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# Effects of probiotic saccharomyces bourlardii on cytokine levels and outcomes of childhood severe malaria: Study protocol for a randomized controlled trial

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**Abstract:** *Background:* Severe malaria is acute malaria with signs of organ dysfunction and/or hyperparasitaemia. About 90% of the world's severe and fatal malaria is estimated to affect young children in sub-Sahara Africa. Systemic complications and neuropathology in severe malaria include 'cytokine storm hypothesis'. Probiotic immunomodulation may show therapeutic benefits in severe malaria.
*Aim:* This study aims to evaluate the effects of probiotic saccharomyces bourladii on serum cytokine levels (IL-6 and TNF), clinical course and outcome of children with severe malaria.
*Methods:* Participants shall be recruited based on WHO diagnostic criteria for severe malaria and their clinical-demographic data shall be obtained with a pretested questionnaire. The study design is randomized controlled trial (RCT); participants shall be randomized into two study sub-groups (intervention vs. control). All participants shall receive standard anti-malarial therapies. In addition, probiotic saccharomyces bourladii 250mg twice a day shall be administered to those in the intervention sub-group for 3 days. There after, their serum cytokines (IL-6 and TNF) shall be measured quantitatively by a Sandwich enzyme-linked immunosorbent assay (ELISA) technique. Primary outcomes shall be cytokine levels and length of stay (LOS) while secondary measures shall be coma scores, seizure, neurological deficits and mortality. The data shall be analyzed using SPSS version 26.0 statistical software for Windows. Both 'intention-to-treat analysis' and 'analysis as per protocol' shall be done. P-value< *0.05* shall be considered significant in all tests.
*Discussion:* At the end of this clinical trial, it is expected that the potential benefits of probiotic saccharomyces bourladii in modulating pro-inflammatory cytokines, averting systemic complications and reducing mortality in childhood severe malaria would have been verified.
*Protocol No:* ADM/E22/A/ VOL.VII/148312145; University of Benin Teaching Hospital's Health Research Ethics Committee Approval; August 5th, 2022.

**Key words**: Probiotic Saccharomyces bourladii, severe malaria, cytokines, outcomes

## Introduction

Malaria is a priority disease in public health, especially in sub-Saharan Africa. It constitutes a great economic and disease burdens on families.[1] Severe malaria is acute malaria with signs of organ dysfunction and/or hyperparasitaemia. The World Health Organization (WHO) has established revised criteria for the diagnosis of severe malaria including clinical manifestations and laboratory values that portend a poor prognosis in semi-immune patients.[1,2] Cerebral malaria is the specific form of severe malaria with altered consciousness or coma. Young children are at high risk for severe malaria in endemic areas while others acquire partial immunity following repeated infections reducing their susceptibility to complicated or severe malaria.[2,3]About 90% of the world's severe and fatal malaria is estimated to affect young children in sub-Sahara Africa. Severe malaria accounted for 25% of infant mortality and 30% of under -5 mortality.[1,2] This highlights the need to explore therapies that can reduce the severity of the disease, especially in resource-limited settings.

Several theories have been proposed for the systemic complications in severe forms of malaria including the

'cytokine storm hypothesis' in the neuropathogenesis of cerebral malaria.[4] This comprises peripheral inflammation, neutrophil activation and increased circulations of multiple serum cytokines such as TNF, IFNγ, and IL-2, IL-6, IL-8, and IL-10.[4] The pathogenesis of cerebral malaria is characterized by damaged vascular endothelium by parasite sequestration, inflammatory cytokine production and vascular leakage, which result in brain hypoxia. There is also possible rupture of the blood-brain barrier, leading to micro-haemorrhages and neurological sequelae.[4,5] Haemolysis and cytokine-related depression of bone marrow contribute to severe malarial anaemia. There is a need to reduce the pro-inflammatory state associated with severe malaria.[6,7]

Therefore, in order to reduce childhood deaths from severe malaria, there is a need to identify interventions that can complement existing therapies and hasten recovery. Optimal anti-malarial drugs alone may not guarantee an intact survival in severe malaria due to its multi-factorial systemic complications and neuropathology.[8-10] Based on the cytokine theory, TNF is a major contributor to coma in cerebral malaria. Pharmacologic reduction of TNF levels showed some improvements in survival.[4,5] Also, neuregulin, a neurotrophic growth factor was shown to be protective of experimental cerebral malaria (eCM) reducing CNS tissue injury. Administration of neuregulin-1 resulted in a 73% increased survival in eCM, as well as a decrease in systemic and CNS cytokines.[11] However, these therapies are not available for routine clinical use. Hence, there remains a dire need for a suitable adjunctive therapy that can provide optimal systemic and neuro-inflammation modulation in cerebral malaria and other forms of severe malaria.

*Justification for the study*

Probiotic immunomodulation is proven in the literature to be beneficial. A systematic review of 30 randomized controlled trials (RCTs) by Manzanares *et al*[12] found that probiotics were associated with a significant reduction in infections in critically-ill patients. Although probiotic reduces the risk of severe bacterial infection, its usefulness has not been previously investigated in severe forms of malaria in children, to the researchers' knowledge. Gut microbiota also influences the adaptive immune response of the host, the arm of the immune system necessary for *Plasmodium* clearance and sustained *Plasmodium* immunity as well as vaccine efficacy.[13] Therefore, gut microbiota modulation is a novel method of reducing the severity of malaria. Timely and appropriate treatment with an adjunctive therapy may have a significant effect on the clinical course and outcome of an illness.

Consequently, probiotics such as saccharomyces bourladii is a promising adjunctive treatment in severe malaria since it modulates pro-inflammatory cytokines. It will potentially lessen the severity of the illness as well as improve its clinical course and outcome. This implies a reduced length of hospital stay and improved cost effectiveness in the management of cerebral malaria. This is a step towards achieving malaria-related targets in the Sustainable Development Goal (SDG). The findings of this study may also highlight the possible relevance of probiotic yogurt in reducing malaria severity in the future.

**Research Question:** What is the effectiveness of probiotic saccharomyces bourladii adjunctive therapy in childhood severe malaria?
**Hypothesis:** The following hypothesis shall be tested in the course of this study:
**Ho:** Probiotic saccharomyces bourladii does not affect the clinical course/outcome of children with severe malaria.
**Ha:** Probiotic saccharomyces bourladii affects the clinical course/outcome of children with severe malaria.

**Aim and objectives**

The goal of this study is to evaluate the effects of probiotic saccharomyces bourladii on the cytokine levels (IL-6 and TNF), clinical course and outcome of children with severe malaria (*cerebral malaria/severe malaria anaemia/others*).

*Specific objectives*

1. To determine the effect of the probiotic on the meancytokine levels (*IL-6 and TNF*) of children with severe malaria;
2. To determine the effect of the probiotic on the clinical course (*durations of coma and/ or admission*) of children with severe malaria;
3. To determine the effect of the probiotic on theneurologic outcome (*developmental milestone regression/ seizures/ cortical blindness*) of children with severe malaria;
4. To determine the effect of the probiotic on overall outcome (*discharged /died*) of children with severe malaria;

**Methods**

*Study Area*

The study shall take place in the Children Emergency Room (*CHER*) of the University of Benin Teaching Hospital (*UBTH*), in southern Nigeria.

*Study design*

This is an interventional study using a randomized controlled trial design. It is an active control superiority trial; the control arm shall receive intravenous artesunate while the intervention arm shall receive both intravenous artesunate and probiotics. This prospective study complies with the Consolidated Standards of Reporting Trials (CONSORT) checklist. (Figure1).

*Randomization and blinding*

This shall be achieved by using a computer-generated random number set from 1 to 140; participants shall be allocated to the 2 sub-groups (intervention vs. control) using the randomized numbers (*https:// www.random.org/sequences*). Based on the computer-generated random numbers, prepared probiotic packs shall be supplied from CHER pharmacy to the intervention sub-group using a decoding record of participants' serial numbers and study sub-groups that shall be kept in the pharmacy precluding access by the researcher and managing team. There shall be no bedside labelling of participants' study groups. Laboratory analysis shall be blinded (*only the randomized code written on the sample bottle*). Also, data analyst will be blinded; trial arm will not be decoded until analysis is completed.

*Participants' recruitment and allocation*

Following a clinical diagnosis of severe malaria, rapid diagnostic testing for malaria (mRDT) shall be done for all participants (*and malaria microscopy for those with negative mRDT*); confirmed cases of severe malaria shall be recruited for the study. A pack of paper cards (4x4cm) shall be labeled (from 1 to 140) and kept in a large envelope shall be used to allocate participants' serial numbers at recruitment; a card shall be randomly drawn after shuffling to enroll participants into the 2 study sub-groups (intervention vs. control).

*Intervention*

Probiotic saccharomyces bourladii 250 mg twice a day shall be orally administered to the children in the interventional group for 3 days, (or via oro-gastric tube if indicated). If a participant in the control group required a probiotic in the course of the study (e.g. for gastroenteritis), he/she shall be excluded from the study. All participants shall receive standard intravenous artesunate dosages.

*Study Population*

**Subjects:** Children aged between 3 months and 18 years admitted into *CHER* with severe malaria based on the inclusion criterion;

**Inclusion criteria:** All children aged between 3 months and 18 years with a diagnosis of severe malaria, and whose parents consent to the study. Severe malaria shall be defined based on WHO diagnostic criteria.[1]

**Exclusion Criteria:** Children with malaria who do not meet the standard diagnostic criteria for cerebral malaria during the study period; children younger than 3months (being relatively protected from severe malaria (e.g. by the presence of fetal haemoglobin and maternal antibodies) shall be excluded; also, children with gastroenteritis.

**Sample Size Determination:** This shall be done using the formula for testing a hypothesis or clinical trial:[22]

$N = 2(Z_\alpha + Z_{1-\beta})^2 SD^2/d^2$ Where N= minimum sample size for the study.

$Z_\alpha$ = normal standard deviation for confidence level of 95% = 1.96

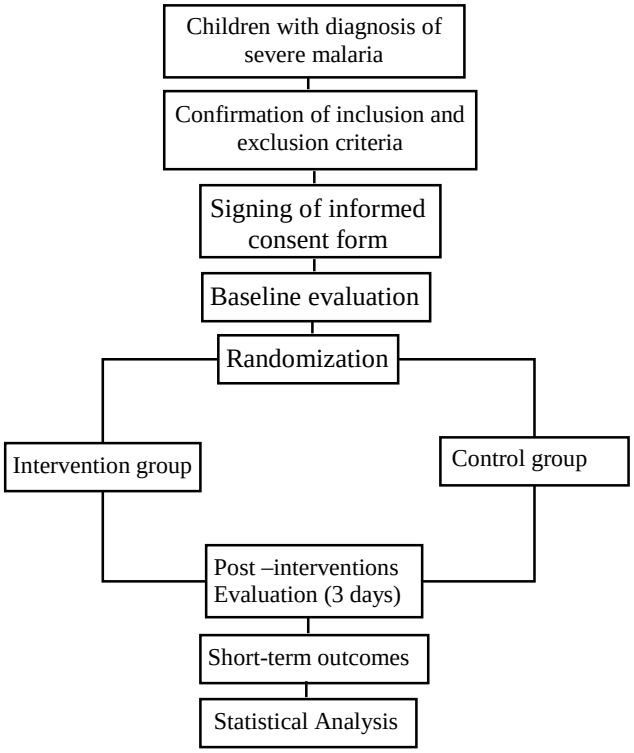
$Z_{1-\beta}$ = Power, which is set at 80% = 0.842 (β=20%)

SD = standard deviation of duration of admission (*2days in AQUAMAT study*[21])

$d = d_1 - d_2$ = detectable difference expected with the probiotic intervention (30%);[22]

$d_1$ = mean duration of admission of children with cerebral malaria receiving artesunate in AQUAMAT study = 3days;[21]$d_2$ = expected duration of admission of children with cerebral malaria receiving artesunate and probiotic = 2days;$d = d_1 - d_2 = 1$

**Fig 1:** Consort diagram on flow of the participants during the study




Therefore, substituting the values in the formula, N = 63, the minimum sample size required in each group. An attrition rate of 10% (i.e. 7) is added to each group. Therefore, a total of 140 children with severe malaria (*70 in intervention group and 70 in control group*) shall be recruited in this study.

**Data collection**: Data on each participant shall be extracted using a structured pretested questionnaire comprising baseline clinical-demographic features at presentation, clinical course and outcome.

**Variables**: Independent clinical-demographic variables among the participants include age, sex, socioeconomic class, duration of illness and severity criteria of malaria (Table 1).Primary outcome variables shall be cytokine levels and length of hospital stay while secondary measures shall be duration of coma, seizure, complications and survival till discharge (Table 2). Clinical evaluation

of the participants shall be done at admission into CHER and 12 hourly thereafter by the researcher/ attending paediatricians. A thorough physical examination shall detect altered consciousness using Blantyre Coma Scale/ Glasgow Coma Scale. Clinical notes of the participants shall be reviewed to ascertain the frequency of evolving morbidities as well as their outcome.

**Malaria parasite tests**: Malaria rapid diagnostic testing (mRDT) and microscopy of all participants shall be done in CHER using standard procedures.

**Laboratory Analysis for Cytokines:** Three milliliters (3mL) of blood shall be taken from every participant after 72 hours on admission using aseptic techniques. The resulting serum (after clotting and centrifugation) shall be frozen at −8◦C until analysis for two pro-inflammatory cytokines [interleukin 6 (IL-6) and tumour necrotic factor (TNF)] at the chemical pathology laboratory. IL-6 and TNF shall be measured quantitatively by a Sandwich enzyme-linked immunosorbentassay (ELISA) technique in batches of 80 samples, using kits from My BioSource (San Diego, United States) with a sensitivity of 1pg/ml, specificity 100%, detection range: 300 pg/ml-4.7 pg/ml. The end products shall be read spectrophotometrically at 450nm using a micro-well DiareaderELX800UV (Dialab GmbH Vienna/Austria).

*Statistical Analysis*

The data shall be analyzed using SPSS version 20.0 statistical software for Windows (IBM, Armonk, N.Y., United States). Fisher's Exact test or Chi-square shall be used to compare categorized data between the intervention and control groups. The Student *t*-test shall determine any significant difference between the mean cytokine levels (IL-6 & TNF), coma scores, duration of illness and lengths of hospital stay between the intervention and control sub-groups. Binary analysis shall determine the factors associated with improved survival in the intervention group. Multivariate logistic regression shall be done to assess probiotic therapy as an independent predictor of an improved clinical course or survival among the subjects. Receiver operating characteristic (ROC) analysis shall be used to evaluate the usefulness of the probiotic intervention in ensuring survival till discharge among the participants. Both 'intention-to-treat analysis' and 'analysis as per protocol' shall be done. The level of significance of each test shall be set at $p < 0.05$.

*Ethical consideration*

Ethical clearance was obtained from the Health Research Ethics Committee of the University of Benin Teaching Hospital. **Protocol No**: ADM/E22/A/ VOL.VII/148312145; August 5th, 2022.

All participants shall be managed with the same standard treatment protocols for severe malarial morbidities in the unit. All questionnaires shall be coded (using the allocated random number) to ensure confidentiality.

**Table 1:** Independent clinical-demographic variables

| Variables | Values |
|---|---|
| Age | Years |
| Sex | Male/Female |
| Socioeconomic class | Upper/middle/lower |
| Duration of illness | Days |
| *Severity criteria of malaria:* | |
| Cerebral malaria | Yes/No |
| Severe malarial anaemia | Yes/No |
| Others criteria | specify |
| Prehospital care | Yes/No |

**Table 2:** Outcome variables of the study

| Outcome Variables | Evaluation period | | Measurement method |
|---|---|---|---|
| | Pre-intervention | Post-intervention | |
| IL-6 level | x | x | ELISA |
| TNF level | x | x | ELISA |
| Duration of coma | x | x | BCS/GCS |
| Length of hospital stay | | x | DOA |
| Complications | x | x | Clinical assessment |
| Survival till discharge | | x | Clinical assessment |

Interleukin 6 (IL-6), Tumour necrotic factor (TNF), Enzyme linked immunoassay (ELISA), Blantyre Coma Scale (BCS), Glasgow Coma Scale (GCS), Duration of admission (DOA); optional pre-intervention cytokine levels.

## Discussion

This clinical trial is expected to verify the beneficial effects or otherwise of probiotic saccharomyces bourladii in modulating pro-inflammatory cytokines that promote systemic complications in childhood severe malaria. The details of participants' severity criteria, clinical course and outcomes will be documented forming a baseline in this novel area of interventional study in malaria therapy. Specific clinical-laboratory criteria define the various forms of severe malaria including cerebral malaria.[1] Since severe malaria is a multi-systemic disease, children frequently present with a combination of the classical clinical phenotypes: cerebral malaria (CM), severe malarial anaemia (SMA), respiratory distress, and hypoglycaemia. The first two, CM and SMA, are the most common complications of malaria in children. Although most children with CM regain consciousness within 48 hours and seem to make a full neurological recovery, approximately 20% die and up to 10% have persistent neurological sequelae.[14] These are particularly associated with protracted or multiple seizures which may cause cognitive deficiency and/ or epilepsy. Severe malarial anaemia (defined as haemoglobin concentration < 5 g/dl in the presence of *P.falciparum* parasitaemia) is more common in children than in adults.[2,14] Hypoglycemia, pulmonary oedema, hepatic dysfunction, disseminated intravascular coagulation, hypotension, cardiovascular shock and multi-organ

failure are less common complications of severe malaria.[14,15]

Cytokines contribute significantly to the pathogenesis of major acute childhood severe malaria but specific anti-cytokine therapies are not available for malaria. Probiotics are "Generally Recognized As Safe" (GRAS). Their benefits have been initially limited to the gastrointestinal disorders, but recognizing 'the gut-brain axis', they are now known to have extra-intestinal modulatory effects on inflammatory and immune responses.[16-18] In a systematic review of 30 randomized controlled trials (RCTs), Manzanares *et al.*[12] found that probiotics were associated with a significant reduction in infections in critically-ill patients. Although probiotic reduces the risk of severe bacterial infection, its usefulness has not been previously investigated in severe forms of malaria in children. Gut microbiota also influences the adaptive immune response of the host, the arm of the immune system necessary for *Plasmodium* clearance and sustained *Plasmodium* immunity, and vaccine efficacy.[13] Therefore, gut microbiota modulation is a potential method of reducing the severity of malaria.

Consequently, probiotics such as saccharomyces bourladii is a promising adjunctive treatment in severe malaria treatment since it reduces this pro-inflammatory cytokine. The mean IL-6 and TNF levels will become known in both the intervention and control sub-groups in this study. Timely and appropriate treatment with an adjunctive therapy like probiotics will potentially lessen the severity of the illness as well as improve its clinical course and outcome. This implies a reduced length of hospital stay, cost effectiveness, less neurological complications and improved survival till discharge. This is a step towards achieving malaria-related targets in the Sustainable Development Goal (SDG). The findings of this study will also highlight the possible relevance of probiotic yogurt in reducing malaria severity in the future.

Severe malaria is a leading cause of childhood death and survivors often suffer complications including neurobehavioral difficulties and milestone regression. Delayed and sub-optimal therapy can contribute to malaria mortality especially in resource-limited settings.[3,19] Only few African children will have access to critical services and, therefore, rely on simple supportive treatments and parenteral anti-malarials.[20] There has been some progress on defining best practice for anti-malarial treatment with the publication of the AQUAMAT trial in 2010, showing that in artesunate-treated children, the relative risk of death was 22.5% (95% confidence interval (CI) 8.1 to 36.9) lower than in those receiving quinine.[21] This proposed study will evaluate the impact of adjuvant probiotic therapy on the outcome of children with severe malaria receiving standard intravenous artesunate dosages

The clinical relevance of this proposed study is that it can lead to the identification of an effective adjunctive therapy that will shorten length of hospital stay, increase survival till discharge and enhance neurocognitive outcome of cerebral malaria survivors. The findings of this study shall be reported based on CONSORT guidelines for clinical trials for quality assurance.[23] This study will also furnish a scientific basis for the use of probiotic-rich diet like yoghurt in the nutritional care of children with severe malaria. This will eventually translate to the use of cheaper probiotic preparations, enhancing cost-effectiveness in severe malaria treatment.[24,25] Overall, if study demonstrates the effectiveness of Saccharomyces bourladii, it will reduce the economic and clinical burden of childhood severe malaria in our setting and sub-regions.

**Authors' Contribution**

Author MTA conceptualized the study; authors ANO and EFOE reviewed an early version of this protocol. All authors critically reviewed and approved the final manuscript.

**Conflict of Interests:** None

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