c	477.63 ± 6.33^c	479.33±3.11^c
WBC (×103/µl)	18.59 ± 0.27	18.89 ± 0.16	20.91 ± 0.36^a	22.61 ± 0.89^b	24.59 ± 0.63^b	18.91 ± 0.29	19.25 ± 0.05
Neutrophils (%)	23.84± 0.46^a	19.19 ± 0.51	26.10 ± 0.67^a	27.66 ± 0.16^a	28.14 ± 0.47^b	24.51 ± 0.66^a	24.65 ± 0.71^a
Eosinophils (%)	2.59 ± 0.16	1.78 ± 0.09	3.87 ± 0.24^a	4.76 ± 0.22^b	5.76± 0.21^c	3.22± 0.23^a	3.35 ± 0.24^a
Basophils (%)	00 ± 0.00	00 ± 0.00	00 ± 0.00	00 ± 0.00	00 ± 0.00	00 ± 0.00.	00 ± 0.00
Lymphocytes (%)	69.50 ± 0.20	45.02 ± 0.43	74.16 ± 0.58^b	76.60± 0.47^b	77.40 ± 0.70^c	71.78± 0.54^c	71.98 ± 0.61^c
Monocytes (%)	5.75± 0.08	3.98 ± 0.11	7.49± 0.35^a	8.43 ± 0.18^b	9.52 ± 0.26^b	6.28 ± 0.05^a	6.4 ± 0.18^a

RBC (Red blood cell) WBC (White blood cell) MCV (Mean corpuscular volume), MCH (Mean corpuscular haemoglobin), MCHC (Mean corpuscular haemoglobin concentration) and differential leucocytes count (neutrophil, eosinophil, basophil, monocytes and lymphocytes) of Plasmodium berghei infected mice compared with that of Plasmodium berghei mice who previously received Chloroquine, sufladoxine pyrimethamine and different doses of. Pediococus ethanalidurans). All the results are expressed in term of mean ± standard error, for n = 6 mice per group. Data were analyzed by one-way ANOVA and Post hoc Tukey test. $^a p< 0.05$, $^b p < 0.01$, $^c p < 0.001$ significantly different compared to t negative control group.

3.2.4 Effect Of Pediococcus Ethanolidurans On The Cytokine Profile Of Plasmodium Berghei Infected Mice During Prophylactic Activity

The cytokine profile of of plasmodium berghei infected treated with different doses of *Pediococcus etthanolidurans*, chloroquine and sulfadoxine pyrimethamine respectively before infection is showed in table 2. The result of this study revealed that there were a significant (p<0.05) increase on the level of anti-inflammatory cytokine (Il-10) and significant (p<0.05) decrease on the level of proinflammatory cytokines (TNF-α, INF-γ, IL-1β and IL-6) of the treated mice in dose dependent manner when compared with that of untreated mice.

Table 2. Effects of isolate UB20 (Pediococcus ethanolidurans) on the inflammatory cytokines profile of plasmodium berghei infected treated and untreated mice during prophylactic activity

UB20		**Normal group**	**Negative Control**	**Treatment**			**Positive control**	
Cytokines		NoG	NeG	Dose 1	Dose 2	Dose 3	CHL	SPT
TNF-α	7 days	163.33 ±6.15	357.78±13.37	285.87±13.01^a	260.94±9.36^a	249.79±4.8^a	187.59±4.92^c	182.27±11.4^c
	14 days	168.99±6.1	378.76±15.39	256.57±16.46^a	239.22±12.62	213.16±4.64^b	178.09±4.15^c	171.05±7.4^c
INF-	7 days	171.62±7.18	377.88±15.21	289.33±6.77^a	264.25±16.48^b	226.08±7.84^b	179.5±6.85^c	175.45±7.42^c
	14 days	175.45±7.42	364.64±18.44	287.85±9.82^a	243.89±11.92^b	180.4±5.91^b	186.69±7.79^c	176.43±11.5^c
IL-1β	7 days	165.46±13.06	364.64±18.44	280.85±9.82^a	233.89±11.92^b	186.42±5.91^b	180.69±7.79^c	176.43±11.3^c
	14 days	176.49±7.85	176.49±7.85	265.44±17.99^a	194.82±7.35^c	177.09±7.27^b	188.3±8.01^c	185.04±10.6^c
IL-6	7 days	357.18±12.41	469.52±25.78	365.36±14.61^a	353.11±5.97^b	336.74±9.26^b	336.74±9.26^c	364.12±9.34^c
	14 days	360.62±7.36	479.71±24.58	344.1±12.44^a	323.78±7.57^b	313.5±11.03^b	365.07±14.9^c	363.09±13.8^c
IL-10	7 days	163.32± 7.1	118.29±9.89	172.42±17.25^a	181.22±14.07^c	181.22±14.07^b	166.73±9.76^c	168.5±13.14^c
	14 days	164.67±13.69	113.29±7.205	170.35±14.29^a	192.8±7.06^c	208.45±85.25^b	170.01±11.4^c	1755±14.19^c

TNF-α (Tumor necrosis factor alpha), INF-γ (Interferon gamma), IL-1β (Interleukin 1 beta) Il-6 (Interleukin six) and IL-10 (Interleukin ten) of Plasmodium berghei infected mice compared with that of Plasmodium berghei mice who previously received Chloroquine, sufladoxine pyrimethamine and different doses of Pediococcuc ethnolidurans for seven and for fourteen days before infection. All the results are expressed in term of mean ± standard error, for n = 6 mice per group. Data were analyzed by one-way ANOVA and Post hoc Tukey test. $^a p< 0.05$, $^b p < 0.01$, $^c p < 0.001$ significantly different compared to the negative control group.

4 DISCUSSION

In this study, we successfully isolated a probiotic bacterium *Pediococcus ethanolidurans* from Cameroonian Mborroro Fulani traditionally fermented milk, molecularly characterised by sequencing its 16S rRNA gene and tested its prophylactic effects on malaria infection in *Plasmodium berghei ANKA* infected mice. The Chloroquine sensitive *Plasmodium berghei* ANKA was used in this study to induce malaria infection in mice because of its ability to produce rodent malaria that is similar to human malaria infection. (Cissy et al., 2016). Prophylactic activity carried out in this study is one of the model currently used and accepted as viable model in the evaluation of the effect of candidate agents in early malaria infection (Esthetie *et al*., 2017).

Pediococcus ethanolidurans shown no toxic effect to infected mice even at the highest dose 2.7 10^9 cfu/ mL since according to WHO and FAO probiotic Lactic acid bacteria are generally regarded as save (WHO and FAO, 2002).

The current study demonstrated that *Pedicoccus ethanolidurans* significantly (p<0.05 prevented malaria disease severity in all the doses in treated mice when compared with the untreated mice since there was a significant (p<0.05) decrease of the level of parasitemia of treatment mice in dose dependent manner as compared to that of the untreated mice. Also, all the mice treated with this *probiotic bacterium* isolate and standard drugs showed high percentage suppression as from day 5 till the end of the experiment. These results could be due to the ability of this bacterium strain to modulate the immune system of the treated mice that in turn fight against the parasite by reducing the level of parasitemia since the immune system of these mice was previously stimulated with the probiotic bacterium before infection. These results are similar to that obtained by Martinez *et al*., (2006) who reported that mice previouly treated with Lactobacillus casei ssp. Rhamnosus for seven and for fourteen days before infection showed significant decrease on theirs level of parasitaemia.It is also in agreement with that of vallarino *et al*., (2016) who demonstrated that mice treated with *lactobacillus and bifidobacterium* displayed a decreased parasite burden and with that of Yilmaz et al., (2014) who revealed that gut microbiome modulates malaria severity in human. The latest authors therefore conluded that the severity of malaria is profoundly affected by the composition of the gut microbiota and that differences in the gut microbiota may explain why some humans infected with Plasmodium progress to severe disease and others do not.

Chanaid *et al*., (2019) reported that body weight loss is one of the general symptoms of malaria infection in human and rodents. The decrement of body weight in malaria has been associated with decreased food intake, disturbed metabolic function and hypoglycemia (Basir et al., 2012). Therefore, a potential antimalarial is expected to ameliorate anemia, prevent body weight loss and stabilize temperature in infected mice (Bottgeret al., 2013). The results of this study demonstrated that *pediococcus ethanolidurans*, chloroquine and sulfadoxine pyrimethamine successfully prevented body weight loss and reduction of body temperature in all the treated mice. This could be due to the capacity of this probiotic bacterium to prevent loss of appetite, increase food intake and prevent disturbed metabolic function associated with malaria. These results are in agreement with those obtained by Oyoma *et al*., (2019). However, there was significant decrease of the body weight and body temperature of untreated mice during the experiment. These results correlate with one of the features of rodent's malaria which is characterized by body weight loss and hypothermia as reported by Chanaid *et al*., (2019). This significant reduction of body temperature and body weight

of untreated mice could also be due to the high level of parasitaemia which tends to disrupt the body's temperature-regulating center and increase loss of appetite. These results are in agreement with those obtained by Misganaw et al., (2019), Oyoma et al., (2019) and Vallarino et al., (2019).

Apart from reducing parasitaemia, preventing body weight loss and reducing body temperature, this probiotic bacterium also increases survival time of all the treated mice as compared to untreated mice (53.6%) with the mean survival time of the treated mice been 100% in all the doses.

Haematological studies are valuable diagnostic tools in evaluating human health (Etim, 2015) The assessments of haematological and differential parameters reveal the effects of foreign compounds in blood constituents in living system. (Ogbole et al., 2016). Malaria infection is usually associated with increased risk of severe anaemia, thrombocytosis and leucocytosis. A potential antimalarial agent should therefore be able to prevent or reduce symptoms in infected mice (Chang and Stevenson, 2004). Low level of haemoglobin, red blood cells, Haematocrit, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration in untreated mice observed in this study, could be due to RBC destruction caused either by parasite multiplication or by spleen reticuloendothelial cell action or by the immune system in response to infection. This result correlates with the results obtained by Kolepui et al., (2014), (Arome et al., 2016) and), (Omoya et al., 2019). The significant increase of the level of red blood cells, haemoglobin and mean corpuscular haemoglobin concentration observed in this study, could be due to the capacity of this probiotic bacterium to stimulated the production of these blood parameters. However, sulfadoxine pyrimethamine and chloroquine highly increase the level of red blood cells, haemoglobin and mean corpuscular haemoglobin concentration in this study. These results are in the same line with those obtained in the studies lead by Kolepui et al., (2014), Arome *et al*., (2016) and Omoya et al., (2019). The results also revealed significant increase in the level of WBC, monocytes, lymphocytes, neutrophils and eosinophils in treated mice as compared to that of untreated mice, this could also be due to the ability of this probiotic bacteria to modulate the immune system that in turn stimulate the production of these immune cells. The increase level of WBC is in the agreement with the study carried by (Malik et al., 2010) who shown that malaria infection could contribute to the localization of leukocytes away from the peripheral circulation, the spleen and the other marginal pools rather than the actual depletion and stasis. The higher level of eosinophil in treated mice concord with the result obtained by (Khan et al., 2015 who revealed that eosinophilia occurs after initiation of anti-malaria treatment. However, these results are in contrary to that of Saidu et al., (2015) who reported that increase in parasitaemia is accompanied by increase in eosinophil count. Although, this author reported that increase in eosinophil level correlates with the level of parasitaemia and is determinated by individual difference in the strength of the immune system to mount an effective immune response against the parasite. This could also be due to the duration of exposure to parasite or to other factors that can induced the production of eosinophil other than malaria infection omoya *et al*., (2019).

Malaria infection is associated with the stimulation of the immune responses that are controlled by the balance between pro- and anti-inflammatory cytokines, whose absolute levels are recognized to impact susceptibility to infection and clinical disease outcome (Clark *et al*., 2006). Animal models and human clinical trials show that probiotics affect cytokine release and reduce inflammation (Kuar *et al*., 2003). It has recently been shown that the gut microbiota induces macrophages and dendritic cells TNF-α and IL-6 play important roles in some immune functions and metabolic disorders. (Karameşe *et al*., 2016). The significant increase of the level of antiflammatory cytokine (Il-10) and significant decrease of the level of anti-inflammatory cytokines (Il-6, Il-1β, TNF-α, INF- **γ)** in this study could be due the capacity of *Pediococcus ethanolidurans* to induce the production of anti-inflammatory molecule IL-10 which is produced mainly by monocytes, T cells, B cells, natural killer cells, macrophages, and dendritic cells, and regulates the inflammatory process by inhibiting the over expression of many pro-inflammatory cytokines, chemokines, and chemokine receptors. Thus, this Probiotic strain possesses anti-inflammatory properties, which may account for its efficacy against acute inflammatory conditions induced by malaria infection in mice. These isolates stimulated both innate and cell mediated immune response in mice and prevented malaria disease severity.

5 CONCLUSION

The aim of this study was to isolate, select, molecularly characterise using the 16S rRNA gene sequensis, a Lactobacillus isolate and evaluate its protective effects on malaria infection in *Plasmodium berghei* infected mice. The results of this study revealed that *Pediococcus ethanolidurans* is a probiotic bacterium with protective effects on plasmodium berghei infected mice since it was able to significantly reduce the level of parasitaemia, prevent body weight loss and reduction of body temperature and significant increase some haematological parameters such as the level of red blood cells, haemoglobin and mean corpuscular haemoglobin. Also, it significantly increases the level of WBC, monocytes, lymphocytes, neutrophils and eosinophils in mice treated as compared to untreated mice in dose dependent manner and successfully prevented inflammation associated to malaria infection in plasmodium berghei infected mice.

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