University of Rwanda – Biomedical Laboratory Sciences Department

Université catholique de Louvain – Louvain Drug Research Institute

Relationships between malaria, helminth infections and the gut microbiota composition in Rwanda

Jean d'Amour Mutoni

Thesis Directors
Professor Nadine Rujeni
Professor Patrice D. Cani
Professor Jean-Paul Coutelier

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Jury composition

Thesis Directors
Professor Patrice D. Cani
Professor Nadine Rujeni
Professor Jean-Paul Coutelier

President of the Jury
Professor Thomas Michiels, UCLouvain, Brussels, Belgium

Jury Members
Professor Niko Speybroeck, UCLouvain, Brussels, Belgium

Professor Sophie Lucas, UCLouvain, Brussels, Belgium

Professor Ezera Agwu, University of Rwanda, Kigali, Rwanda

Associate Professor Conor Meehan, Nottingham Trent University, Nottingham, UK

Dr Emmanuel Hakizimana, Rwanda Biomedical Center, Kigali, Rwanda

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Summary

Malaria has consistently remained on the list of top causes of morbidity and mortality especially in sub-Saharan Africa, for decades. Its clinical presentation is heterogeneous with asymptomatic, mild and severe phenotypes. That clinical presentation is partly determined by host immune responses which, themselves, are subject to altering by several factors. In recent years, research has increasingly reported the gut microbiota as one of such factors. On the other hand, Soil Transmitted Helminths (STHs) have been shown to affect both the immune responses to malaria and the gut microbiota (GM) composition. The latter can also be shaped by genetics, geographic location, age, nutrition and other factors. However, to our knowledge, there in not a single study about malaria and the GM in Rwanda. Thus, our project comprised two studies and was aimed at investigating malaria-GM relationships, considering multiple counfounding factors, in Rwanda.

The first study was conducted in 3 malaria-endemic provinces of Rwanda: East, South and West. The terms 'West' and 'Western' province were used intercheangably to represent a geographic region of the Republic of Rwanda. We explored associations between malaria and the gut microbiota across the three geographic regions, considering host's nutritional habits, STH coinfections and age. We used questionnaire-derived qualitative data for demographic analysis while malaria and soil-transmitted helminth diagnosis was assessed by microscopy. The gut microbial composition was analyzed based on bacterial 16S rRNA gene amplicon sequencing. We observed that preschool children had a significantly lower microbiota diversity compared to both school children and adults. Unlike age, infection status (uninfected, malaria alone, soil-transmitted helminth alone or coinfection) was not significantly associated with the gut microbiota. Moreover, using Bray-Curtis distances, we found a significantly differential gut microbial beta diversity in the West province compared to the other provinces. This geographic difference was not explained by any change in energy intake, protein, lipids, or carbohydrates consumption but was likely due to lower dietary fibre intake in the West compared to the South and the East (results' chapter 1).

The second study was conducted in the central plateau of Rwanda to compare the gut microbiota profiles of malaria patients with different clinical presentations

(severe, mild, asymptomatic and uninfected). Results (chapter 2) showed significantly lower alpha diversity in severe malaria patients compared to other malaria severity groups across all measured metrics. Furthermore, beta diversity analysis using Bray Curtis distance showed that severe malaria samples were significantly different and formed a unique cluster visualized by PCoA compared to other groups. In the same direction, ANCOM-BC differential analysis at genus level revealed that genera *Suterella* and *Veillonella* were depleted in asymptomatic and mild samples respectively, while both *Lactobacillus* and *Bacteroides* were among the top 6 genera depleted in both groups compared to severe malaria group.

In summary, our research project analyzed malaria-gut microbiota relationships by age, geographic location, parasitic infection and/or coinfection status and malaria severity in Rwanda. We reported significant differences in the gut microbiota composition of participants by age, geographic location – with possible connection to low-fibre food intake, and most importantly by malaria severity. Interestingly, we have not found significant links between gut microbiota composition and the presence or absence of parasitic infection. Further studies are recommended to unravel malaria-GM interactions towards discovering novel interventions to defeat severe malaria.

List of abbreviations

ACT	Artemisinin-based Combination Therapy
AL	*Ascaris lumbricoides*
ANCOM	Analysis of Compositions of Microbiomes
ANOVA	ANalysis Of VAriance
ASV	Amplicon Sequence Variant
BMI	Body Mass Index
CDC	Centers for Disease Control and Prevention
CDI	*Clostridioides difficile* Infection
CMHS	College of Medicine and Health Sciences
DC	Dentritic Cells
DNA	DeoxyriboNucleic Acid
EBI	European Bioinformatics Institute
EMBL	European Molecular Biology Laboratory
ENA	European Nucleotide Archive
FFAR	Free Fatty Acid Receptor
FFQ	Food Frequency Questionnaire
FISH	Fluorescence In Situ Hybridization
FMT	Fecal microbiota transplant
GIT	GastroIntestinal Tract
GLP1	Glucagon-Like Peptide 1
GM	Gut Microbiota
GPR	G Protein-coupled Receptor
HW	HookWorm
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IFN	Interferon
IL	Interleukins
IRB	Institutional Review Board
IREC	*Institut de Recherche Expérimentale et Clinique*
LDA	Linear Discriminant Analysis
LDRI	Louvain Drug Research Institute

LEfSe	Linear discriminant analysis Effect Size
MAC	Microbiota-Accessible Carbohydrate
MNUT	Metabolism & Nutrition
MS	Mass Spectrometry
NA	Non-Applicable
NISR	National Institute of Statistics Rwanda
NK	Natural Killer
NMDS	Non-Metric Multidimensional Scaling
NMR	Nuclear Magnetic Resonance
NTD	Neglected Tropical Disease
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PYY	Peptide YY
QC	Quality Control
RCT	Randomized Controlled Trial
RDHS	Rwanda Demographic and Health Survey
RDT	Rapid Diagnostic Test
RNA	RiboNucleic Acid
SCFAs	Short-Chain Fatty Acid
SSA	Sub-Saharan Africa
STH	Soil Transmitted Helminth
T2DM	Type 2 Diabetes Mellitus
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TT	Trichuris Trichiura
UCLouvain	*Université catholique de Louvain*
UR	University of Rwanda
USA	United Stated of America
WHO	World Health Organization

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INTRODUCTION

Background of the Study

For decades, malaria has been among the top causes of morbidity and mortality, especially in sub-Saharan Africa [1]. Its clinical presentation ranges from asymptomatic, to mild and severe infection [2]. Malaria clinical presentation is partly determined by host immune responses which, themselves, are subject to altering by several factors. Among other factors, the gut microbiota has been shown to modulate host's immune system and its response to pathogens. For example, the severity of malaria in mouse models, which involved immunopathological anti-*Plasmodium* immune responses, was shown to be modulated by increased abundances of *Lactobacillus* and *Bifidobacterium* in resistant mice [3]. Interestingly, Soil-Transmitted Helminths (STHs) – commonly coendemic with malarial causative agents, were reported to affect the host's gut microbiota with which they coevolved and shared gastrointestinal niche for years [4]. Therefore, STHs make an important factor to consider in studying associations between malaria and the gut microbiota (GM) as does the GM in analyzing host's immune responses in malaria infection. In addition to STHs, several other factors at play include genetics, geographic location, nutrition habits, age, antimicrobials and deworming [5–7].

Malaria-STH coinfection remains particularly significant in tropical and subtropical regions where sanitation and access to healthcare is limited [8]. Sub-Saharan Africa carries more than 90% of the disease burden caused by *Plasmodium falciparum* – responsible for more than 90% of malaria cases and deaths worldwide [9]. Similarly, STH infections caused mainly by *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms (*Ancylostoma duodenale* and *Necator americanus*) inflict distress on more than a billion people worldwide, resulting in malnutrition, anemia, and compromised immune responses [10]. Coinfection between malaria and soil-transmitted helminthiases yield complex interactions which may determine the direction of pathogenesis, immune responses and overall health status in affected induviduals [11–13]. Indeed, evidence suggest that STHs may promote regulatory immunity to favor their survival within the host [14]. However, such immune modulation can impair inflammatory responses against *Plasmodium* parasites necessary to reduce parasitemia, potentially affecting disease severity [15, 16].

As of recent, thanks to novel high throughput sequencing technologies and bioinformatics softwares, research outputs have increasingly shed light on the role of the GM in health and disease. The GM represents a diverse community of microorganisms such as bacteria, viruses, fungi, parasites and algae present in the host's gastrointestinal tract (GIT) [17]. Their composition (in diversity and abundance) and function have been shown to play crucial roles in the host's homeostasis or dysbiosis [17]. Among other functions fulfiled for their hosts, the gut microbiota have been shown to modulate immune responses by either preventing or predisposing hosts to infections through producing metabolites such short-chain fatty acids (SCFAs) that impact signaling pathways, cytokine production, and the activation of T-cells and other immune cells [18].

In summary, studying relationships between malaria and the gut microbiota composition presents a novel avenue for public health research. Understanding their associations, keeping in mind potential confounding factors, could pave the way to a better understanding of malaria necessary to devise new strategies for its prevention and control. Indeed, microbiota-based interventions such as probiotics and dietary recommendations may contribute new strategies to boost immunity and improve disease outcomes. This thesis, compiling two observational studies, has the potential to inspire the development of new approaches to designing interventions against malaria in endemic regions.

Malaria

Malaria is a parasitic disease that, for centuries, has remained a major cause of morbidity and mortality worldwide. The WHO reported 249 million cases and 608 000 deaths due to malaria worldwide in 2022 [9]. Representing more than 90% of yearly cases and deaths, sub-Saharan Africa is often the most affected region (Figure 1) [19]. Young children and pregnant women are the most vulnerable groups [20]. In Rwanda, highest prevalence of malaria was reported in the West, South and East provinces although the disease epidemiology has been changing and new foci in the North are reported, possibly as a result of migration, climate variability or both [21, 22].

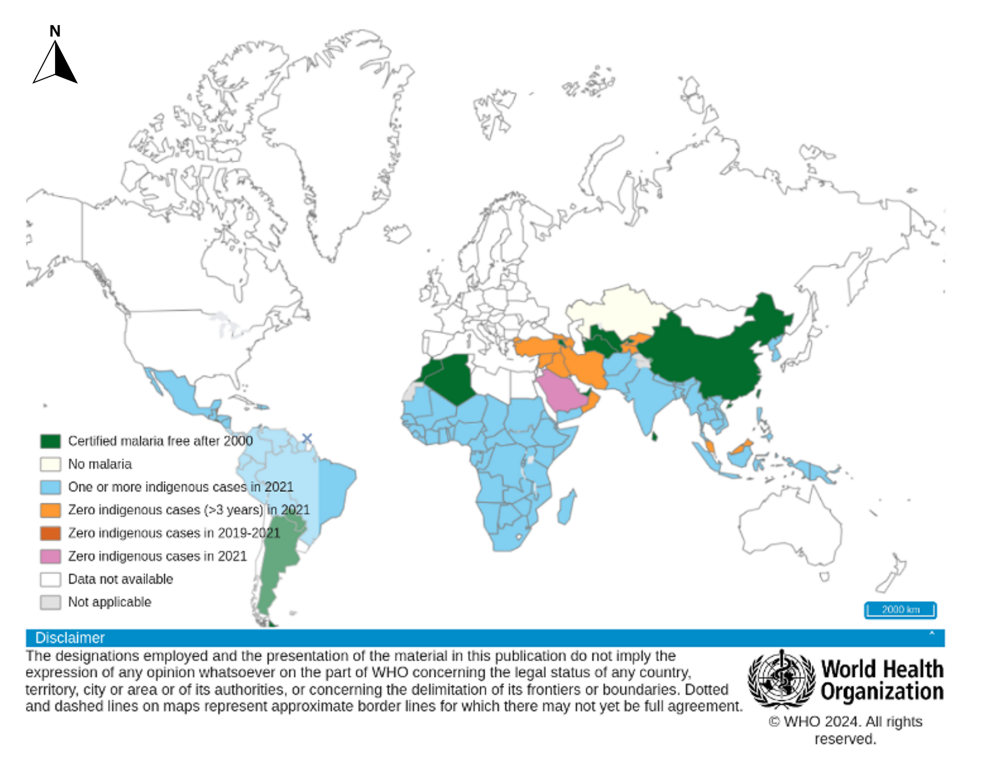


Figure 1. Global status of indigenous malaria cases by country [9]

Malaria is caused by an apicomplexan parasite of genus *Plasmodium* which is transported by a female *Anopheles* mosquito from human to human. *Plasmodium falciparum*, the most common species has been shown to cause more than 90% of the infections [9]. Other species include: *Plasmodium vivax*, *P. malariae*, *P. ovale*

and *P. knowlesi* [23]. Parasite transmission to human host starts in the skin following a mosquito bite which releases midgut-matured sporozoites that travel through bloodstream to reach the liver. Upon successful multiplication, parasites exit the liver as merozoites capable of infecting and multiplying in red blood cells. By evading human immunity, parasites participate in initiating malaria clinical presentation while others mature into male and female gametocytes which would be taken up by the next mosquito to continue the cycle (Figure 2) [2, 24].

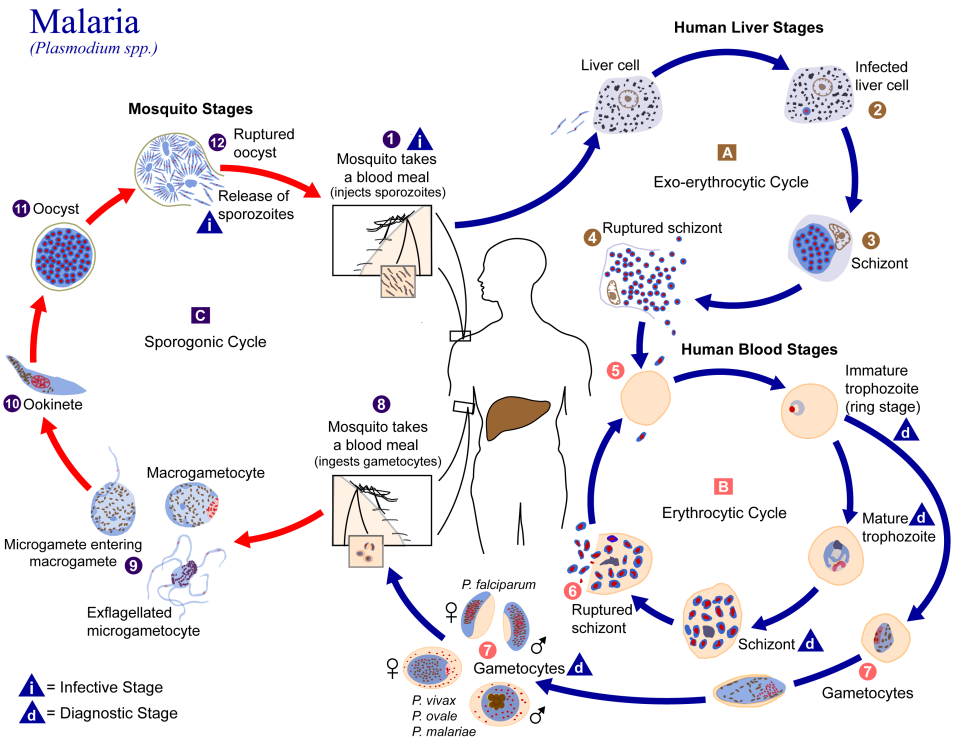


Figure 2. The life cycle of the parasites of the genus *Plasmodium* that are causal agents of malaria [25].

Clinical presentations of the disease are threefold : asymptomatic, mild and severe malaria. While the presence of *Plasmodium* parasite in blood (parasitemia) is a mandatory condition for all types of malaria clinical manifestation, signs and symptoms allow us to distinguish them.

Parasite virulence factors

Key virulence factors of *Plasmodium*, particularly *P. falciparum*, enable the parasite to undermine host immunity through immune evasion, citoadherence and

sequestration of erythrocytes in internal organs. PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1) and REX1 (Ring-exported protein 1) are known for promoting pathology in cerebral malaria [26]. Hemozoin, a toxic byproduct of hemoglobin destruction, has been shown to play central role in the pathogenesis of severe malarial anemia (SMA) [27]. In a complex coordination, these and more virulence factors oppose host immunity through promoting immune evasion, vascular damage and inflammatory responses in *Plasmodium*-infected subjects.

Host immune response to Plasmodium

Proinflammatory T-helper 1 (Th1) initiates immune responses to *Plasmodium* infection. Host immune response to malaria starts with innate, non-specific immune responses such as physical barriers (i.e. skin) and culminates into more specific, adaptive responses corresponding to the progress from infection to blood-stage disease, respectively [28]. Maturation of dendritic cells is followed by the recruitment of neutrophils and monocytes to the initial infection site in the skin [29]. While Th1 and NK (natural killer) cells are the main immune players in the pre-erythrocytic stage of infection in the liver, the erythrocytic stage relies heavily on T-helper 1 (Th1) and T follicular helper (Tfh) which lead to the activation of cytokines such as IFNγ, TNFα, IL-4, IL-5, and IL-10, resulting in inflammation and antibody production [30]. It has been argued that successful type 1 response to malaria requires a well-timed and proportional release of interleukin (IL)-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α to minimize parasitemia and preserve erythropoiesis [27]. For regulation purposes, a Th2 response (e.g. Il-10 and Tregs) is also activated to dampen excessive inflammatory reactions without [2]. Th1/Th2 balance is particularly important in determining malaria clinical outcomes.

Depending on host's immune status and parasite virulence, *Plasmodium* infection can result into asymptomatic, mild or severe malaria [2]. In addition, malarial clinical manifestation is dependent on many other factors ranging from genetics to age, pregnancy, gut microbiota, diet and coinfection (eg. Soil-Transmitted Helminth) [2].

The gut microbiota has been shown to affect malaria transmission and clinical presentation outcomes by modulating host immune responses through mechanisms such as regulating systemic inflammation [6]. Therefore, investigating

gut bacterial profiles in the context of malaria severity has the potential to improve our understanding, prevention or management of the disease.

Asymptomatic and mild malaria

Once parasitemia is confirmed, the lack of observable symptoms is characteristic of asymptomatic infection [31] while simple, general symptoms (e.g. fever, chills, headache, myalgia, nausea and vomiting) characterise mild infections [32, 33]. Pre-asymptomatic malaria covers the incubation period after infection, yet before symptom onset. Different from asymptomatic individuals who serve as infection reservoirs, pre-asymptomatic subjects are not yet infectious [26]. Asymptomatic infection is a result of repeated exposure leading to gradual acquision of clinical immunity in endemic settings [32].

Severe malaria

Severe malaria is characterized by specific symptoms (jaundice, acidosis, coma, renal failure, anaemia, convulsions, shock and hypoglycaemia) and can be either anaemic or cerebral [32]. With the mortality exceeding 30%, severe malarial anaemia (SMA) involves red blood cell (RBC) destruction driving hemoglobin concentration to as low as below 5.0 g/dL. SMA is the most common clinical manifestation of severe malaria among children living in holoendemic areas [27]. In contrast, cerebral malaria (CM) affects adults more, with lethal signs ranging from ischemic anoxia, acidosis, metabolic irregularities and coma [34].

According to the WHO, effective strategies to fight malaria rely on combining methods for prevention, early diagnosis and treatment as well as chemoprophylaxis drugs. Insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) are essential methods used in vector control [9]. Nevertheless, an ever emerging insecticide resistance presents a threat to these two interventions [32]. Alternatively, insecticide-independent vector control methods such as genetic modification (i.e. gene drive) have recently been added to the pipeline while limitations of ethical nature and more persist [35]. While microscopy remains the gold standard, rapid diagnostic tests (RDTs) have been shown to improve early detection and treatment outcomes [9]. As per treatment, parenteral artesunate treats severe malaria whereas artemisinin-based combination therapies (ACTs) are recommended first-line treatment for mild malaria, but parasite resistance, mainly against the latter, has been reported in almost every endemic country [32]. Intermittent preventive treatment is recommended for high-risk groups such as

pregnant women and immunologically naïve travellers from non-endemic to endemic settings [26]. Finally, new WHO-recommended vaccines (RTS,S and R21) marked a significant milestone towards elimination [36].

Soil-Transmitted Helminthiases

STHs present one of the most serious causes of morbidity worldide. Almost a billion people are infected with at least one species worldwide [10]. STHs belong to the neglected tropical disease (NTD) group which primarily affect populations in low- and middle-income countries in regions with poor sanitation infrastructure [37]. In Rwanda, their reported combined prevalence was estimated to reach 65.8% [38]. There exist three main STH types of medical importance : hookworms (*Ancylostoma duodenale* and *Necator americanus*), whipworms (*Trichuris trichiura*) and roundworms (*Ascaris lumbricoides)* (Figure 3a) [37]. They affect school children disproportionately compared to other age groups, hence, negatively affecting their education performance in addition to malnutrition, anemia, and impaired cognitive development. Preschool children have recently been added to the list of high risk groups [10, 39, 40].

The life cyles of the three medically important species have a lot in common with subtle differences. *Ascaris lumbricoides* (AL), the largest and most prevalent of all STHs, infects humans who ingest fertilized eggs that release new worms in the intestines. AL worms can then go on to infect others organs – from the liver to lungs and trachea – before returning to the Gastrointestinal tract (GIT) where they mature into males and females. New embryonated eggs are released in the soil upon defecation to continue the cycle. *Trichuris trichiura* (TT) is the most common whipworm. Unlike AL, TT's eggs are fertilized in the environment before being ingested to reach human small intestines as larvae and the colon as adult worms which release new unembryonated eggs to restart the cycle. For hookworms, the portal of entry is the skin through which, via bloodstream, Filariform larvae regain GIT to mature and release embryonated eggs that, once in the soil, produce Rhabditiform larvae preceeding the infective stage [37, 41].

STH clinical manifestation, diagnosis and control are relatively well understood. Clinically speaking, AL is known for causing obstruction in small intestines, bile and pancreatic ducts [37]. TT may cause colitis, inflammatory bowel disease and *Trichuris* dysentery syndrome in the large intestines [37]. Hookworms' ability to bite and employ enzymes to cause physical damage results in considerable host's blood loss and anaemia [37]. Together, the three STH types are known to cause abdominal pain, nausea and vomiting. While they may cause chronic and serious morbidity, soil transmitted helminthiases rank low in their contribution to

global mortality [10]. Wet mount on a light microscope is used for their diagnosis. The control of STH in endemic countries involve periodic mass drug administration (MDA) with albendazole or mebendazole and there is no approved vaccine [37, 42].

An STH-modified T-helper 2 (Th2) coordinates efforts to suppress both Th2 and Th1 cytokines and promotes both tolerogenic dendritic cells (DCs) and Tregs during host STH infection (Figure 3b) [37]. Thus, although STHs are not commensal organisms, they enjoy some level of immunological tolerance by the host, thanks to their ability to modulate host immune responses [10]. Consequently, in most cases, hosts fail to mount required immune responses to eliminate STHs and clear infection [37]. For example, in the case of Hookworms, STHs release products that block the IL-33 pathways, thus impairing innate immunity in the skin [43]. As per *Ascaris*, it was shown to use its pseudocoelomic fluid to repeal DC response, minimise inflammatory cytokine production (Th1) and reduce DC's ability to stimulate T cells [44]. Indeed, modified Th2 immune response enables STH to survive in the hosts and may influence immune response to other infections such as malaria.

The same STH's immunomodulatory properties have been shown to play important roles in protecting hosts against the pathogenesis of autoimmune, allergic and metabolic disorders. Therefore, scientists see STH's metabolites as potential sources of therapeutic products against these inflammatory diseases [37]. For example, in an randomized controlled trial, *N. americanus* was shown to improve symptoms of gluten intolerance and promote Treg response in humans with coeliac disease [45]. In contrast, *Trichuris suis* ova failed to prove therapeutic efficacy in patients with IBD – Crohn's disease and ulcerative colitis in phase 2 trials [46]. Nevertheless, studies are looking at individual STH-derived products (e.g. proteins, microRNAs, etc) to manage different diseases (asthma, colitis and ischemic stroke) through immunomodulation [47, 48].

Annual mass drug administration save many children's lives and education while a vaccine is yet to be found [10].

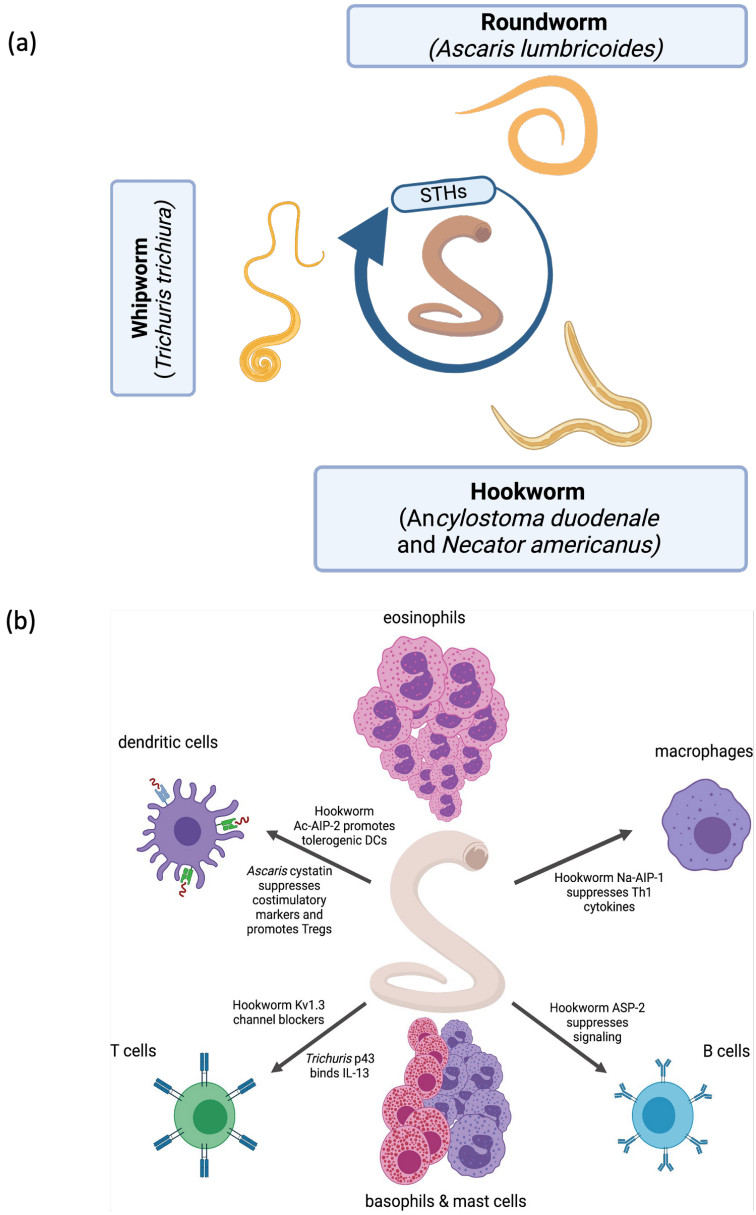


Figure 3. (a) Three types of soil-transmitted helminths (STHs). (b) Mechanisms by which STHs defined recombinant proteins modulate the host immune system [37]. Ac/Na-AIP-2/1, Acquired/Non-acquired Anti-Inflammatory Protein; ASP-2, Ancylostoma Secreted Protein-1/2; Kv1.3, voltage-gated potassium channels ; p43, protein 43. Created with Biorender.com

In summary, soil transmitted helminthiases, as major NTDs, continue to pose a serious morbidity problem, especially in low- and middle-income countries. STH's immunomodulatory properties allow them to survive, inflicting chronic morbidities in hosts, although they may downregulate excessive inflammatory responses in metabolic diseases and co-infections.

Malaria-STH Coinfection

Malaria and Soil-Transmitted Helminthiases are both parasitic diseases, and overlap in their geographical distribution, which facilitates coinfection. However, research findings remain divergent about STH coinfection's effects on malaria clinical presentation outcomes [49]. Their opposing immune responses (Th1 for malaria and Th2 for STHs) modify pathology onset, development and severity for each or both. Here we are interested in the case of malaria-STH coinfection's effects on host's immune responses to *Plasmodium* and how these affect malaria clinical presentation.

The clinical outcomes of STH-malaria co-infection are controversial. For example, in Rwanda and Cameroon, both parasite coinfection was associated with and predicted worse anaemia parameters more than isolated single infections [13, 50]. However, in Nigeria, *Ascaris* coinfection neither exacerbated nor ameliorated the severity of malarial anaemia [51]. Such data are examples of contrasting findings about the effects of malaria-STH coinfection on general health outcomes.

Malaria-infected hosts tend to mount pro-inflammatory Th1 immune response – dominated by cytokines like IFN-γ and TNF-α – to clear *Plasmodium* parasites off [27]. In contrast, STHs promote a modified anti-inflammatory Th2 immune response which encompasses TGF-β, IL-4, IL-5, and IL-10 that favor their survival in the host [52]. This immune response antagonism provides the basis for studying possible clinical outcomes in co-infections.

Malaria clinical presentation is heterogeneous (mild, severe or asymptomatic) and immunomodulation plays a critical role [27]. STH have been shown to play a role in the latter. On the one hand, there is evidence to support the worsening of malaria clinical presentation by STH coinfection. In Ethiopia, a study showed that helminth coinfection was associated with increased *Plasmodium* parasitaemia levels [14]. Another study findings communicated that severe malaria and *A. lumbricoides* were strongly associated in a cohort of rural Senegalese children [53]. From that angle, researchers argue that the STH-elicited Th2 immunity may downregulate Th1 anti-inflammatory immunity against malaria, allowing parasitemia to increase. On the other hand research findings show that helminth coinfection protect hosts against malaria severity. A study conducted in Ghana communicated that malaria immune response was modified towards

expressing more anti-inflammatory markers (increased Treg cell numbers and IL-10 concentration levels) in rural children with helminth coinfection compared with their STH-unfected peers [54]. In the same direction, helminth infections were reported to protect hosts against cerebral malaria in Thailand [55]. Such finding suggest STH's ability to modulate immune responses to malaria, thus limiting disease severity. In any case, despite such contradicting findings, one may argue that studying immune responses to malaria would likely be impossible if disconnected from STH in their coinfection context.

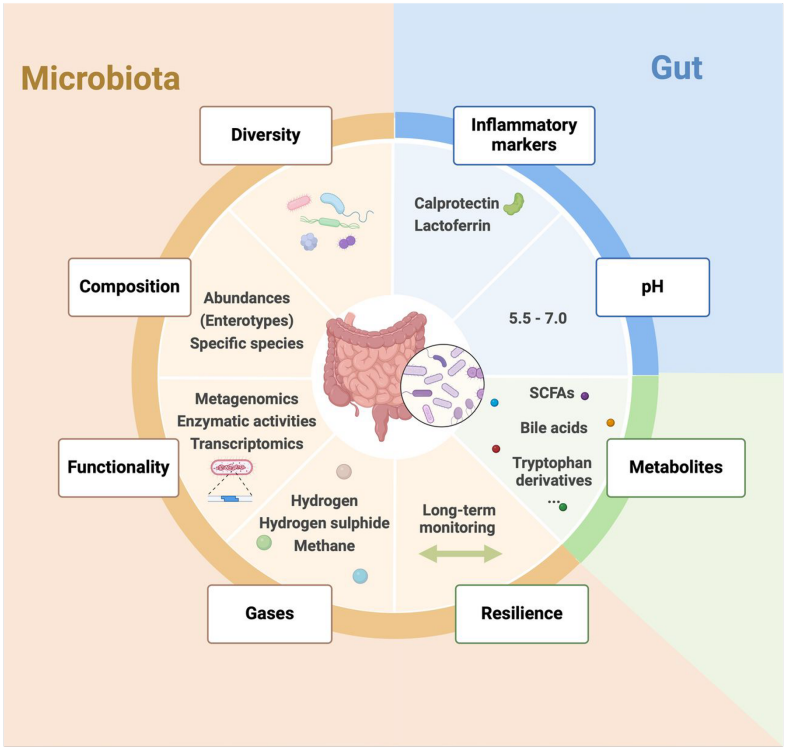
The Gut Microbiota

The Gut Microbiota (GM) is as an essential full fledged organ as the brain or other critical organs. The famous human microbiome project decribed it as 'a vital organ-like entity, critical to immune function, nutrition and metabolic balance, highlighting its systemic importance' [56]. It is a relatively novel science field and thanks to the development of novel high throughput sequencing technologies and data analysis softwares, the last few years have been characterized by an incredible awareness of the potential of the gut microbiota. Here, we attempt to summarise current knowledge about its definition, composition, function and role in health and disease.

The GM is a collection of all taxa constituting microorganism communities, such as bacteria, viruses, archaea, fungi and protists within the GIT [57]. In an individual human, its total microbial cell count is estimated at 10^{14} which outnumbers the total own human cell count of 10^{13} [58]. However, given the currently available technologies, bacteria are the most commonly studies of all the GM members [59]. With that in mind, it is commonly accepted that six main phyla dominate the microbiota composition: Firmicutes (renamed Bacillota), Bacteroidetes (renamed Bacteroidota), Actinobacteria (renamed Actinomycetota), Proteobacteria (renamed Pseudomonadota), Fusobacteria (renamed Fusobacteriota) and Verrucomicrobia (renamed Verrucomicrobiota) [60]. The human GM composition is diverse, complex and dynamic (Figure 4a). Different factors (e.g. host genetics, geographic location, age, diet, etc) can alter the GM composition (Figure 4b) [7, 60, 61]. Our understanding of the GM depends on and evolves with available study methods (i.e. sequencing and data analysis technologies).

The GM is essential to human host and its functions in ways including, but are not limited to metabolic, immune modulation and more. Firstly, metabolic functions of the GM are primarily related to the fermentation of of endogenous intestinal mucus as well as dietary fibers, functionally known as microbiota-accessible carbohydrates (MACs), to produce metabolites such as short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate ; bile acids; etc. These metabolites interact with host's receptors (e.g. FFAR3, GPR119, AhR, TGR5, lipolysaccharides, etc) on entero-endocrine cells to release hormones like GLP1 and PYY which regulate different metabolic functions [58, 62] (Figure 4a & b).

(a)



(b)

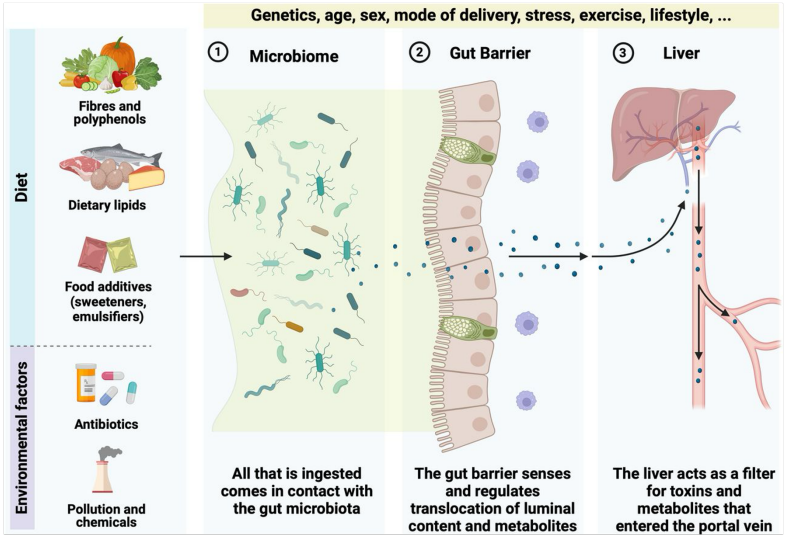


Figure 4. Potential markers of a healthy gut microbiota (a) and the three lines of defence (b) [58].

Example GM's functions include: (1) butyrate is central in the maintenance of a healthy gut in the intestines, (2) acetate is involved in the balance between appetite and satiety in the brain as well as in regulating insulin resistance in mucles while propionate is sent to the liver where it regulates gluconeogenesis and satiety signalling [57, 59, 62–64]. Furthermore, the microbiota is essential for developing and maintaining a balanced immune system. Certain commensal bacteria promote regulatory T cell development, modulate IgA secretion, and contribute to mucosal immunity, helping the body to distinguish between harmful and non-harmful agents [65]. Finally on this point, research has shown the GM's function in neurological and mental health also known as the gut-brain axis: the communication between gut microbes and the central nervous system which impacts mood, cognition and behavior [66].

Therefore, these Gut Microbiota functions serve as key determinants of health and disease.

While the GM's role in health can easily be comprehended through its functions described above, its role in disease represents deviation, also sometimes referred to as dysbiosis. Circumstances of dysbiosis have been observed and described in disorders such as inflammatory bowel diseases (IBD), metabolic disorders, autoimmune and allergic diseases, and cancer to mention just a few [58]. For example, Lloyd-Price et al., demonstrated how specific bacterial population dynamics such as the reduction of *Subdoligranulum* species are linked to inflammation and exacerbate symptoms in IBD - Crohn's disease and ulcerative colitis [67]. As per metabolic disorders like obesity and type 2 diabetes mellitus (T2DM), there are several research publications showing the causal relationship of the GM [68, 69]. Indeed, GM imbalance can result in ill health.

Leveraging and modulating the GM, researchers and other actors in healthcare have managed to reverse some abnormal health conditions. Gut microbiota-based interventions including probiotics, prebiotics, synbiotics and fecal material transplation (FMT) have shown the potential to prevent or reverse dysbiosis in its many forms. Probiotics are live microorganisms (e.g. *Lactobacillus* species) that can confer a health benefit on the host if used the right way and in the right amount [70]. They can be used in clinical management of metabolic disorders such as irritable bowel syndrom (IBS), inflammaroty bowel disease (IBD) and

antibiotic-related diarrhea [70]. As per prebiotics, they are fibers or MACs that promote the development of beneficial GM members. For example, inulin, fructooligosaccharides, and resistant starches are considered prebiotics and are found in foods like garlic, onions and bananas [71]. They can enhance the growth of *Bifidobacterium* and *Faecalibacterium* genera [71, 72]. Conversely, diets richs in sugar, fats and processed foods can lead to imbalance in gut microbial composition associated with metabolic and inflammatory deseases such as T2DM and obesity [59]. While FMT is explained with an example below, synbiotics are a combination of probiotics and prebiotics that enhances probiotic viability and colonization in the gut [71]. Finally, in recent years, increasing number of research outputs have taken a step further to look at individual, specific gut bacterial remedies termed 'next generation beneficial bacteria'. *Akkermansia muciniphila* is an example bacterium which has been shown to prevent or improve health conditions in obesity and T2DM [69]. In summary, different forms of GM members and inputs can be leveraged to preverve good health and reverse abnormal health conditions.

Overall, the GM represent a diverse, complex community of microorganisms residing in the GIT. The GM plays essential roles in host' health as much as it can be associated with pathologies. Probiotics, prebiotics, synbiotics, FMT and next generation beneficial bacteria are examples of GM-targeted health interventions.

1.5.1. Gut Microbiota-Malaria Interplay

Malaria-gut microbiota research focuses on how microorganisms sheltered in GIT might affect host's protection againt, susceptibility to and the progression of malaria. In this section, we explore what's known and what remains unknown in that field: from malaria immunomodulation to gut microbiota modification and GM-target interventions against malaria.

Research about the GM's role in infectious diseases lags behind. Indeed, authors of a recent publication acknowledged that many review articles remain silent on the role of the GM in infectious diseases [59]. Arguably, as of today, the best achievement made in the application of the GM to manage infections is that of using FMT as a recommended therapy for *Clostridioides difficile* infections (CDI)

[73]. That is a drop in the ocean given the multitude of infections facing humanity. In fact, there is an even longer way to go in terms of understanding malaria-microbiota relationships to devise GM-targeted remedies to improve malaria clinical presentation. Here, we summarise the few, yet commendable research outputs.

The gut microbiota modulates plasmodium infection risk and transmission

Malaria life cycle in humans starts with the injection of sporozoites (infective stage of *Plasmodium*) from the female *Anopheles* mosquito's salivary gland into the mammalian skin. These **'**sporozoites' have a glycan called 'Gala1-3Galb1- 4GlcNAc-R (α-gal)' which they share with a member of the gut microbiota community '*E. coli* O86: B7'. A study by Yilmaz et al. demonstrated how *E. coli* O86: B7 elicits the production of anti–α-gal antibodies which cross-react and block the transmission of *P. berghei* sporozoites beyond the skin within mice, potentially stopping infection altogether [74].

Applied to humans, the same antibodies showed capacity to affect prospective risk of infection (Figure 4). A cohort of 195 Malian children and adults was enrolled in a study just prior to an intense *P. falciparum* transmission season to assess whether specific compositions of the human gut microbiota could modulate the risk of malaria infection [75]. In their report, Yooseph et al. argued that strategic modulation of commensals in the human intestinal tract could decrease the risk of *P. falciparum* infection in malaria-endemic areas. According to the authors, favorable composition of the gut microbiota included 3 key members of the gut bacterial community: genera *Bifidobacterium* and *Streptococcus*, and family *Ruminococcaceae*. They proposed that the administration of pre- and/or pro-biotic nutritional supplements can promote the growth of commensal organisms like *Bifidobacterium* which would make a difference in the context of malaria prevention.

The gut microbiota modulates malaria disease severity

The GM was shown to play a role in determining the extent of malaria clinical presentation. In a recent study conducted on children in Uganda, authors reported that gut microbiota, specifically bacteria, have the capacity to modulate the severity of malaria through regulating the spleen germinal centers [76]. The same study was extended to testing the effects of selected antibiotics on gut

microbial communities in ways that affect host's immunity to malaria. Surprisingly, dependent on pre-existing/baseline gut microbiota composition (*Bacteroides* in this case), selected antibiotics were shown to lead to changes in the gut microbiota composition that enhance immunity to *Plasmodium* infection.

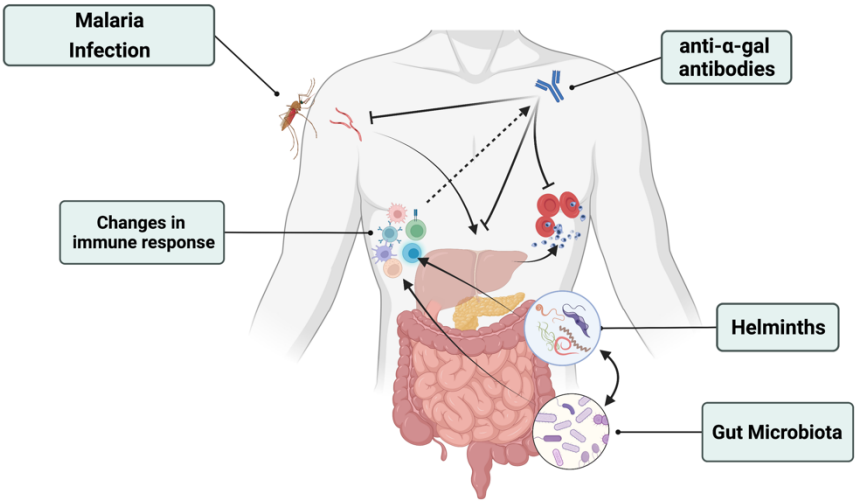


Figure 4. Helminth - Gut Microbiota – Malaria interactions affect malaria transmission and severity [77]

Villarino et al. [3] demonstrated that specific members (*Lactobacillus* and *Bifidobacterium*) of the gut microbiota were associated with resistance to malaria severity in a murine study. Two groups of genetically similar mice from different commercial vendors were infected with *Plasmodium* species. Mice from one vendor emerged as 'resistant' while those from the other vendor were 'susceptible' to the infection. The analysis of cecal bacterial communities of the resistant group showed two distinguished population members: *Lactobacillus* and *Bifidobacterium*. Followup efforts, during the same study, showed that when germ-free (GF) susceptible mice were treated with cecal content rich in *Lactobacillus* and *Bifidobacterium* or equivalent probiotics, the parasite burden dropped, further supporting the argument. These study findings suggest that specific composition of the gut microbiota or its treatment with supplements rich in specific bacterial content have the potential to reduce *Plasmodium* parasite burden which leads to the modulation of malaria severity.

Malaria changes the gut microbiota composition and the gut barrier integrity

Plasmodium infection is systemic and can lead to changes across the entire body including the gut microbiota and the mucosal barrier. For example, a study conducted by Yilmaz et al. [74] argued that *Plasmodium* infection altered both the GM composition and the gut permeability, thus rendering experimental murine hosts more susceptiple to diseases. Similar observations were made but in an indirect way through the effects of antibiotics on the GM. Selected antibiotics' effects on the GM were assessed to find out that they led to alterations in gastrointestinal bacterial communities which, in turn, modifed microbial diversity to weaken host defenses against malaria [76]. Furthermore, a study conducted in mice demonstrated how a *Plasmodium* infection was linked to dysbiosis and gut barrier alterations, thus exercerbating gastrointestinal symptoms of the disease [78]. Taken together, such research findings suggest direct and indirect effects of *Plasmodium* infection on the gut microbiota and gut barrier integrity.

Therapeutic interventions to modulate immune response to malaria

In this nascent field of research, a few publications talk about leveraging the GM as therapy to improve host immune responses. *Lactobacillus casei and Bifidobacterium longum* probiotics administered to mice showed potential to improve neurological outcomes in *Plasmodium berghei* infection [79]. Although still new, this approach presents a potential solution for areas with high malaria transmission and malnutrion - where the GM composition is already drained.

Summarising the whole topic, malaria-microbiota research is still in its early years. On the one hand, initial publications have shed light on the potential of the GM to impact *Plasmodium* infection risk and transmission as well as disease severity. On the other hand, malaria was shown to alter the gut microbiota composition and barrier integrity. Additionally, we are starting to see promising candidate probiotic therapies for malaria although only tried out in mice. There is still a long way to go in this new malaria-microbiota research field, especially when you consider external factors such as STH-coinfection.

1.5.2. The Gut Microbiota in Malaria-STH Coinfection Context

Studying malaria-STH coinfection is important in malaria-microbiota reseach for two reasons : (1) the overlaping geographic distribution of the two parasitic diseases and their opposing immune responses (Th1 for malaria and Th2

for STH). In that regard, researchers have often advocated for combined interventions to address both NTDs [8, 80–84]. Therefore, GM-targeted interventions should be no different vis-a-vis that fact.

In the course of malaria-STH coinfection, the gut microbiota plays a hypothetically crucial role. In our short review published earlier on this topic, we communicated that there is growing evidence that helminths can alter the gut microbiota by favoring specific bacterial communities [77]. STH infections, such as *Ascaris lumbricoides*, Trichuris trichiura, and hookworms (*Ancylostoma* spp. and *Necator* spp.) are as common as *Plasmodium* spp. in Sub-Saharan Africa and Southeast Asia. Targeting the interplay between these parasitic infections, mediated by the gut microbiome, is motivated by their effects on immunity, disease severity, and potential for combined interventions [6].

STHs are known for their immunomodulatory properties by modifying type 2 host immunity. STHs, through Th2 immunity, have been shown to confer resistance to pathogenic colonization and to alleviate inflammation in Crohn's Disease within hosts [85]. That ability can be handy in the case of malaria intestinal pathology as discussed above.

Gut microbiota composition analysis in the context of malaria-STH coinfection was recently conducted in different settings. *P. vivax* – microbiota association was shown in a Colombian study [86]. In India, higher levels of *Lactobacilli* were reported among the microbial communities of *P. vivax* –infected people [87]. While no association was reported between coinfection and the GM in both studies, authors communicated that other factors may have been at play. For example, Easton at al., [86] speculated that geography could be a possible reason for non-differential gut microbiota composition between STH-coinfected and -uninfected groups in Colombia because *T. trichiura* infection was associated with greater microbial diversity among infected individuals in their previous Malaysia-conducted study.

Anti-parasitic therapy for both malaria and soil transmitted helminthiases, in their individual action as well as their interaction, can affect gut microbiota composition. For example, a study conducted on Kenyan children reported that anti-malaria treatment was associated with minimal changes in gut microbial profiles [88]. With that in mind, we can hypothesize that Artimesinin-based

Combination Treatment (ACTs) may alter the GM composition which would weaken host immune integrity. Similarly, albendazole – a common treatment for STHs – has been associated with dysbiosis in a study carried out in Ghana [89].

Concluding on this point, we can highlight that this section emphasized the importance of considering coinfections in microbiota research as well as the potential of microbiota-targeted approaches to improve health outcomes in settings endemic for both malaria and soil transmitted helminthiases.

Methodologies of Studying the Gut Microbiota

Methodological approaches involved in GM composition studies include old ones which existed for centuries and novel ones which brought true revolution to the field. Particularly, novel methods vary and come with unique strengths and limitations. This complexity is due to the vast diversity of microbes within the gut as well as the interactions not only between GM members but also with hosts. Here, we cover common and emerging methods, putting an emphasis on DNA-based techniques.

1.6.1. Sample Types and Collection Approaches

To study microorganisms in the GIT, one can use different types of samples such as stool or fecal material and mucosal biopsies.

Stool samples are the most common for two main reasons : easy to collect for research teams and non-invasive for research participants . Stool samples are collected in tubes designated for GM analysis (e.g. OMNIgene GUT, DNA Genotek, Ottawa, ON, Canada), kept on ice or preserved with anaerobic methods (e.g. BD GasPak EZ anaerobe pouch system) for maximum 24h ahead of the long-term −80◦C freezing [90, 91]. However, stool samples represent one part of the communities – luminal microbes, leaving behind mucosa-associated ones.

Mucosal biopsies are prefered for studies aimed at analyzing mucosa-associated microbiota, particularly in conditions like IBD. However, this method is invasive which may deter a good number of both researchers and study participants [90].

DNA extraction for either sample follows kit manufacturer's instructions. There are several manufacturers. Instructions may be slighly modified to optimize results by adding steps such as bead-beating [92].

Sample collection is the first and probably most critical step in the process. Standardization and principles should be followed as variations in collection, storage (e.g., freezing at −80°C), and processing could introduce biases in microbial composition analysis, interpretation and reporting later [92].

1.6.2. Culture-Based and Histotechnology Techniques

Microbial culture methods are almost as old as the field of microbiology itself. They have been used for centuries to study, discover and diagnose microorganisms. They involve growing, isolating and identifying specific microorganisms – including gut microbes on selective media and under anaerobic conditions for GM members. The same approach is also use for antimicrobial susceptibility testing. They limitations lie in the fact~~r~~ that some microbes are demanding too fastidious growth conditions while others are totally unculturable [93].

Fluorescence In Situ Hybridization (FISH) is a histology technique used as a solution to the unculturability of some microbes. FISH used fluorescent probes to target, visually identify and measure microbial populations in a sample. Results are easier to see, thus requiring less know how to interprete. Nevertheless, only known probe-matching sequencing can be target and it can provide a low throughput despite being work-intensive [94].

1.6.3. 16S rRNA Gene Sequencing

To identify and classify GM bacterial communities, this method amplifies and sequences one of the most conserved bacterial DNA regions: 16S ribosomal RNA gene [90].

With DNA extract samples available, the process start with identifying the gene's target region(s) – commonly V3 and/or V4 and/or V5. Next, according to manufacturer's instructions, target region(s) are amplified by PCR (Polymerase Chain Reaction) with the use of DNA polymerase enzyme, two bacterial primers (forward and reverse) specifically designed, adapters and error-correcting barcodes unique to each sample to allow running multiple sample in a pool. After being pooled into a single library and quantified fluorometrically, samples are cleaned up and normalized using a high- throughput normalization kit. 16S rRNA gene sequencing method is often chosen for its broad bacterial coverage, being cost-effective, suitable for general profiling of bacterial taxa at genus or family levels and for providing high-throughput [90].

As per disadvantages, in addition to not reaching species level in classification, it does not apply to fungi, viruses, or archaea [90, 95].

1.6.4. Metagenomic Sequencing

Also known as Shotgun sequencing, this method is definitely more powerful and informative than 16S rRNA Gene Sequencing. It offers researchers an opportunity to analyse whole genomes, thus generating a bigger picture about microbial diversity, gene identity and potentially, metabolic functions. Generated outputs are presented in the form of gene and species abundance tables. But, its downside is characterised by narrow bacterial coverage (low throughput), high cost, requiring extra computational resources and calling for more powerful bioinformatics infrastructure – majory of which are not yet standardised [96] [97].

1.6.5. Metatranscriptomics, metabolomics and more

In this rapidly changing and exciting research field, there are other methods in use and more in the making. Here we briefly talk about metatranscriptomics, metabolomics and single-cell genomics.

Metatrancriptomics use RNA, not DNA as with the previously discussed methods. This method helps researchers to analyze gene expression and interprete metabolic pathways, potentially determining functions of specific members of the studied microbial community. Challenges associated with method include the relatively higher instability of RNA which degrades faster and also requiring more complex, not yet standardised bioinformatics [98].

Metabolomics is a sequencing method used to study individual metabolites produced by gut microbes. Two technologies are commonly use to complete metabolomic tasks: mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. The latter is more recommended for lower costs, requiring no extra sample preparation and providing quantitative results. The methods yields information about microbial activity and host-microbe interactions, towards determining roles of key metabolites in health and disease. However, generated massive data require robust big data analysis skills and technologies [99].

Single-cell genomics is an emerging method promising to solve the challenge of uncultivable species' genetics. Its higher resolution is commendable in providing cell composition and itentity details. Thus, it is commonly used in studying rare and new organisms. But, as a new method, it remains expensive and challenging as long as big data generation and analysis methods haven't caught up.

1.6.6. Computational and Statistical Approaches

Computational and statistical approaches are essential to make sense of generated data in processing microbiota samples. They are employed to analyse big datasets, make patterns and generate meaning necessary to answer initial research questions about the GM composition and functions.These methods are used to preprocess data, to control data quality, to conduct exploratory and taxonomic analyses, to measure diversity metrics and calculate relative abundance and to test differential abundance. Here, we shall share more details about 16S rRNA gene sequencing-related methods while talking briefly about other more advanced methods.

Data preprocessing and quality control is the first step after availing sequencing data in the right format (e.g. FASTQ files). The purpose of this step is to cut low-quality reads and remove chimeric sequences along with other contaminants. You use tools such as QIIME2 [100] and DADA2 [101] to achieve your filtering, denoising and dereplicating goal with accuracy and efficiency . Next, BBTools and Trimmomatic tools are handy for quality control. If done correctly, this step will save you from biases in taxonomic and functional annotation, impacting downstream analysis [100].

Exploratory and taxonomic analyses give a bigger, general picture of the data. Exploratory tests helps researchers to understand the data structure and patterns in order to generate hypotheses ahead of proper analyses. Taxonomic classification is performed using a pre-trained classifier (e.g. naive Bayes) against a reference databases (e.g. silva138_AB) which are sometimes incorporated in QIIME2 [102].

Diversity metrics are tested to gain insights about how different samples and groups of sample compare where higher diversity is often associatiate with healthier gut microbiota. There are two main types of such metrics : alpha and beta. On the one hand, alpha diversity measures within-sample diversity in terms of OTU number, richness, eveness and phylogeny. There are five most common alpha diversity metrics : Observed OTUs/ASVs, Shannon Diversity Index, Simpson's Diversity Index, Chao1 Diversity index and Faith's Phylogenetic Diversity (PD). On the one hand, beta diversity tests between-sample differences and similarities in order to compare species shared or not between two or more communities (e.g.

healthy vs diseased). There two major groups of beta diversity metrics : Non-phylogenetic (e.g. Bray-Curtis Dissimilarity, Jaccard Index, etc) and Phylogenetic (e.g. Unweighted UniFrac, Weighted UniFrac, Generalized UniFrac, etc). Conventional ordination techniques such as PCoA or NMDS are used to visualize beta diversity results. More often, diversity analyses are performed together with relative abundance calculation by diving each OTU/ASV reads by the total number of reads in the sample. Commonly used tools to measure diversity (both alpha and beta) include : QIIME2, Phyloseq (R package) and scikit-bio (Python). The latter two can help with calculating relative abundance too [103].

Differential abundance testing is aimed at determining statistically signficant differences between sample groups (e.g. healthy vs diseases) by comparing taxa or genes. There are three commonly used methods : (1) LEfSe (Linear discriminant analysis Effect Size) that compares biomarkers such as sex or age-group or else infection status based on linear discriminant analysis (LDA) scores ; (2) DESeq2 which was initially developed for RNA-seq analyses but later was extended to analyzing count data and (3) ANCOM (Analysis of Composition of Microbiomes) that accounts for the compositional nature of microbiome data [104] [105].

There are more advanced tests such as machine learning and predictive models, network analyses, time-series analysis, multi-omics integration as well as causal inference and mediation analysis. While we did not use these methods in the current work, they are widely used in metagenomics, metabolomics and other genomics research projects [106–109].

To summarise, here, we shared an overview of 16S rRNA gene sequencing-related methods used in computational and statistical analysis of data from preprocessing and quality control, to measuring diversity and relative abundance and determining differential abundance. In addition, we hoghlighted other more advanced methods and shared references you can check just in case. Neither the list nor the content is claimed to be exhaustive in this rapidly evolving field of research.

AIMS AND OBJECTIVES

2.1. Problem Statement

Malaria is a parasitic disease that, for many years, has remained a leading cause of morbidity and mortality in the developing world. Through its global technical strategy for malaria 2016–2030, WHO makes malaria a top global health priority.

The overlaping geographic distribution of malaria and soil transmitted helminths guarantees their co-infection in endemic areas. Immunologically, it has been shown that, during co-infection, some helminths confer protective effects against (or exacerbate) malaria, potentially affecting its clinical presentation as asymptomatic or mild or else severe disease. However, little is known about the mechanism behind that outcome.

The human body, especially the gut, is naturally inhabited by trillions of microbial cells which, as a group, are referred to as the 'gut microbiota'. In recent years, significant evidence has been generated about the ability of human microbiota to modulate host immunity against infectious pathogens. But, the role of the gut microbiota in malaria clinical presentation remains largely unknowly.

Research has shown a plethora of factors that contribute in determining the clinical presentation of malaria : from the parasite virulence to host immunity, genetics, age, pregnancy, gut microbiota, nutritional habits, coinfection and more. Here, we are attempted to evaluate relationships between the gut microbiota and malaria, keeping in mind potential confounding effects of STH coinfection, host age, nutritional habits and geographic location in Rwanda.

2.2. Research Objectives

The main aim of this project was to analyse the relationships between malaria and the gut microbiota composition, considering confounders such as STH coinfection, in Rwanda.

Specific objectives were to:

I. Analyse gut microbiota profiles among the general population living in malaria-endemic regions, considering host's nutritional habits, STH coinfections and age;

II. Evaluate relationships between the gut microbiota and malaria clinical presentations (asymptomatic, mild and severe disease), considering confounders, in Rwanda;

In other words, we attempted to answer the following questions:

I. Is the gut microbiota composition affected by *P. falciparum* infection in Rwanda? What are the effects of other factors such as STH coinfection, geographic location, nutritional habits or age?
II. Are there links between malaria severity and the gut microbiota composition in Rwanda? Are those links affected by confounders such as geographic location or age?

2.3. Research Hypothesis

This study sought to test the hypothesis that, accounting for key confounding factors, specific gut microbiota profiles can be associated with different malaria clinical presentations.

RESULTS

Chapter 1 - Gut microbiota composition differences are associated with geographic location and age in malaria-endemic regions of Rwanda

RESEARCH ARTICLE

Gut microbiota composition differences are associated with geographic location and age in malaria-endemic regions of Rwanda

Jean d'Amour Mutoni[1,2], Matthias Van Hul[1,3], Aline Uwimana[1,2], Camille Petitfils[1,3], Giselle C. Wong[1,3], Anthony Puel[1,3], Amandine Everard[1,3], Hélène Alexiou[4], Leon Mutesa[5#], Jean-Paul Coutelier[6☯], Nadine Rujeni[2☯*], Patrice D. Cani[1,3,7☯*]

1 Louvain Drug Research Institute (LDRI), Metabolism and Nutrition Research Group (MNUT), UCLouvain, Université catholique de Louvain, Brussels, Belgium, **2** Biomedical Laboratory Sciences Department, College of Medicine and Health Sciences, University of Rwanda, Kigali, Rwanda, **3** WELBIO Department, Walloon Excellence in Life Sciences and BIOtechnology (WELBIO), WEL Research Institute, Wavre, Belgium, **4** Dietetics Department, Haute Ecole Leonard de Vinci, Health Sector, Brussels, Belgium, **5** Centre for Human Genetics, College of Medicine and Health Sciences, University of Rwanda, Kigali, Rwanda, **6** De Duve Institute, UCLouvain, Université Catholique de Louvain, Brussels, Belgium, **7** Institute of Experimental and Clinical Research (IREC), UCLouvain, Université Catholique de Louvain, Brussels, Belgium

☯ These authors contributed equally to this work.
* n.rujeni@ur.ac.rw (NR), patrice.cani@uclouvain.be (PDC)

1.1. Abstract

Evidence suggests that a significant interplay exists between the host gut microbiota and both the transmission and severity of malaria. Therefore, we explored the association between malaria and the gut microbiota across various geographic regions, considering host's nutritional habits, helminth coinfections and age. This observational study was conducted in 3 malaria-endemic provinces of Rwanda: West, South and East. Demographic data, blood and fecal samples were collected from 169 participants (85 females and 84 males) aged between 2-78 years. We used questionnaire-derived qualitative data based on geographic regions, age, and nutrition. Malaria and soil-transmitted helminth diagnosis was assessed by microscopy. The gut microbial composition was analyzed based on bacterial 16S rRNA gene amplicon sequencing. We observed that preschool children had a significantly lower microbiota diversity compared to both school children ($q=0.027$, K-Wallis) and adults ($q=0.011$, K-Wallis). Unlike age, infection status (uninfected, malaria alone, soil-transmitted helminth alone or coinfection) was not significantly associated with the gut microbiota. However, using Bray-Curtis distances, we found a significantly differential gut microbial beta-diversity with a convergent distribution in the Western province compared to the other provinces ($q=0.0045$, pairwise PERMANOVA). This geographic difference was not explained by any change in energy intake, protein, lipids, or carbohydrates consumption but was likely due to lower dietary fibre intake in the West compared to the South ($q<0.0001$, ANOVA) and the East ($q=0.07$, ANOVA). In conclusion, we have not found significant links between infection and gut microbiota. However, we showed a significant difference in the gut microbiota composition of people living in different geographic locations in Rwanda, possibly due to their nutritional habits.

Keywords: Malaria, Gut microbiota, Geographic location, Age, Rwanda.

1.2. Introduction

Malaria is classified among the so-called group of 'poverty-related diseases', representing a major health problem predominantly in the global south [110]. The World Health Organization (WHO) reported 249 million cases and 608,000 deaths due to malaria worldwide in 2022 [9]. *Plasmodium falciparum* is the primary cause of severe malaria and is responsible for more than 90% of global malaria fatalities, with the Sub-Saharan African (SSA) region carrying over 90% of the burden [9].

The gut microbiota has been shown to play a major role in health and disease. In the context of malaria, existing evidence shows trends but no causal roles have been established. A groundbreaking study published in 2014 (Yilmaz et al.,) discussed the role of gut microbiota-elicited alpha-gal antibodies in blocking *Plasmodium* transmission . The latest publications of 2023 presented the genus *Bacteroides* as a key player in predisposing hosts to severe malaria in both human and murine subjects [6, 111]. Importantly, the malaria-gut microbiota associations may be shaped by several factors such as geographic location, nutrition, coinfections (e.g., soil-transmitted helminths), age, antimalarials, deworming and antibiotic exposure [6].

Geographic variation is a critical factor shaping the host microbiota diversity and composition ′[112, 113]. Specifically, in malaria-gut microbiota research, Yooseph et al., (2015) have shown differential microbiome composition by geographic regions ranging from Mali to Malawi and around the world. According to the Center for Disease Control and Prevention (CDC, Atlanta, Georgia, USA), malaria distribution has wide geographic variations, even within a country [114]. In Rwanda, malaria predominantly occurs in the West, South and the East provinces (Fig 1A), but there are no studies analyzing gut microbial composition in these regions.

Malaria-microbiome interactions may be influenced by coinfection with Soil Transmitted Helminths (STH) resulting in divergent and still poorly understood effects on gut bacteria. Indeed, STH are among the most common parasitic infections worldwide and their distribution overlap with that of malaria in several regions, primarily affecting the poorest and most vulnerable populations [77]. There is a growing evidence that helminths (or the immune response to helminths)

may alter the gut microbiota by favoring specific bacterial communities [77]. A recent study conducted in Ethiopia showed that people infected with *Trichuris trichiura* exhibit lower alpha diversity than uninfected peers [115]. On the other hand, the gut microbiota might influence the host's immune response towards certain helminths, potentially modulating the severity and outcome of infections [116–118]. Additionally, interactions between *Plasmodium* and helminth infections may alter immune responses and susceptibility of the infected host; thus causing impact on clinical outcome by either worsening (synergism) or reducing (antagonism) the severity of infection and disease [8, 84, 119]. Immunomodulation between malaria and STH is a result of two opposing immune response types produced by the two parasites. Malaria-infected hosts mount pro-inflammatory Th1 immune response – dominated by cytokines like interferon-γ (IFN-γ) and tumor necrosis factor-alpha (TNF-α) – to clear *Plasmodium* parasites. In contrast, STH promote a modified anti-inflammatory Th2 immune response which encompasses Transforming growth factor beta (TGF-β), Interleukin-4 (IL-4), IL-5, and IL-10 that favor their survival in the host [120]. It remains poorly understood whether STH immunomodulates malaria responses through altering the gut microbiota. Although coinfections are common, significant gaps remain in our understanding regarding the nature and extent of these interactions, including their directionality and magnitude.

Taken together, these findings highlight an incomplete understanding of the impact of malaria on the gut microbiota and vice versa. To assess how different factors could affect gut microbiota composition in malaria-endemic regions, we used a multidimensional approach to investigate malaria-gut microbiome associations within the context of geographic regions, diet, parasitic coinfection and age in Rwanda.

Specifically, this study's first objective was to analyze the gut microbiota composition by parasitic coinfection, age and geographic regions using 16S rRNA gene amplicon sequencing. Our second objective was to assess the potential role of nutritional habits in observed gut microbial differences.

This study's findings have the potential to enrich the limited gut microbiota literature in Rwanda. We also shed light on key factors to consider in designing malaria-gut microbiota studies. Most importantly, our work contributes to the

WHO’s call for more research to inspire innovations for malaria prevention, control and management in endemic settings [1].

1.3. Materials and Methods

Ethics and consent

Our cross-sectional research project was reviewed and approved (reference number 031/CMHS IRB/2021 issued on the 2nd of February 2021 and reference number 217/CMHS IRB/2022 issued on the 2nd of February 2022) by the Institutional Review Board (IRB) of the College of Medicine and Health Sciences (CMHS) at the University of Rwanda (UR). For both participation and publication of all clinical data, and other data included in this manuscript, written informed consent was obtained from study participants aged 18 and above, and from parents, relatives or guardians of younger participants.

Study design, participants and samples

This cross-sectional study was conducted in the Republic of Rwanda. The covered territory included 11 out of 30 districts - belonging to Western, Southern and Eastern provinces - of Rwanda which are classified as malaria-endemic with stable transmission (Fig 1A). Samples were collected from 169 participants between the 1st of November 2021 and the 30th of September 2022 (Fig 1A). Study participant recruitment took place at either a health facility (cases) or within their household (controls). We used a convenience sampling strategy given our time and budget limitations and in response to the commendable depletion of malaria cases, thanks to robust interventions (e.g. indoor residual spraying) deployed by the government of Rwanda in targeted areas at the time of our study. Cases of malaria-infected participants were found at either district hospitals or health centers. Control participants, who had to be from the same households as cases, were met in their homes on the same day. Study cases were malaria-positive patients found in health facilities within our study area who had not been given antimalarials, antibiotics, and/or antihelminthics in the past two weeks prior to sample collection. Study controls were malaria-negative people, found in the same households of the already recruited cases, preferably with similar age, gender and nutritional habits.

Upon receiving their signed consent forms, a demographic questionnaire was filled for each participant. On the same day, for each participant, a confirmatory (for cases met in health facilities) or screening (for controls met in households) rapid diagnostic test (RDT) was performed, blood smears were prepared for final malaria diagnosis and stool samples were collected for helminth screening and gut bacterial DNA extraction for bacterial 16S rRNA gene sequencing.

For malaria diagnosis, blood smear microscopy was considered gold standard, thus its results were considered final to avoid false negative and positive preliminary results of the RDT. That resulted in four comparison groups based on infection status: Uninfected (Neither), *Plasmodium*-infected (Single_P), STH-infected (Single_H) and Co-infected. Age groups (0-4 aged preschool children, 5-14 aged school children and 15+ aged adults) were assigned based on questionnaire-derived qualitative data which was also used to compare nutritional intake and geographic location based on provinces (West, South and East) (Fig 1). Malaria patients identified in health facilities were treated by these same facilities while for other malaria-positive people identified in households (not included in controls), our team provided treatment according to the guidelines of the Ministry of Health effective in the Republic of Rwanda at the time of diagnosis. All samples were collected before any treatment was administered to patients.

Nutritional questionnaires and Body Mass Index (BMI)

Food intake was evaluated using a 7-day Food Frequency Questionnaire (FFQ) and 24 hour recall questionnaire. FFQ and 24h recall questionnaires were combined to minimize error in recording data related to food items consumed and to enhance complete and accurate food recall [121]. Study participants (both cases and controls) were challenged to recall and list all the foods and drinks they had consumed the day before, using visuals aids provided in "Photographic Food Atlas for Kenyan adolescents (9-14 years)" to approximate the serving sizes of various foods. For the frequency of consumption, four categories were generally available (never or rarely, 1 – 3 times per week, 4 – 7 times per week coupled with once or twice per day and 3 times per day). Total quantinties of items consumed by study participants were recorded per 7 days. To obtain daily quantities, recorded amounts were divided by 7 before multiplying the result by the number of times the food item was consumed during the week. To translate the quantities of each food consumed in nutrients (macronutrients), we established a Rwanda Food Composition Table (FCT), made from the West Africa FCT 2019 supplemented with the Kenya FCT 2018. Finally, to complete the Rwanda FCT with few items which were not present in the two previously mentioned tables, we used the 7th edition of Belgian FCT 2022. The three FCT were chosen based on the closest proximate in food items, preparation for any given food composition. Thereafter, the Rwanda FCT generated was used to translate each food in macronutrients (protein (g), lipids (g), carbohydrates (g) and fibres (g)) that were then converted into energy intake

(kcal). Finally, the nutrition analysis was undertaken using GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

The BMI was calculated by dividing the participant's weight (in kg) by the square of their height (in meters) and was expressed in kg/m². As recommended by the WHO, sex- and age-specific BMI-for-age Z-scores were used for participants aged 2-19 [122–124]

Rapid test and blood film malaria diagnosis

Trained laboratory technicians used sterile, single-use lancets to draw finger-prick blood used to perform a malaria RDT for on-site immediate confirmation or screening. Additionally, thick and thin blood films on glass slides for the identification of *Plasmodium* species and the determination of parasite density (parasitemia) at the National Reference Laboratory. Prepared blood smears were stained with Giemsa as previously described [125]. A compound microscope was used to determine *Plasmodium* species and parasite density on stained smears fully covered with immersion oil type A. Following complete positive diagnosis combined with questionnaire generated data, participants were classified according to clinical manifestations as: having 'severe' malaria (cerebral malaria, respiratory distress, severe malarial anemia, malaria with complicated seizures, and prostration); having 'mild' malaria (fever and flu-like illness, including one or all of the following symptoms: shaking chills, headache, muscle aches, tiredness, nausea, vomiting, and diarrhea) and having 'asymptomatic' malaria (no symptoms). Control participants with a laboratory negative diagnosis and with no symptom were classified as 'uninfected' with malaria.

Stool samples and Soil-Transmitted Helminths (STH) screening

After technicians had collected malaria blood samples, adult participants or guardians were given clear instructions to collect stool samples on single-use aluminum plates. Upon reception from the participant/guardian, stool samples were divided into two parts. Part one was transferred into a formalin-ether container for helminth screening. Part two was divided into three aliquots (5mL per tube), and kept under anaerobic conditions by GasPak EZ Anaerobe Pouch System with Indicator, 20 (BD Diagnostic Systems, USA) for a maximum of 24 hours during

sample transport. Both parts were transported the same day from field to the laboratory where part one was kept at room temperature until STH screening and part two frozen (−80 °C) until bacterial DNA extraction. STH screening results were recorded as negative or positive plus causative species (i.e., *Trichuris trichiura*, *Ascaris lumbricoides*, and hookworm (*Ancylostoma duodenale* and or *Necator americanus*). All STH-positive participants received screening results and antihelmintic treatment through community health worker channels in maximum two weeks after sample collection. Treatment followed the guidelines of the Ministry of Health effective in the Republic of Rwanda at the time of diagnosis.

Stool bacterial DNA extraction, sequencing and gut microbiota composition analysis

Bacterial DNA extracted from fecal samples was sequenced for gut microbiota composition analysis. Samples were kept frozen at −80°C until DNA extraction. The extraction of metagenomic DNA was carried out using QIAamp Fast DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions with the addition of a homogenization step by bead-beating. DNA purity (A260/A280) and concentration were determined using a NanoDrop2000 (Thermo Fisher Scientific, USA).

Samples were diluted in TE buffer to a concentration of 20ng/µl and sent to MrDNA (www.mrdnalab.com; Shallowater, TX, USA) for sequencing. The V4 region of bacterial 16S rRNA gene was amplified using the primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') [103]. Purified amplicons were sequenced using the Illumina MiSeq platform (2x250 bp PE) according to the manufacturer's guidelines, followed by data demultiplexing.

The analysis of demultiplexed paired-end FASTQ files provide by MrDNA was performed using QIIME2 (version 2024.10-amplicon) [100] on an Apple M2 Max system. Primer sequences were trimmed, and the quality of the sequences were assessed through visualization of interactive quality plots. Denoising and merging of paired-end reads were conducted using the QIIME2 DADA2 plugin, generating amplicon sequence variants (ASVs) [101]. Truncation and trimming parameters were optimized based on the quality plots to maintain the highest sequence quality (quality score of 37 or more for the 25th, 50th, 75th, and 91st percentiles). To enhance the reliability of downstream analyses, low-abundance features (<5 counts across samples) and poor-quality samples (<40000 sequence

reads) were filtered out. Features were clustered de novo based on sequence similarity using the QIIME2 VSEARCH cluster plugin with a 99% identity threshold. This clustering step reduced computational complexity and enhanced the accuracy of subsequent chimera detection. Chimeric sequences were identified and removed using the UCHIME [126] algorithm within the VSEARCH plugin [127]. Taxonomic classification was performed using a pre-trained classifier based on the SILVA v138 reference database . Finally, we filtered out chimeric sequences and unwanted sequences (Eukaryotes, choloroplasts, mitochondria) (S1 Table). For phylogenetic analyses, a phylogenetic tree was constructed to enable diversity assessments. This step involved sequence alignment using MAFFT [128] and tree building with FastTree [129]. Alpha diversity metrics (Observed features, Shannon entropy, Faith's phylogenetic diversity, Chao1 and Pielou evenness) were calculated using rarefied feature tables (threshold at 47000 reads) (S1 Fig) [130–133]. Beta diversity metrics (Bray-Curtis – used for PCoA visualization, Weighted Unifrac, Unweighted Unifrac and Jaccard) were also computed to examine community composition differences between samples [134–136]. To identify differentially abundant taxa across experimental conditions, Analysis of Composition of Microbiome with Bias Correction (ANCOM-BC) was employed [137]. This analysis was conducted at multiple taxonomic levels, including phylum, family, and genus. Finally, the processed data were exported for further statistical analysis and qza files generated in QIIME2 were used for visualization in R (version 4.4.1, R Core Team, 2024) [138]. With the package qiime2R, we imported QIIME2-generated distances into R to produce principal coordinate analysis (PCoA) beta diversity plots using ggplot2, glue, tidyverse, ggrepel, dplyr and ggExtra packages [139–144]. GraphPad Prism (version 10) was used to create alpha diversity figures using QIIME2-generated metrics [145].

Statistical analysis

Statistical significance of group comparisons were analyzed by QIIME2 (version 2024.10-amplicon), GraphPad Prism (version 10) and R (version 4.4.1). In GraphPad, we used the ROUT method to remove outliers (Q=1%). Next, for alpha diversity and nutritional data, we used ANOVA (analysis of variance) or Kruskal Wallis tests if data were normally distributed or not (by Shapiro-Wilk test) respectively. Follow-up multiple pairwise comparisons were determined by Dunn's test (alpha diversity) and post hoc Bonferroni (nutrition). QIIME2's PERMDISP test was performed to determine sample homogeneity (non-significant result), before

running PERMANOVA and Pairwise PERMANOVA (999 permutations for both) for beta diversity. Group comparisons were considered significantly different at $p < 0.05$.

For further efficient exploratory purposes, Spearman's correlations were tested to assess associations between differentially abundant, fully identified bacterial genera (identified in the ANCOM-BC analysis) and other key variables. The latter included age, macronutrient values, *Plasmodium* parasitemia and alpha-diversity metrics. Correlations were computed in R using the package 'Psych' (version 2.3.6) with FDR multiple testing correction [146]. The corresponding r-score, p-value and adjusted p-values were listed in a table (S2 Table). A correlogram representing the correlation and statistical significance levels was generated using the R package 'Corrplot' (version 0.92) [147]. Overall, specific statistical tests and significance cutoffs are described in figure legends. Group comparisons were considered significantly different at p-value/adjusted $p < 0.05$.

1.4. Results

Study design

This cross-sectional study was designed to assess the gut microbiota composition within the context of geographic regions, nutrition, parasitic coinfection, and age in malaria-endemic regions of Rwanda (Fig 1A). Therefore, we recruited participants from Rwanda's Western, Southern and Eastern provinces. Demographic data, blood and fecal samples were collected from 169 participants (85 females and 84 males) aged between 2-78 years. Malaria diagnosis was followed by soil-transmitted helminth (STH) screening which enabled us to make four comparison groups based on infection status: Uninfected (Neither), *Plasmodium*-infected (Single_P), STH-infected (Single_H) and Coinfected (Fig 1B). Socio-demographic characteristics of the study population are summarized in Table 1.

The bacterial 16S rRNA gene (V4 region) amplicon sequencing carried out on 169 samples yielded 126,748,404 paired-end reads (S1 Table). After quality control, denoising and filtering, we obtained 3,166 amplicon sequence variants (ASVs) with an average of 387,370 reads per sample across the dataset.

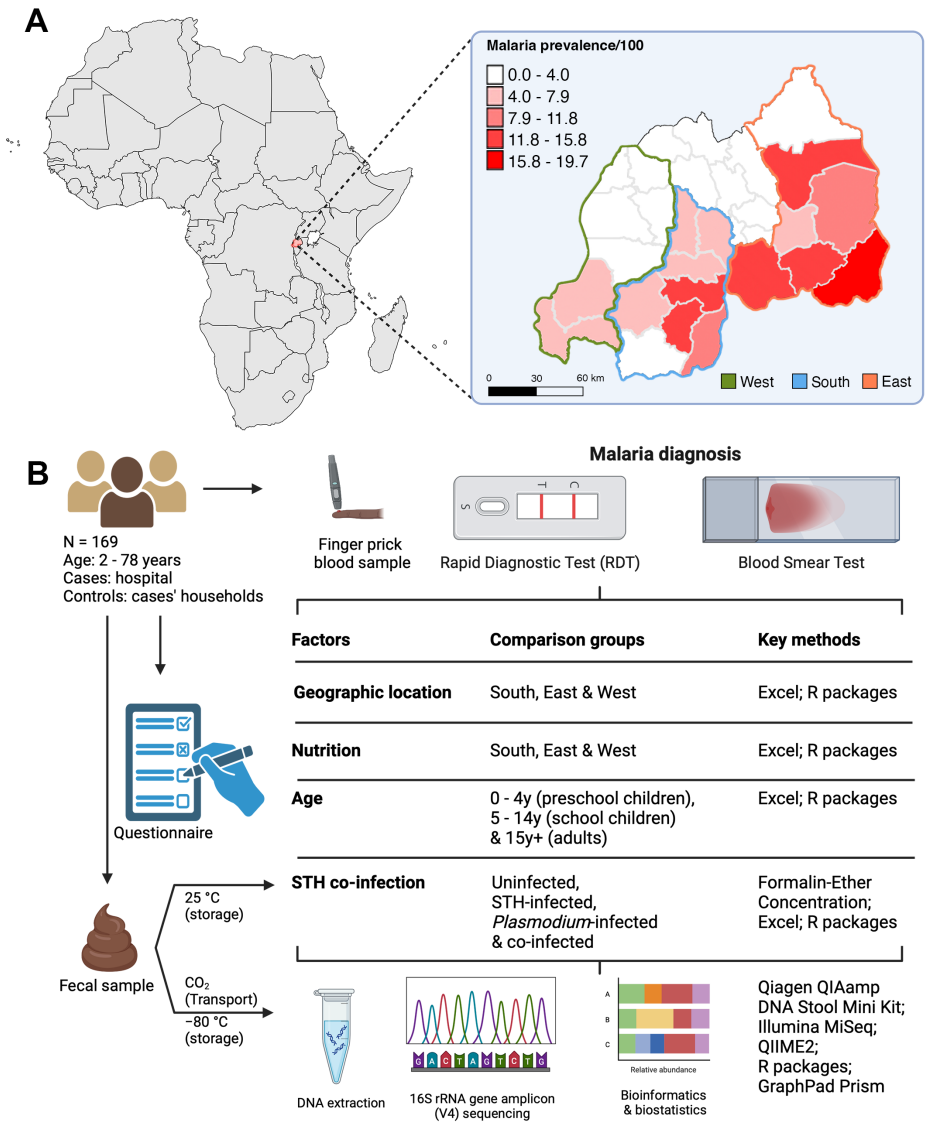


Fig 1. Study area and design. (A) Study area showing three malaria-endemic provinces of Rwanda: West, South and East. (B) Study design summary including, but not limited to: participant recruitment, sample and data collection, malaria diagnosis, STH screening, key methods of testing, bioinformatics and biostatistical analyses for group comparisons. N, number of participants; STH, soil-transmitted helminths. Created with BioRender.com.

Table 1. Characteristics of the study population

Characteristics: units	*Provinces*				
	Overall	**East**	**West**	**South**	***p*-value**
Participants: n (%)	169	73	50	46	
Females: n (%)	85 (50.3)	38 (52)	26 (52)	21 (45.6)	
Age: median (range)	16 (2-78)	16 (2-78)	15 (4-73)	2.5 (2-64)	0.15 (Kruskal-Wallis)
BMI: mean±SD kg/msq	19.13.8	19.6±3.9	18±3.7	19.6±3.7	0.03* (Kruskal-Wallis)
Uninfected: n (%)	37 (21.9)	24 (32.9)	4 (8)	9 (19.5)	
Only Plasmodium-infected: n (%)	129 (76.3)	48 (65.7)	45 (90)	36 (78.3)	
Plasmodium parasitemia: mean±SD parasites/ul	16,058±32,330	23,818±38,891	17,376±33,762	6,495±18,401	0.18 (Kruskal-Wallis)
Plasmodium-uninfected: n (%)	40 (23.7)	25 (34.2)	5 (10)	10 (21.7)	
Asymptomatic Plasmodium-infected: n (%)	2 (1.2)	2 (2.7)	0 (0.0)	0 (0.0)	
Mild Plasmodium-infected: n (%)	121 (71.6)	40 (54.8)	45 (90)	36 (78.3)	
Severe Plasmodium-infected: n (%)	6 (3.5)	6 (8.2)	0 (0.0)	0 (0.0)	
Coinfection: n (%)	14 (10.8)	8 (16.7)	4 (8.9)	2 (5.5)	
Only STH-infected: n (%)	3 (7.5)	1(4)	1(2)	1(10)	
Total STH-infected: n (%)	17 (10)	9 (12.3)	5 (10)	3 (6.5)	
AL-infected: n (%)	10 (5.9)	6 (8.2)	1 (2.2)	3 (6)	
TT-infected: n (%)	3 (1.8)	1 (1.4)	1 (2)	1 (2.2)	
HW-infected: n (%)	2 (1.2)	1 (1.4)	1 (2)	0 (0.00)	
Double (TT and AL) STH-infected: n (%)	1 (0.6)	0 (0.00)	1 (2.8)	0 (0.00)	
Triple (TT, AL and HW) ST-infected: n (%)	1 (0.6)	1 (1.4)	0 (0.00)	0 (0.00)	

Study participants were recruited from the Western, Southern and Eastern provinces of Rwanda. n, number; %, percent; STH, Soil-Transmitted Helminths; TT, *Trichuris trichiura*; AL, *Ascaris lumbricoides*; HW, Hookworm (*Ancylostoma duodenale* and/or *Necator americanus*). SD, standard deviation; Statistical significance: *: $p < 0.05$.

Unique gut microbiota profiles observed in the Western province

While all alpha diversity metrics tested showed non-significant differences, beta diversity analyses demonstrated notable differences between provinces at

genus level. Using Bray-Curtis distances, a Principal Coordinates Analysis (PCoA) was performed to test and visualize beta-diversity between provinces. PC1 and PC2 principal coordinates' explained variance is 13.51% and 10.37% respectively. A non-significant PERMDISP test result ($p > 0.05$) excluded the possibility of a sample dispersion bias, allowing us to perform a PERMANOVA test which confirmed significant differences between provinces ($p < 0.05$). The pattern observed in the central confidence ellipses and the marginal density plots indicates the degree of variability between provinces; with samples from the West clustering relatively together compared to the South and the East (Fig 2A).

By pairwise comparisons, significant differences were observed between the East and West regions using Jaccard (pseudo-F = 1.809, $q = 0.024$), Unweighted UniFrac (pseudo-F = 2.530, $q = 0.015$), and Bray-Curtis (pseudo-F = 2.431, $q = 0.0045$). However, Weighted UniFrac did not reach statistical significance (pseudo-F = 1.956, $q = 0.1$).

Similarly, the South and West regions differed significantly with Jaccard (pseudo-F = 1.524, $q = 0.0405$), Unweighted UniFrac (pseudo-F = 1.896, $q = 0.0405$), and Bray-Curtis (pseudo-F = 2.010, $q = 0.0045$), while Weighted UniFrac did not pass the statistical threshold (pseudo-F = 2.047, $q = 0.1$). In contrast, comparisons between the East and South regions did not reveal significant differences across any metric.

The Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) returned differentially abundant taxa between the West and the East at phylum, family and genus levels while only one family was differentially abundant between the West and the South. At phylum level, *Verrucomicrobiota* were enriched while *Bacillota* (formerly called '*Firmicutes*') were depleted in the East compared to the West. At family level, *Sutterellaceae* and unidentified (Bacteria_NA) were enriched while *Erysipelotrichaceae* and *Coriobacteriaceae* were depleted in the East versus the West. Out of five, three fully identified genera (*Lachnospiraceae*_NK4B4_group, *Eubacterium_xylanophilum*_group and *Tyzzerella*) were encriched in the East while only one family (*Sutterellaceae*) was enriched in the South (Fig 2B).

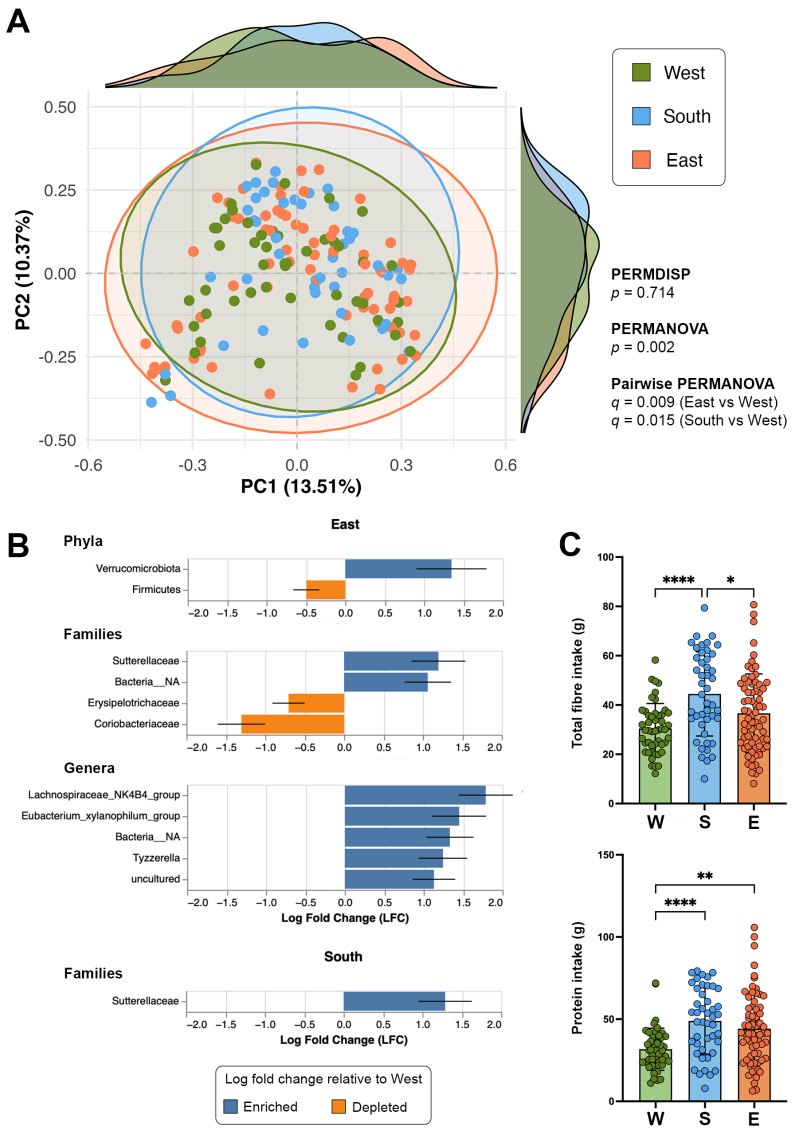


Fig 2. Panels show results of gut microbiota and nutritional intake analyses by provinces. (A) Beta diversity analyses, visualized by a PCoA, reveal statistically significant differences between the West and both the East and the South; (B) ANCOM-BC analysis results show differentially abundant taxa between the West and the East at phylum, family and genus levels while only one family was differentially abundant between the West and the South. (C) Nutritional intake analyses show decreased levels of both total fibre and proteins in the West compared to the East and South. For panels A and C, each point represents an individual sample. For panel C, box plots represent the mean with standard deviation (SD) of the samples. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

Generally poor nutritional intake observed in the Western province

Nutritional intake analyses revealed differences between the Western, Southern and Eastern provinces. The three groups were compared by the values of total fibre, total energy, proteins, carbohydrate and total lipid intake. As summarized in Table 2, overall significant differences were reported between fibre intake ($p < 0.001$) and protein intake ($p < 0.002$). Pairwise comparisons revealed that total fibre intake were significantly lower in the West compared to the South ($q < 0.0001$), while a tendency was observed compared to East ($q = 0.07$) by one-way ANOVA. Furthermore, we found that protein intake was significantly lower in the West compared to both the South ($q < 0.0001$) and the East ($q < 0.002$) (Fig 2C). For all the other nutritional intake types measured, we observed lower values in the West compared to other two provinces, but differences were not statistically significant (Table 2). Taken together, these findings paint a picture that distinguishes the Western province from both the Eastern and Southern provinces.

Table 2. Nutritional intake values by provinces

Nutritional intake type	***Provinces***			
	West (n=50)	**East (n=72)**	**South (n=46)**	***p*-value (ANOVA)**
Total fibre intake in grams (Mean± SD)	31.4±12.1	37.4±18.3	44.7±17.1	< 0.001
Total Energy intake in kilocalories (Mean±SD)	1406.3±449.9	1535.5±858.9	1667.9±537.0	0.16
Proteins intake in grams (Mean±SD)	34.1±16.8	46.9±32.5	49.1±19.9	0.006
Carbohydrates intake in grams (Mean±SD)	243.8±71.8	247.3±95.5	280,65±94.5	0.08
Lipids intake in grams (Mean±SD)	25.8±12.1	30.6±49.7	27±11.1	0.71

The table summarizes nutritional intake values for the three provinces: West, East and South. n, number of participants; SD, standard deviation; Statistical significance: $p < 0.05$.

Specific gut microbiome profiles were associated with age

Alpha diversity analysis contributed most of the differences observed in the gut microbiota profiles between age groups with a minor contrast observed in beta diversity. Additionally, ANCOM-BC returned differentially abundant taxa between specific age groups. Alpha diversity analysis showed statistically significant

differences between the preschool children group and both school children and adults groups. Observed features showed that preschool children exhibited significantly lower microbial diversity compared to school children (H = 5.571, q = 0.027) and adults (H = 8.488, q = 0.011) (Fig 3A). No significant differences were observed between school children and adults (p = 0.594, q = 0.594). Shannon diversity index analyses indicated significant differences between preschool children and adults (H = 5.379, p = 0.020, q = 0.061), though these differences did not persist across all comparisons after correction for multiple testing. Faith's phylogenetic diversity showed that preschool children differed significantly from adults (H = 9.086, q = 0.008), with differences between preschool and school children approaching significance (H = 4.428, p = 0.035, q = 0.053) (Fig 3A). Evenness analyses revealed no significant differences in microbial community evenness among age or province groups (all q > 0.1).

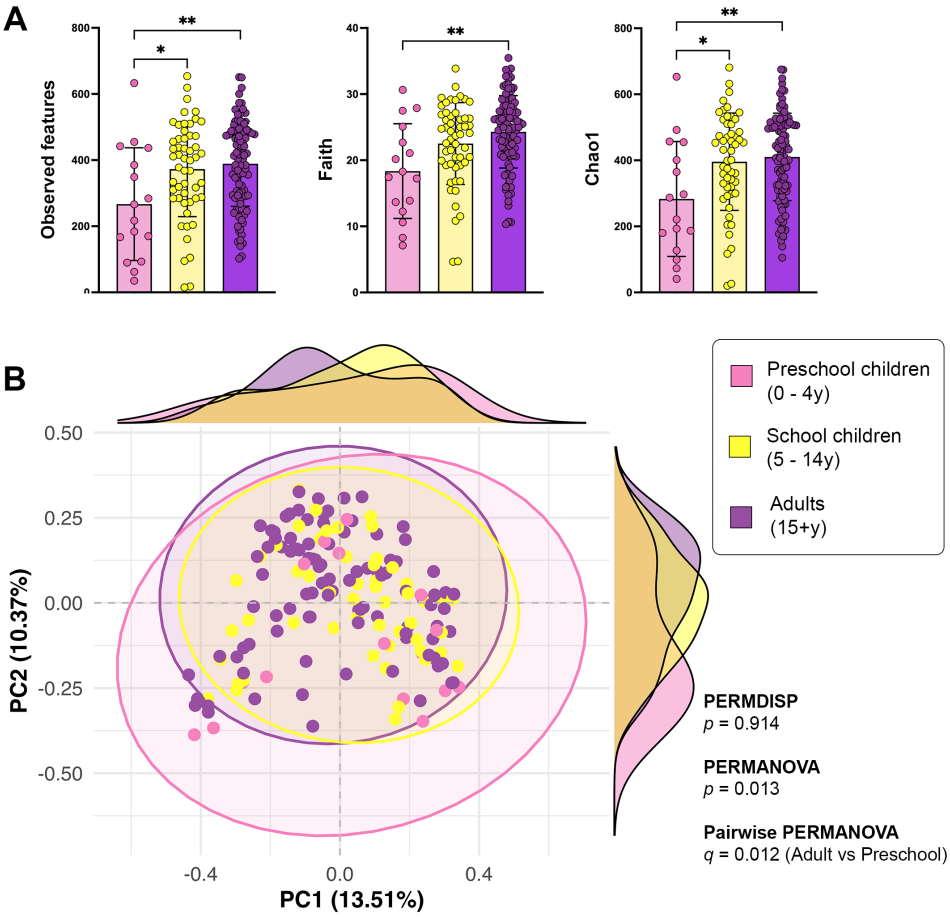


Fig 3. Alpha and beta diversity analyses by age-groups. (A) Observed features, Faith phylogenetic diversity and Chao1 metrics revealed significantly lower alpha diversity in preschool children than in adults and school children. (B) By beta diversity analyses, the adults group is statistically different from the preschool group. For both panels, each point represents an individual sample. Box plots represent the median (bar), interquartile range (box), and 95% confidence interval (whiskers). Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

Beta-diversity analysis was conducted and a PCoA plot was generated to visualize differences between the adults, school children, and preschool children age groups using Bray-Curtis distances. Results of the PERMDISP and PERMANOVA tests allowed us to perform a pairwise PERMANOVA test whose results confirmed differences in beta-diversity between preschool children and adults ($p < 0.05$) (**Fig 3B**). This suggests that the microbial community composition differs notably between these two age groups. The marginal density plots show distinct

distributions for the three groups, particularly between preschool children and adults. However, overlap in the confidence ellipses and density plots indicates minimal degree of variability within groups, particularly for school children, which appear intermediate between preschool children and adults (**Fig 3B**).

ANCOM-BC analysis of differential taxa relative to preschool children revealed 5 phyla, 15 families and 8 genera unique to adults whereas only two genera were unique to school children. While majority of the taxa were enriched, phylum *Campilobacterota*, family *Campylobacteraceae* and genus *Campylobacter* were depleted in adults relative to preschool children. The same analysis returned two genera (*Lachnospiraceae*_uncultured and *Moryella*) uniquely enriched in school children relative to preschool ones (**Fig 4**). This observation implies that major and minor differences were observed in the adults and school children, respectively, in comparison to preschool children.

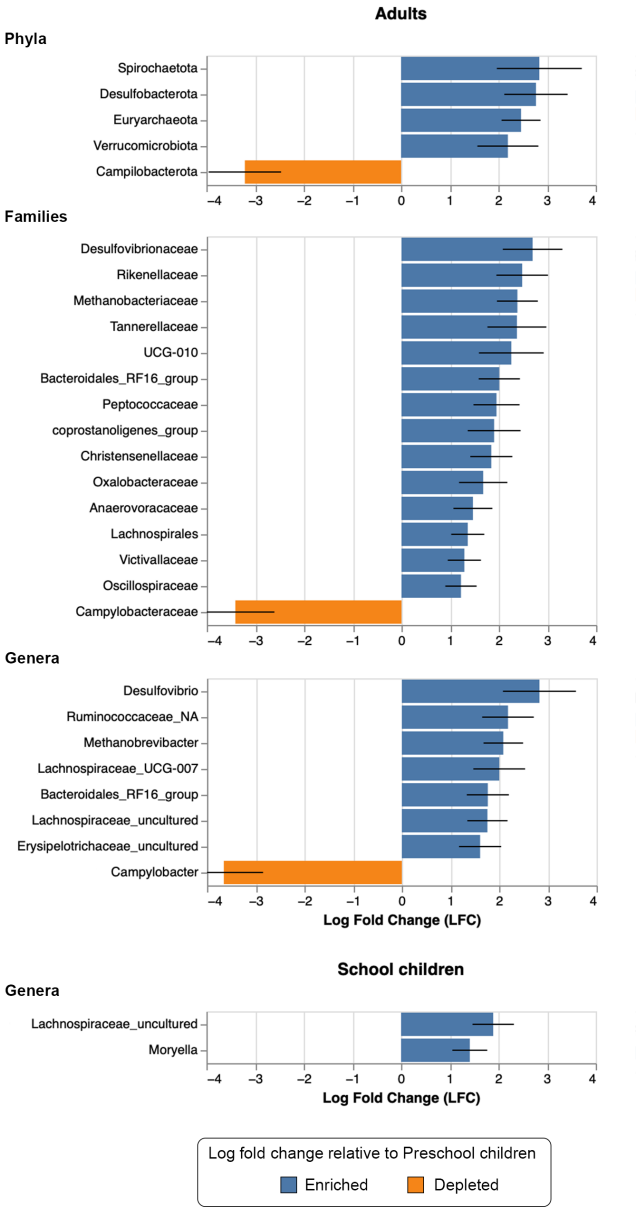


Fig 4. ANCOM-BC differential abundance analysis by age-groups. Results show differentially abundant taxa between adults and preschool children at phylum, family and genus levels while only two genera were differentially abundant between school children and preschool ones.

No association observed between infection status and gut microbiota profiles

Malaria infection alone or its coinfection with STH were not associated with specific gut microbiota signatures. Malaria was confirmed in 129 participants while 40 tested negative. Average *Plasmodium* parasitemia (parasites per microliter of blood) per province was 23,818 in the East; 17,376 in the West and 6,495 in the South (Table 1). Differences were not statistically significant between provinces ($p > 0.05$). However, observed variations may be explained by other factors. For example, all six (6) severe cases reported in this study were from the East which exhibited higher average parasitemia compared to the other two provinces. Notably, we could not compare malaria diagnosis results based on severity because, apart from mild cases whose distribution was not significantly different between provinces, asymptomatic and severe cases were detected in the East only (Table 1).

STH screening results revealed that 17 participants were infected with STH which represents 10% of the studied population (n=169). Affecting 5.91 % of all the studied population, *Ascaris Lumbricoides* (AL) was the most prevalent STH species, and it was found to affect more people in the East than in both the West and the South combined. The second most prevalent STH species was *Trichuris trichiura* (TT) followed by Hookworm (HW) - *Ancylostoma duodenale* and/or *Necator americanus*. Double (TT and AL) and triple (TT, AL and HW) STH infections were rare. Only 3 participants were infected with STH alone in our study (Table 1).

Coinfection affected 14 participants. Among provinces, coinfection prevalence was 16.7%, 8.9% and 5.5% in the Eastern, Western and Southern provinces respectively (Table 1).

Gut microbiota analysis by infection group revealed no statistically significant differences among the four compared groups: Coinfection, Uninfected, *Plasmodium*-infected and STH-infected. Alpha diversity analyses results were statistically non-significant between groups by all metrics used in this study. Beta diversity analysis using Bray Curtis distances were also non-significant ($p = 0.992$ by PERMANOVA).

A multifactorial analysis yielded significant positive correlations

Correlational analysis of multiple factors showed important relationships between differentially abundant genera (identified in the ANCOM-BC analaysis), alpha diversity metrics, nutritional intake values, age, and BMI (Fig 5). Differentially abundant genera showed remarkable positive correlation with alpha diversity metrics. Significant positive correlations were observed between six genera (*Methanobrevibacter*, *Desulfovibiro, Eubacterium_xylanophilum*_group, *Bacteroidales*_RF16_group and *Ruminococcaceae*_NA, *Lachnospiraceae*_UCG.007) and all five alpha diversity metrics (Chao1, Pielou evenness, Faith's pd, observed features and Shannon entropy) while Bacteria_NA only showed correlation with Faith's pd and *Moryella* with Chao1 and observed features (Fig 5).

Two genera,*Campylobacter* and *Tyzzerella*, did not exhibit significant correlations with any metric. These results suggest that specific bacterial genera play essential roles in determining intestinal community differences within samples. Age and BMI significantly correlated with nutritional intake types (energy, proteins, lipids, carbohydrates and fibres). Age was also positively correlated to four bacterial genera: *Lachnospiraceae*_NK4B4_group, *Desulfovibrio*, *Methanobrevibacter* and *Lachnospiraceae*_UCG.007. This highlights the evolution of the gut microbiota with age. *Plasmodium* parasitemia showed no statistically significant correlation across all other factors. This observation reinforces the lack of association reported between malaria infection and the gut microbiota composition in our study. All nutritional intake types were significantly correlated with one or multiple of the following differentially abundant genera: *Lachnospiraceae*_NK4B4_group, *Tyzzerella* and *Methanobrevibacter*. Such observation shows the association between nutritional intake and the gut microbiota composition. Interestingly, *Desulfovibrio* was the only genus that correlated significantly with only one nutritional component (total lipid intake), suggesting a potentially specific relationship between these two variables (Fig 5).

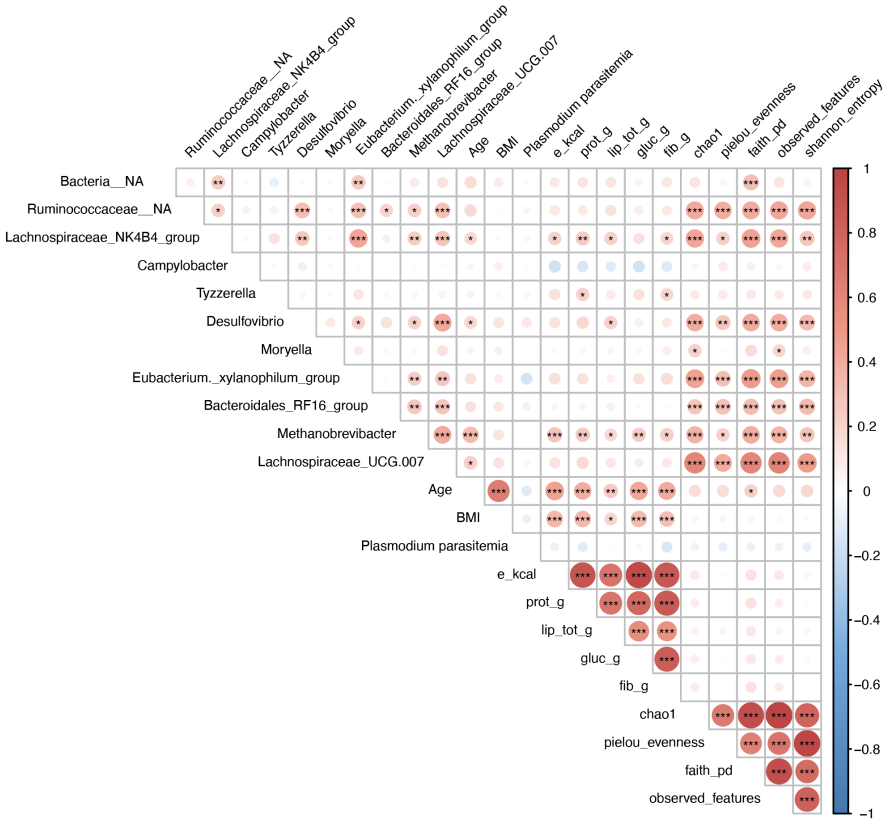


Fig 5. Spearman's correlations between key differentially abundant bacterial genera (by ANCOM-BC), nutritional intake values, alpha diversity metrics and key metadata variables (age, BMI and *Plasmodium* parasitemia). The Correlogram shows positive (red) and negative (blue) correlation with, where applicable, statistical significance. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. lip_tot_g, lipid (total) intake in grams; gluc_g, glucose/carbohydrates intake in grams; fib_g, fibre intake in grams; prot_g, protein intake in grams and e-kcal, energy intake in kilocalories.

1.5. Discussion

In this study, we demonstrate significant differences in the gut microbiota composition of people living in three different malaria-endemic provinces of Rwanda. Our multifactorial analysis allowed us to assess the contribution of different factors likely to influence the gut microbiota composition such as the host geographic location, nutritional intake, parasitic coinfection and age. In our settings, geographic location and host age showed differences in the gut microbial composition differences whereas infection status (including malaria-STH coinfection) showed non-significant results.

Indeed, we discovered a geographic variation associated with a different beta-diversity of the gut microbiota in the Western province of Rwanda. These findings are consistent with the results of a study conducted by Yooseph *et al*. (2015) in Mali which identified significant differences in the gut microbiota composition between the Malian cohort and cohorts from Malawi and other countries around the world. The relationship between geography and microbiome profiles has also been shown by studies conducted in Tanzania and Botswana as well as in South Africa [148–150]. Our study stands out by using primary data to identify gut microbial differences among regions within one, small territory with relatively less host genetic variations and taking into consideration the host age, infection/coinfection status and nutritional habits. Additionally, the Western province of Rwanda presents unique environmental aspects such as being separated from the rest of the country by the Nyungwe Forest National Park which was recently added to UNESCO's world heritage [151]. Consequently, that makes the West particularly disconnected and relatively more rural compared to the South and the East which are directly connected with Kigali – the capital city of Rwanda.

Nutrition plays a fundamental role in shaping the gut microbiota composition. Evolutionarily, it has been shown that dietary intake is directly linked to gut microbial diversity of mammals including humans [152]. In their review, Wiertsema *et al*. discussed the role of nutrition in modulating the effects of the gut bacterial communities on infections such as malaria [153]. In the present study, nutritional analyses revealed that, regardless of infection status, a low-fibre intake may explain the differentially unique gut microbial beta diversity observed in the Western Province compared with the East and the South. Indeed, total fibre intake was 20 to 30 % lower in the Western than in the two other provinces. In addition,

compared with Southerners and Easterners, participants from the Western province were characterized by significantly lower BMI levels and lower total energy intake, although not statistically different. Interestingly, the Spearman's correlation analysis showed that the BMI significantly correlated mostly with all nutritional intake types (energy, proteins, lipids, carbohydrates and fibres) followed by selected bacterial genera. Hence, compared to other studies, our findings confirm the association between low dietary fibre intake and lower gut microbiota diversity in human cohorts. However, there were other studies which reported statistically non-significant differences by alpha and beta diversity analyses [154, 155]. Therefore, the impact of fibre intake is commonly seen as a factor that may influence the diversity of gut microbiota [156], although not alone nor always [69].

In Africa, low dietary fibre intake is often reported as a characteristic of common forms of malnutrition such as Kwashiorkor [157]. Thus, in our case, lower fibre among other generally low nutritional intake types may be linked to the malnutrition and food insecurity reported in the Western province by the Rwandan national institute of statistics [158]. Consistent with our results, a study conducted in Bangladesh communicated that malnutrition impaired the maturation and diversification of the gut microbiota [159]. Moreover, lower gut bacterial diversity can be associated with a less varied diet in cultures. For example, this was observed in the nomadic, pastoral Fulani people living in rural settings compared to Jawara ethnicity who, despite dwelling in urban Nigeria, consume fibrous and fermented foods in addition to processed diets [5]. Taken together, the findings of this and previous studies argue that low-fibre nutrition can lead to a comparatively different gut microbiota composition like we observed in the Western province of Rwanda. Exposure to a large variety of environmental microbes associated with a high-fibre diet could increase potentially beneficial bacteria and enrich microbial diversity. A reduction in the gut microbial richness, because of low fibre intake, has been associated with poor health outcomes [160]. Hence, our findings call for further investigations in this regard.

The differences observed in the gut microbiota composition were not associated with infection status. Neither beta diversity nor alpha diversity analyses revealed any statistical differences associated with infection status. Consequently, differential abundance as well as correlational analyses revealed no relationship between gut bacteria and infection status (i.e., *Plasmodium* parasitemia). Similar

findings were reported by Yooseph *et al* in their study which failed to observe an association between gut microbiome composition and febrile malaria after *Plasmodium* had reached blood stage of infection. Another study conducted in Kenya reported that only the number of malaria episodes and antimalarial treatment explained differences, although minimal, in microbial profiles [88]. In the context of STH-*Plasmodium* coinfection, *P. vivax* – microbiome association was shown in a Colombian study [86] and higher levels of *Lactobacilli* were reported among the microbial communities of *P. vivax* –infected people in India [87]. These findings inform us that, in general, apart from mild infection with *Plasmodium vivax,* available studies about *Plasmodium* and STH parasites have shown limited potential to modify the human gut microbiota. These discrepancies could be explained by differences in the genetics of the host, parasite (*Plasmodium vivax*) or even vectors plus environmental factors (e.g. geographic location) which are obviously different between Africans and American or Asians. In particular, Easton *et al.* [86] speculated that geography could be a possible reason for non-differential gut microbiota composition between STH-infected and -uninfected groups in Colombia whereas *T. trichiura* infection was associated with greater microbial diversity among infected individuals in Malaysia.

We reported associations between specific gut microbiota profiles and age groups with preschool children showing significantly lower alpha diversity than school children and adult. Our ANCOM-BC differential abundance analyses showed that, relative to preschool children group, genus *Campylobacter* was depleted in the adults group while two genera (*Lachnospiraceae*_uncultured and *Moryella*) were enriched in school children group. Furthermore, relationship assessment by Spearman's correlation shed light on significant positive relationships between age and specific bacterial taxa as well as alpha diversity metrics. In accordance with the present results, a study conducted in Mali reported that age may be a stronger predictor of gut microbiota composition than *P. falciparum* infection status . Consistently, Palmer *et al*. showed that clearly noticeable changes take place during preschool (below 5) stage, arguably due to the shift from breastfeeding to consuming solid foods [161]. Furthermore, the alpha diversity has consistently been demonstrated as the right measurement of the effects of age on the gut microbiome.

Amongst the differentially abundant bacterial genera (identified in the ANCOM-BC analysis), three genera belonging to the *Lachnospiracea* family (*Tyzzerella, Lachnospiraceae NK4B4 and Eubacterium xylanophilum* group*)* were increased in the Eastern province relatively to the West. *Lachnospiraceae* as a family is an abundant component of the human digestive tract and has been involved in the production of butyrate from dietary fibres [162]. This has been shown specifically for these three genera in *in vitro* and animal models of fibre supplementation [163]. This is in line with our results, as *Tyzzerella, Lachnospiraceae NK4B4 and Eubacterium xylanophilum* group were depleted in the Western province, which had a lower fibre intake. Genus *Desulfovibrio* was enriched in adults relative to preschool children. Although one of the most prevalent genera of the human microbiota, both beneficial and detrimental associations with health and diseases were described for this genus. While associations with a low-fat diet and exercises in humans or a protective effect on non-alcoholic fatty liver disease in a murine model have been observed, its increased abundance has been associated with intestinal and extra-intestinal diseases in clinical and pre-clinical settings (e.g. cancer, metabolic diseases and Parkinson's disease) [164]. In our study *Desulfovibrio* was positively correlated with total lipid intake which is similar to an observation in a mouse model of high fat diet [165]. *Bacteroidales RF16* group, was another genus enriched in the adult group in our study. While little information is available about this genus in human cohorts, it has been linked to fibre consumption in ruminants [166]. This association was not found in our study, suggesting that further studies are needed to understand how nutrition and this genus are associated. Finally, *Campylobacter* was enriched in preschool children when compared to adults in our study. This bacterial genus is the most common cause of gastroenteritis in the world with children being particularly affected [167]. Overall, associations between the identified genera, with the exception of *Campylobacter,* and health and diseases are only at the level of association and no causality has been established yet which warrants future investigations.

This study presents limitations as to the generalizability of its findings. One is related to low STH infection prevalence and the limited number of cases of extreme malaria severity (asymptomatic and severe). Another limitation is related to the recall bias experienced when answering the food frequency questionnaire [168]. Also, we believe that longitudinal studies could generate more insights

needed to assess how age and diet influence microbiota in malaria-endemic regions. In addition, instead of the basic 16S rRNA gene, metagenomic sequencing methods would certainly add insights regarding gut microbial functions. Therefore, we recommend more studies to inform our understanding of associations between gut microbiota and malaria as well as other infections in various demographic groups and geographic settings. Finally, given the role of nutrition, it would be of interest to link the microbiota composition and potential microbial functions with specific micronutrients.

In summary, our results demonstrate that microbial diversity is significantly influenced by age, particularly in preschool children, who exhibit distinct microbial communities compared to adults. Geographic differences, though present, were primarily observed between East and West regions, while the Southern region displayed less pronounced variability. Nutrition intake analyses added another layer of contrast between geographic regions with the West showing lower intake of fibre and proteins compared to the South and East. We observed no link between infection groups and the gut microbiota composition. Finally, a multifactorial analysis revealed significant correlations between differentially abundant genera, alpha diversity metrics, age, BMI and nutritional intake.

1.6. Conclusions

In conclusion, our study contributed to the limited body of literature about the gut microbiota composition in Africa and more specifically in Rwanda. Using a multifactorial approach, we were able to demonstrate that unique microbial profiles observed in the Western province of Rwanda could be linked to a low-fibre nutrition intake. However, unlike age, infection status was not associated with significant differences in the composition of the gut microbiota of the studied population. This study's findings have the potential to pave the way for research-driven alternative innovations (i.e. microbiota-modulating diets) to control malaria in endemic settings.

1.7. Acknowledgements

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Chapter 2 - Gut microbiota composition differs across malaria severity spectrum

Full manuscript draft

Jean d'Amour Mutoni[1,2], Matthias Van Hul[1,3], Jean-Paul Coutelier[4,¶], Nadine Rujeni[2*,¶], Patrice D. Cani*[,¶,1,3,5]

[1] Louvain Drug Research Institute (LDRI), Metabolism and Nutrition Research Group (MNUT), UCLouvain, Université catholique de Louvain, Brussels, Belgium,
[2] Biomedical Laboratory Sciences Department, College of Medicine and Health Sciences, University of Rwanda, Kigali, Rwanda,
[3] Walloon Excellence in Life Sciences and BIOtechnology (WELBIO), WELBIO department, WEL Research Institute, Wavre, Belgium,
[4] De Duve Institute, UCLouvain, Université catholique de Louvain, Brussels, Belgium,
[5] Institute of Experimental and Clinical Research (IREC), UCLouvain, Université catholique de Louvain, Brussels, Belgium.

**corresponding authors:*
E-mail: n.rujeni@ur.ac.rw *(NR),* patrice.cani@uclouvain.be *(PDC)*

[¶]equally contributed to this work

2.1. Abstract

Malaria remains a major cause of morbidity and mortality in sub-Saharan Africa (sSA). Research has shown that the gut microbiota (GM) can modulate malaria severity. Thus, our cross-sectional study assessed GM-malaria severity relationships in Rwanda. We recruited 150 participants (70 females and 80 males) aged 1-57 from 4 districts (Bugesera, Gasabo, Gicumbi and Rulindo) in Rwanda. We compared GM composition between malaria groups (asymptomatic, mild and severe) and to a control group (uninfected), while controlling for confounders such as age and geographic location. Qualitative data about gender, age and geographic location were collected using a questionnaire. Malaria and STH diagnosis was completed using a microscope. The gut microbial composition was analyzed based on bacterial 16S rRNA gene amplicon sequencing. We reported that severe malaria anaemia (SMA) samples had a significantly lower alpha diversity than other malaria severity groups across all measured metrics (observed features, faith_pd as well as Shannon entropy) and compared to uninfected group by Shannon entropy alone ($p<0.05$, K-Wallis). Furthermore, beta diversity analysis using Bray Curtis distance revealed SMA samples as significantly different ($p=0.001$, PERMANOVA) and formed a unique cluster visualized by PCoA compared to other groups. In the same direction, ANCOM-BC differential analysis at genus level retuned genera *Suterella* and *Veillonella* that were depleted in asymptomatic and mild samples respectively, while both *Lactobacillus* and *Bacteroides* were among the top 6 genera depleted in both groups compared to the SMA malaria group. These results suggest that we can leverage the GM to understand and potentially control severe malaria. Moreover, this report makes a new contribution to the limited literature available on the malaria-GM relationships. Longitudinal and mechanistic studies are recommended to assess whether and how identified bacterial genera and more can shape malaria clinical presentation in humans.

Keywords: malaria severity, gut microbiota, Rwanda.

2.2. Introduction

Malaria is a parasitic disease transmitted by *Plasmodium* species through a female *Anopheles* mosquito bite in humans. Response mechanism relies on preventive methods (e.g. LLINs and IRS), early diagnosis, treatment and prophylaxis regimens such as the artemisinin-based combination therapy [33]. The WHO has recently approved the use of two vaccines (RTS,S and R21) but roll out is not systematic yet, possibly because of low production [36]. Malaria clinical manifestation includes asymptomatic, mild and severe forms, the latter being lethal in most cases [32].

Malaria affects Sub-saharan Africa region disproportionately. According to the WHO, in 2022, that region reported 233 million cases equivalent to 94% of the reported global burden, with an incidence of 223 per 1000 population at risk [9]. Recently, individual countries made commendable progress towards malaria elimination. Rwanda has been commended for reducing malaria cases by 88 percent (from 5 million cases in 2017 to 600,000 cases in 2023) thanks to key control interventions such as LLINs and IRS combined with community engagement [169]. However, towards the end of 2024, as a result of antimalarial drug and insecticide resistance, media reported alarming malaria case rise which was, in January 2025, echoed by the Minister of Health call for increased vigilance especially in 7 districts: Bugesera, Gasabo, Gisagara, Kicukiro, Nyanza, Nyamasheke, and Nyagatare [170, 171]. This surge in malaria cases was characterized by a significant rise in the toll of severe malaria, possibly resulting from impaired immunomodulation (Ndoricyimpaye et al, 2023).

The gut microbiota (GM) could be a potential tool for malaria control through immunomodulation. On one hand, evidence demonstrated GM's effects on malaria transmission and infection outcomes. For instance, around ten years ago, studies conducted in both mice and humans reported that the gut microbiota could elicit anti–α-gal antibodies with the ability to sterilize sporozoite forms of *Plasmodium* in the skin of infected subjects , potentially stopping the transmission [75, 172]. Another study showed that gnotobiotic mice exhibited higher and lower parasite burden after receiving fecal transplantation from malaria-susceptible or -resistant Malian children respectively, following an infection with *P. berghei* [173]. On the other hand, the GM has been shown to modulate the host reponses, thus influencing malaria clinical presentation: specific bacterial genera such as

Bacteroides and *Lactobacillus* were shown to modulate immune responses that are involved in determining *Plasmodium* infection outcomes [75]. In 2023, Mandal et al., argued in their publication that *Bacteroides* work in concert with other bacterial communities to predispose hosts to severe malaria infection [111].

From a different angle, the effects of *Plasmodium* infection on the gut microbiota composition was also documented. For example, infections with *P. falciparum* (in humans) and *P. berghei* (in mice) led to similar results: lower microbial diversity and higher aboundance of pathogenic bacterial such as *E. coli* [76, 78].

Although still limited in number and statistical power, published studies show evidence of possible interplay between malaria and the gut microbiota. Importantly, to our knowledge no study has attempted to characterize the gut microbiota composition across the full malaria severity spectrum (asymptomatic, mild and severe forms of the disease) in humans. Thus, our current study aims to fill that knowledge gap.

2.3. Materials and Methods

Ethics statement

This study project was reviewed and approved (reference No 257/CMHS IRB/2023 issued on the 2nd of February 2023) by the Institutional Review Board (IRB) of the College of Medicine and Health Sciences (CMHS) at the University of Rwanda (UR).

For both participation and publication of all clinical data, and other data included in the manuscript, written informed consent was obtained from study participants aged 18 and above, and from parents or relatives or guardians of younger participants.

Study design

Our cross-sectional study was conducted in the Republic of Rwanda. The covered territory included 4 districts (Bugesera, Gasabo, Gicumbi and Rulindo) belonging to the central plateau of Rwanda. Samples were collected from 150 participants between May and December 2023 (Fig 1A).

Study participant recruitment took place at either a health facility (cases) or within cases' household (controls). We used a convenience sampling strategy given our time and budget constraints and in order to prioritise extreme malaria severity (asympromatic and SMA) case availability. Cases of malaria-infected participants were found at either district hospitals or health centers. Control participants, who had to be from the same households as cases, were met in their homes on the same day. Study cases were included if they had not been given antimalarials and/or antibiotics and/or anthelminthics in the past two weeks prior to sample collection. To be included, study controls had to be malaria-negative, from the same households as corresponding cases - preferably with similar age, gender and nutritional habits. Upon receiving their signed consent forms, a demographic questionnaire was filled for each participant. On the same day, for each participant, a confirmatory (for cases met in health facilities) or screening (for controls met in households) rapid diagnostic test (RDT) was performed, blood smears were prepared for final malaria diagnosis. Next, stool samples were collected for helminth screening and gut bacterial DNA extraction for 16S rRNA gene amplicon sequencing. For malaria diagnosis, blood smear microscopy was considered gold standard, thus its results were considered over preliminary RDT

results. That resulted in four comparison groups based on malaria severity: asymptomatic, mild and SMA cases of malaria plus uninfected controls. Age groups (0-4 aged preschool children, 5-14 aged school children and 15+ aged adults) and geographic location groups based on provinces (West, South and East) were assigned based on questionnaire-derived qualitative data (Fig 1). Malaria patients identified in health facilities were treated by these same facilities while for other malaria-positive people identified in households (not included in controls), our team provided treatment according to the guidelines of the Ministry of Health effective in the Republic of Rwanda at the time of diagnosis. All samples were collected before any treatment was administered to patients.

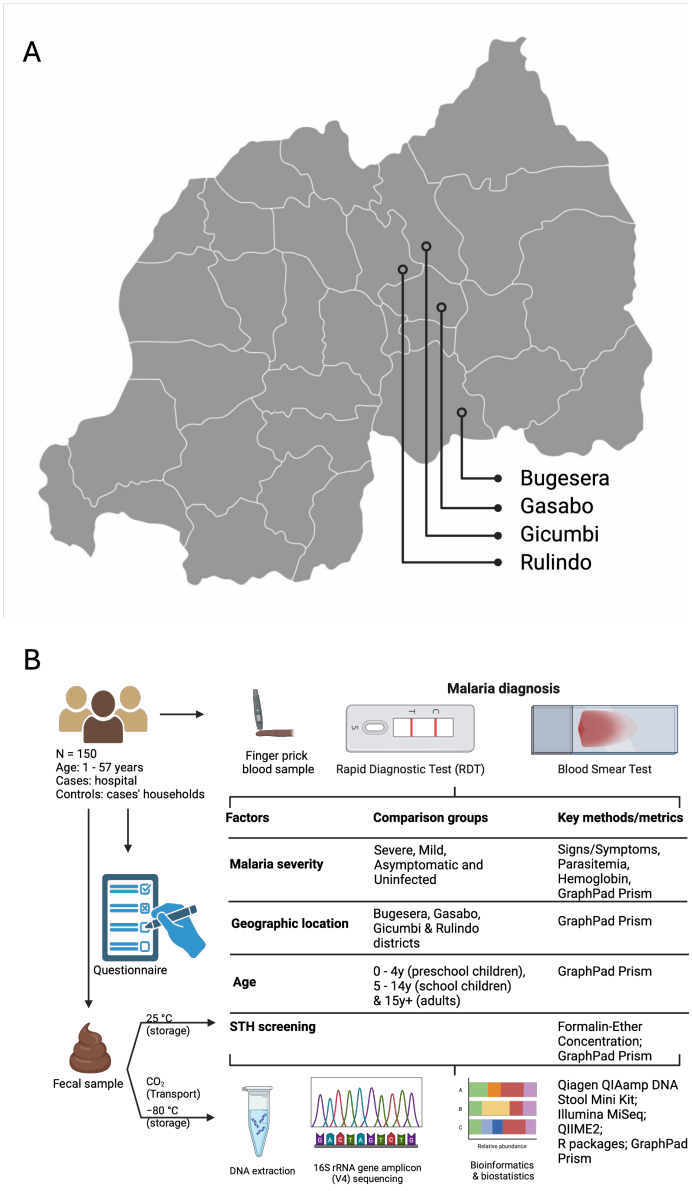

Fig 1. Study area and design. (A) Map of Rwanda showing four districts of our study area. (B) Study design summarizing participant recruitment, sample and data collection, malaria diagnosis, STH screening, key methods of testing, bioinformatics and biostatistical analyses for group comparisons. N, number of participants; STH, soil-transmitted helminths. Created with BioRender.com.

Malaria diagnosis

Trained laboratory technicians used sterile, single-use lancets to draw finger-prick blood used to perform a malaria RDT for on-site immediate confirmation or screening. Additionally, thick and thin blood films on glass slides - for the identification of *Plasmodium* species and the determination of parasite density (parasitemia) - were tested at the National Reference Laboratory according to WHO guidelines [174]. Prepared blood smears were stained with Giemsa followed by air-drying as previously described [125]. A compound microscope was used to determine *Plasmodium* species and parasite density on stained smears fully covered with immersion oil type A. Following complete positive diagnosis combined with questionnaire generated data, participants were classified according to clinical manifestations as: having 'severe' malaria (cerebral malaria, respiratory distress, severe malarial anemia, malaria with complicated seizures, and prostration); having 'mild' malaria (fever and flu-like illness, including one or all of the following symptoms: shaking chills, headache, muscle aches, tiredness, nausea, vomiting, and diarrhea may also occur) and having 'asymptomatic' malaria (no symptoms). Control participants with laboratory negative diagnosis and with no symptom were classified as 'uninfected' with malaria.

STH screening

After technicians had collected malaria blood samples, adult participants or guardians were given clear instructions to collect stool samples on single-use aluminum plates. Upon reception from the participant/guardian, stool samples were divided into two parts. Part one was transferred into a formalin-ether container for helminth screening. Part two, divided into three aliquots (using 5mL tube per each), was kept under anaerobic conditions by GasPak EZ Anaerobe Pouch System with Indicator, 20 (BD Diagnostic Systems, USA) for a maximum of 24 hours during sample transport. Both parts were transported the same day from field to the laboratory where part one was kept at room temperature until STH screening and part two frozen (−80 °C) until bacterial DNA extraction. STH screening results were recorded as negative or positive plus causative species (i.e., *Trichuris trichiura*, *Ascaris lumbricoides*, and hookworm). All STH-positive participants received screening results and antihelmintic treatment through community health worker channels in maximum two weeks after sample collection. Treatment followed the guidelines of the Ministry of Health effective in the Republic of Rwanda at the time of diagnosis.

Gut microbiota composition analysis

Bacterial DNA extracted from fecal samples was sequenced for gut microbiota composition analysis. Samples were kept frozen at −80°C until DNA extraction. The extraction of metagenomic DNA was carried out using QIAamp Fast DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions with the addition of a homogenization step by bead-beating. DNA purity (A260/A280) and concentration were determined using a NanoDrop2000 (Thermo Fisher Scientific, USA). Samples were diluted in TE buffer to a concentration of 20ng/µl and sent to Azenta US, Inc (Corporate Boulevard South Plainfield, NJ, USA) for sequencing. The V3 and V4 regions of bacterial 16S rRNA gene was amplified using 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' forward and reverse primers respectively [101]. Purified amplicons were sequenced using the Illumina MiSeq platform according to the manufacturer's guidelines, followed by data demultiplexing.

The analysis of demultiplexed FASTQ files was performed using QIIME2-amplicon-2024.10 [100] on an Apple M2 Max system. Primer sequences were trimmed, and the quality of the sequences were assessed through visualization of interactive quality plots. Denoising and merging of paired-end reads were conducted using the DADA2 algorithm, generating amplicon sequence variants (ASVs) [101]. Truncation and trimming parameters were optimized based on the quality plots to maintain the highest sequence quality (quality score of 37 or more for the 25th, 50th, 75th, and 91st percentiles). To enhance the reliability of downstream analyses, low-abundance features (<5 counts across samples) and poor-quality samples (<40000 sequence reads) were filtered out. Features were clustered de novo based on sequence similarity using the QIIME2 vsearch cluster plugin with a 99% identity threshold. This clustering step reduced computational complexity and enhanced the accuracy of subsequent chimera detection. Chimeric sequences were identified and removed using the UCHIME algorithm within the vsearch plugin.

Taxonomic classification was performed using a pre-trained classifier based on the SILVA v138 reference database. Finally, we filtered out chimeric sequences and unwanted sequences (Eukaryotes, choloroplasts, mitochondria).

For phylogenetic analyses, a phylogenetic tree was constructed to enable diversity assessments. This step involved sequence alignment using MAFFT and tree building with FastTree. Alpha diversity metrics (Observed features, Shannon entropy, Faith's phylogenetic diversity and Pielou_evenness) were calculated using rarefied feature tables (threshold at 47000 reads). Beta diversity metrics (Bray-Curtis, Weighted Unifrac, Unweighted Unifrac and Jaccard) were also computed to examine community composition differences between samples.

To identify differentially abundant taxa across experimental conditions, Analysis of Composition of Microbiome with Bias Correction (ANCOM-BC) was employed. This analysis was conducted at genus level. Finally, the processed data were exported for further statistical analysis and qza files generated in QIIME2 were used for visualization in GraphPad Prism v10 and R in order to generate alpha and beta diversity figures respectively. With the package qiime2R, we imported QIIME2-generated distances into R to produce principal coordinate analysis (PCoA) beta diversity plots using ggplot2, glue, tidyverse, ggrepel, dplyr and ggExtra packages [34, 37–43]. A correlogram representing the correlation and statistical significance levels was generated using GraphPad Prism v10.

Statistical analysis

Statistical significance of group comparisons was analyzed by QIIME2 version 2024.10, GraphPad Prism v10 software and R version 4.4.1. In GraphPad, we analyzed alpha diversity to determine statistical significance by ANOVA (analysis of variance) for normally- or Kruskal Wallis, if positive, by pairwise comparisons tests for abnormally-distributed data (by Shapiro-Wilk test) respectively. Follow-up multiple pairwise comparisons were determined by Dunn's test (alpha diversity). QIIME2's (1) PERDISP test was performed to determine sample homogeneity (non-significant result), before running 999 permutations for both (2) PERMANOVA and (3) Pairwise PERMANOVA for beta diversity statistical significance testing. Spearman's correlations between key metadata variables (e.g. Plasmodium parasitemia) and alpha-diversity metrics were computed in GraphPad Prism v10 software. Corresponsing statistical details (r-score, p-value and adjusted p-values) were reported. Specific statistical tests and significance cutoffs are described in figure legends. Group comparisons were considered significantly different at $p < 0.05$.

2.4. Results

Age and hemoglobin were associated with differences in malaria severity groups

Our cross-sectional study analysed 150 samples grouped into three malaria severity plus one control groups : severe (SMA), mild, asymptomatic and uninfected (Table 1). It was conducted in four districts (Bugesera, Gasabo, Gicumbi and Rulindo) within the central plateau of Rwanda (Fig 1A). Age ranged between 1-57 for the entire study population while the severe group's differed significantly from the mild (q=0.0004), the asymptomatic (q<0.0001) and the uninfected (q=0.02). *Plasmodium* parasitemia distribution among malaria severity groups showed no statistically significant differences (p=0.923) (Table 1).

Table 1. Characteristics of the study population

	Malaria Severity Groups				
Characteristics	Severe	Mild	Asymptomatic	Uninfected	P-value (test)
Participants (n=150)	26	50	27	47	
Female (n=70)	11	23	13	23	
Age: median [range]	4 [1-44]	11 [1-44]	16 [7-56]	13 [2-57]	<0.0001 (Kruskal-Wallis)
Plasmodium parasitemia (mean+/-SD)	3450+/-9959	3173+/-8358	633+/-1615	0	
Hemoglobin in mg/dl (mean+/-SD)	11.02+/-2.39	12.13+/-2	11.71+/-1.45	12.93+/-1.24	<0.0012 (KruskalWallis)
STH infection (n=14)	1	4	4	5	

Study participants were grouped into three malaria severity plus one control groups: severe, mid, asymptomatic and uninfected. n, number; %, percent; STH, Soil-Transmitted Helminths; SD, standard deviation; NS, non-significant; Statistical significance: $p < 0.05$.

Hemoglobin values were significant different across malaria severity groups (p=0.001) solely driven by the severe versus asymptomatic pairwise comparison (q=0.0007). STH screening was positive in 14 out of 150 participants. Overall malaria-STH coinfection was reported in 5 participants. Hookworm was the most common STH infection (8 out of 14). The second most prevalent STH infection was with roundworms (5 out of 14). Only 1 double STH infection (with hookworm and roundworm) was reported in the asymptomatic group.

Severe malaria patients exhibited significantly lower alpha diversity

Alpha diversity analyses revealed significant differences within malaria severity groups. Shannon entropy painted a picture where severe group samples

were different from all the other comparison groups : mild (q=0.001), asymptomatic (q=0.027) and uninfected (q=0.033). From the observed features angle, severe malaria cases were significantly different from mild (q= 0.0003) and asymptomatic (q=0.0006) malaria samples while other pairwise comparisons showed non-significant differences. Faith_pd showed differences between severe group samples and samples belonging to mild (q<0.0001) and asymptomatic groups while the uninfected group samples were also significantly different from mild (q<0.0001) and asymptomatic (q=0.019) groups' samples. Pielou_evenness metrics revealed that severe malaria samples were significantly different from mild (q=0.015) and uninfected (q=0.042) ones (Fig 2A).

Since alpha diversity differences within gut bacterial communities can be driven by other factors such as age and geography, we run analyses to check for possible biases.

For age, we considered preschool sample subset which dominates the SMA samples and drove all the differences in our previous study (chapter 1). By observed features, preschool children's alpha diversity was similar across malaria severity groups. Similar results were observed by other metrics, except Faith_pd which revealed a significant difference between severe and mild groups (q=0.006) (Fig 2B).

By geographic location, analyses by any alpha diversity metrics revealed no significant differences between districts (Fig 2C).

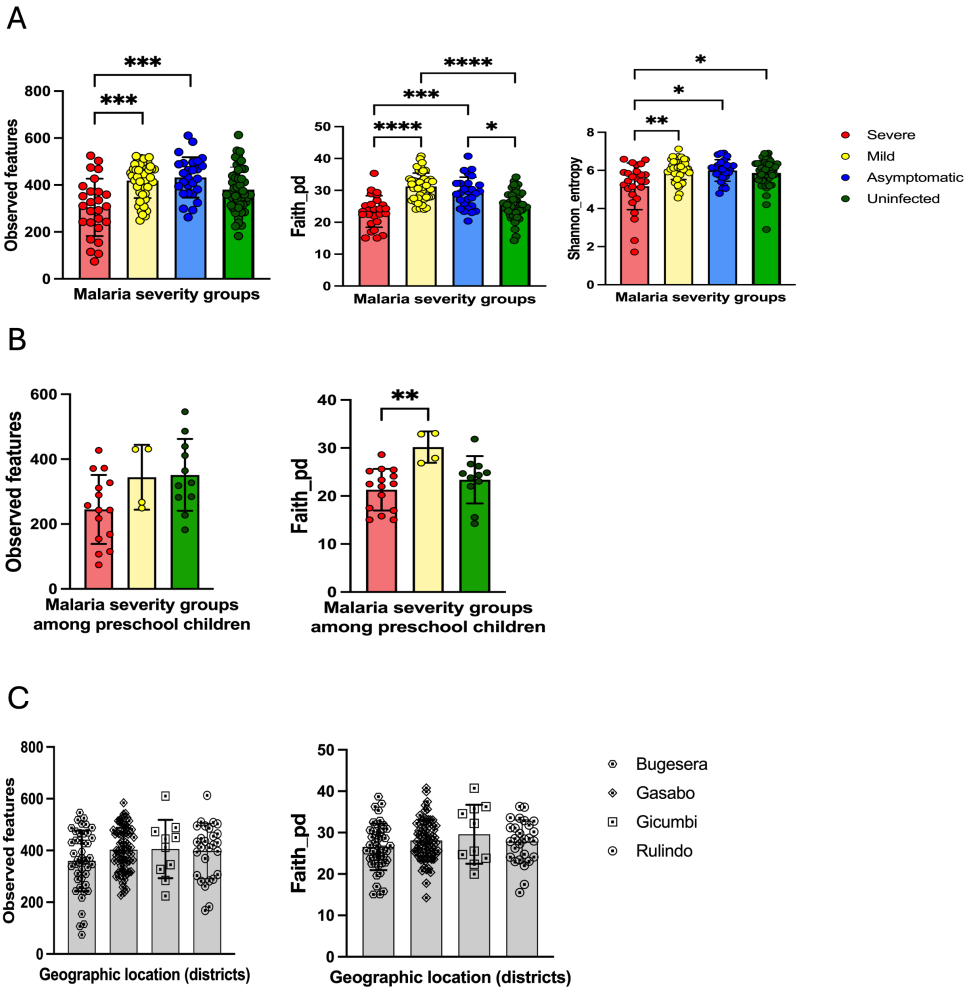

Fig 2. Alpha diversity analyses showed differences in gut microbiota composition between malaria severity groups. (A) By Observed features, faith phylogenetic diversity and shannon metrics, severe malaria samples exhibit significantly lower alpha diversity than other groups. (B) Age was shown to have minimal effects on the observed significant differences among malaria severity groups. (C) Geographic location was shown to have no influence on the reported alpha diversity differences among malaria severity groups. For all panels, each special symbol (e.g. point) represents an individual sample while box plots represent the mean with standard deviation (SD) of the samples. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

SMA group was significantly different from other malaria severity groups

Beta diversity analysis was carried out using Bray-Curtis distances and visualized by a PCoA. Statistical analysis revealed significant differences between SMA and all the other comparison groups: asymptomatic (q=003), mild (q=003) and uninfected (q=003). Uninfected samples formed another unique community

compared to asymptomatic (q=0.036) and mild (q=0.003). Mild-asymptomatic pairwise comparison revealed a statistically non-significant difference (Fig 3).

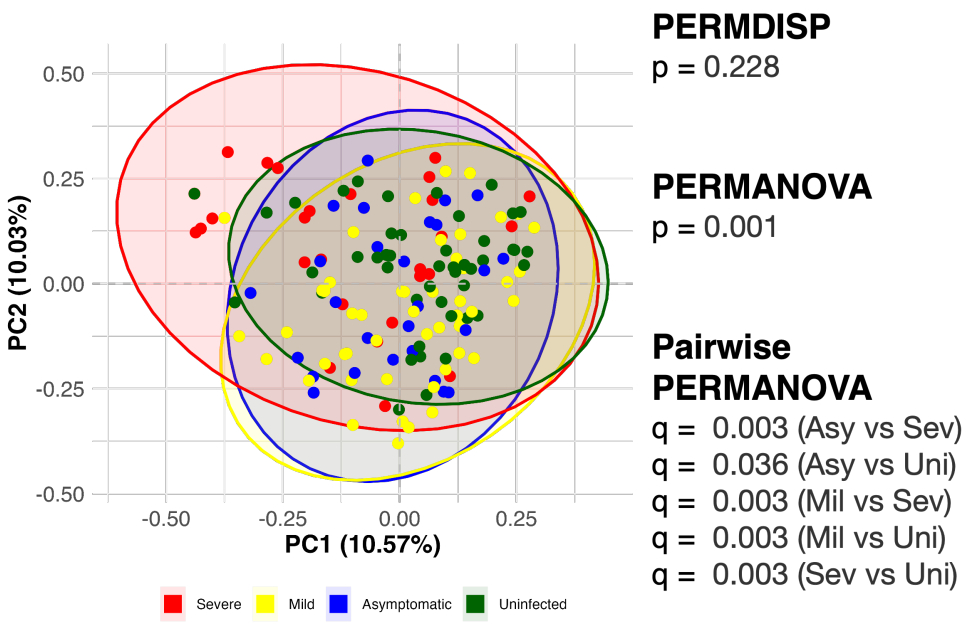

Fig 3. Beta diversity analysis of the gut microbiota composition revealed differences between malaria severity groups as visualized by a PCoA. Each point represents an individual sample. PCoA, Principal Coordinates Analysis. Statistical significance: $p < 0.05$.

Overall, beta diversity analyses showed us significant differences between gut microbiota profiles by malaria severity groups. Notaby, SMA samples were shown distinct from the rest. Another interesting finding is the lack of significant gut bacterial compostion difference between mild and asymptomatic samples, suggesting that the presence or absence of infection may not affect GM composition.

The number of depleted genera decreased as malaria severity increased

As depicted on the panels of Fig 4, Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) at genus level returned differentially abundant taxa (genera) between comparison groups (asymptomatic, mild and uninfected) relative to severe malaria.

Firstly, when the asymptomatic samples were compared to severe ones, ANCOM-BC analysis returned 27 differential genera. On the one hand, just one genus (uncultured) was enriched in the asymptomatic group. On the other hand, 26 genera were depleted in the latter group. Among the 26 genera, 22 were fully identified to genus level: *Sutterella*, *Lactobacillus*, *Bacteroides*, *Ruminococcus_gnavus_group*, *Flavonifractor*, *Veillonella*, *Parasutterella, Erysipelatoclostridium, Family_XIII_UCG-001, Corynebacterium, Eggerthella, dgA-11_gut_group, Incertae_Sedis, UBA1819, Paludicola, Caldicoprobacter, F0332, Serratia, V9D2013_group, Saccharimonadales, Negativicoccus and Hydrogenoanaerobacterium.*

Secondly, compared to severe group, mild group samples showed 11 depleted genera whereas no taxa was enriched in either group. Of the 11 genera, 8 were fully identified to genus level: *Lactobacillus, Veillonella, Bacteroides, Erysipelatoclostridium, Atopobium, Aggregatibacter, Saccharimonadales, F0332 and Negativicoccus.*

Finally, compared to severe, uninfected group analysis by ANCOM-BC returned 1 genus (unidentified) depleted while 4 out of 7 enriched genera were fully identified : *Moryella*, *Marvinbryantia*, *Slackia and Clostridia_UCG-014.*

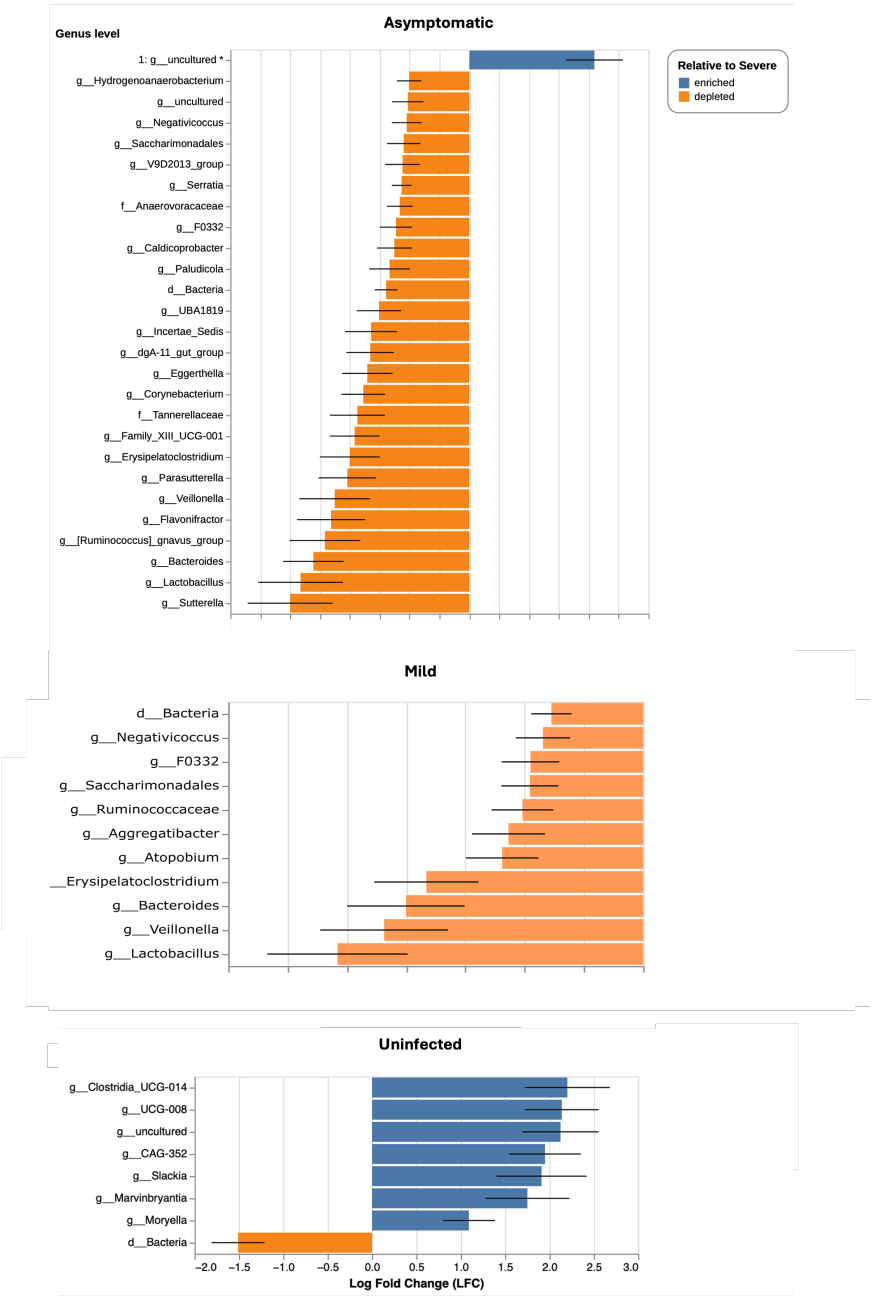

Fig 4. Differential abundance analysis (using ANCOM-BC) of the gut microbiota composition by malaria severity groups at genus level. Differentially abundant taxa were shown between severe and other malaria severity groups. ANCOM-BC, Analysis of Compositions of Microbiomes with Bias Correction.

Taken together, we observed a pattern in terms of malaria severity and differentially abundant genera. The ANCOM-BC analysis results show that, relative to severe group, both asymptomatic and mild groups had a number of shared differentially abundant genera which depleted : *Lactobacillus, Veillonella, Bacteroides, Erysipelatoclostridium, Saccharimonadales and Negativicoccus.* Thus, we speculate that as *Plasmodium* infection severity increased, the number of depleted differential genera decreased. Furthermore, compared to severe malaria group, the uninfected group showed more enriched genera with none depleted.

Plasmodium parasitemia did not correlate with alpha diversity metrics

As demonstrated on Fig 5, we carried out Spearman's correlations to check for associations between key metadata variables and alpha diversity metrics. We used two sample subsets : all positive malaria (pos_m) or only severe malaria (sev_m).

From the all positive malaria (pos_m) angle, we observed overall positive but weak correlations between key metadata variables and alpha diversity metrics. Age correlated positively with all metrics : observed (r=0.38, p=00007), shannon (r=0.37, p=0001), evenness (r=0.32, p=0.0009) and faith (r=0.25, p=0.010). Hemoglobin correlated positively with all metrics too: shannon(r=0.40, p=0.0003), evenness (r=0.40, p=0.0003), observed (r=0.30, p=0.0088) and faith (r=0.24, p=0.036). However, correlations between *Plasmodium* parasitemia and alpha diversity metrics were negative and not statistically significant.

In the severe malaria group, there were positive but weak correlations between key metadata variables and alpha diversity metrics. Age correlated positively with all the alpha diversity metrics in a more or less same way as described in the case of all positive malaria samples above. Hemoglobin showed significant correlations with all metrics but faith_pd: shannon(r=0.47, p=0.015), evenness (r=0.46, p=0.17) and observed (r=0.41, p=0.037). Parasitemia did not correlate with any metrics.

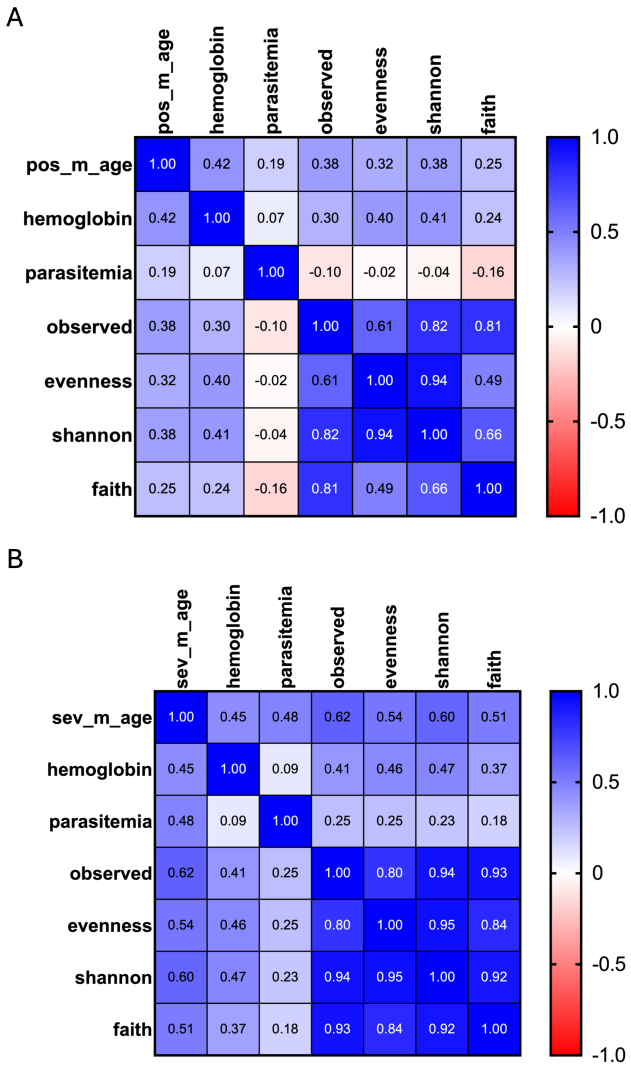

Fig 5. Spearman's correlations between alpha diversity metrics and key metadata variables (age, hemoglobin and *Plasmodium* parasitemia. (A) All positive malaria samples' alpha diversity metrics showed positive correlations with both age and hemoglobin. (B) Severe malaria samples showed similar results, except the absence of correlation between faith_pd metric and hemoglobin. Statistical significance of key correlations is described in the text.

These results may tell us that unlike parasitemia, age and hemoglobin are significantly associated with alpha diversity metrics in our study. Thus, they may be used to predict GM composition and vice versa.

2.5. Discussion

This observational study aimed to analyse gut microbiota compositional differences across malaria severity spectrum in humans. The findings indicate significant, interesting differences between severe and asymptomatic malaria patients based on alpha and beta diversity as well as ANCOM-BC differential abundance analyses. Using Spearman's correlation analysis, significant associations were shown between alpha diversity metrics and patient's age and hemoglobin levels, but no association was observed with *Plasmodium* parasitemia. Limitations such as low STH prevalence forced to exclude malaria-STH coinfection aspect in the analysis due to very low numbers in some compariason groups such as severe malaria which had only 1 STH coinfected participant.

Alpha diversity analysis results indicated that severe malaria samples exhibited significantly lower alpha diversity compared to other groups (mild, asymptomatic and uninfected). Considering both species richness and evenness as measured by Shannon entropy, severe malaria samples showed significantly lower alpha diversity than all the other groups. According to observed features which measure species richness, severe malaria cases were significantly different from mild and asymptomatic. Faith_pd, employed to assess phylogenetic differences, showed differences between severe malaria patients with mild and asymptomatic malaria patients. The same metric showed that uninfected participants were significantly different from mild and asymptomatic patients. The assessment of evenness of species distribution by Pielou_evenness index revealed that severe malaria patients were significantly different from mild and uninfected individuals. These findings are different from the ones reported by a study conducted on children in Uganda. Authors reported significantly lower diversity within *Plasmodium falciparum*-positive-without-symptoms (asymptomatic) samples compared to *P. falciparum*-negative (uninfected) and severe malarial anemia (SMA) samples by both Shannon index and Pielou_evenness [76]. In the same study, observed features metric did not reveal any significant differences while Faith_pd was not reported. Combined, our two studies may suggest that, the gut microbial alpha diversity is significantly lower in severe or asymptomatic groups compared to mild and uninfected groups. Conversely, alpha diversity analysis based on observed features and Shannon index showed non-significant differences in gut microbiota composition between before and after malaria episodes/artemether-lumefantrine

(AL) treament among Kenyan infants [88]. Together, this and our study findings may suggest that the presence or absence of *Plasmodium* infection as well as anti-malaria treatment do not change the gut bacterial diversity while malaria severity does. However, in mice*, P. yoelii* nigeriensis infection was associated with significantly lower alpha diversity which progressively recovered to baseline level [175]. In contrast, murine infection with *P. yoelii* 17XNL was linked to increased [176] or stable [177] alpha diversity in hyperparasitemia-susceptible mice. Taken together, these findings indicate the potential to distingush between malaria severity groups by alpha diversity analysis of the gut microbiota composition. However, there is a huge contradiction between findings.

Beta diversity analysis, carried out using Bray-Curtis distances and visualized by a PCoA, revealed that severe and asymptomatic groups formed significantly unique clusters while no significant difference was observed between mild and asymptomatic groups. The Uganda-conducted study reported similar significant differences between *Plasmodium* status groups by Bray Curtis distances: Pf Neg (uninfected) compared to Pf Pos (asymptomatic), Pf Neg (uninfected) compared to SMA (severe) and Pf Pos (asymptomatic) compared to SMA (severe) – but the cohort did not include mild malaria cases [76]. In contrast, Mandal *et al* (2020) showed that beta diversity (Bray Curtis distance) analysis revealed no significant difference between before and after malaria infection among Kenyan infants [88]. Similar non-significant findings were reported in a murine study [178]. Taken together, these findings suggest that gut microbiota composition differences between communities may influence malaria severity and could be shaped by the latter more than mere absence or presence of infection in humans.

ANCOM-BC differential abundance analysis results showed that, relative to severe malaria group, top 6 genera were depleted in both asymptomatic and mild malaria groups: *Lactobacillus, Veillonella, Bacteroides, Erysipelatoclostridium, Saccharimonadales and Negativicoccus.* Such observation is supported by the findings reported by Wilairatana *et al* (1997) arguing that *P. falciparum*-induced gut bacterial changes lead to transient gastrointestinal permeability which resolves during convalescence [179]. Indeed, such shift in gut bacterial communities may be a result of malaria-induced inflammation. Although we did not include immunological analysis in this investigation, previous publications support that argument. For example, in mice, infection with *P. berghei* ANKA initiated, among

other symptoms: the infiltration of inflammaroty macrophages and cytokines followed by intestinal permeability [78]. Contradicting such findings, a study conducted on Kenyan infants report no difference in gut microbiota composition after malaria episodes [88]. These observations may suggest that gut bacterial differential abundance testing may help us to distinguish between asymptomatic or mild and severe malaria clinical presentations. Moreover, previous research data associated key bactaerial genera with health and disease. Higher abundance of genus *Lactobacillus* has previously been linked to both *P. vivax* and *P. falciparum* infection [87] as well as with resistance to malaria severity measured by parasitemia in mice [3]. Genus *Bacteroides* has been reported to cause, in concert with other bacteria, susceptibility to SMA in Ugandan infants [76]. Such finding is not maintained in other studies which rather associated higher abundance of genus *Bacteroides* with *P. vivax* mild malaria among Columbian school children [86] and with both *P. vivax* and *P. falciparum* in India [87]. Combined, these findings point in the direction of the possibility of employing gut microbiota differential abundance analysis to distinguish between malaria severity groups by specifc bacterial biomarkers. However, there is still a long way to go given limited data and apparent contradiction.

Spearman's correlation analysis revealed that alpha diversity metrics correlated positively with age as well as hemoglobin while the same metrics did not correlated with *Plasmodium* parasitemia in samples with malaria infection. Although reported correlations were generally weak, these data suggest that increasing age and hemoglobin concentration reflect higher alpha diversity of gut bacteria while parasitemia does not. In contrast, bacterial taxa were shown to predict *Plasmodium* parasitaemia among *P. vivax* infected school children in Colombia [86]. The discrepancy may be a result of difference in *Plasmodium* species or methodologies used. In any case, it has been argued that, compared to hemoglobin concentration, the degree of parasitemia is typically a poor indicator of malaria disease severity in endemic settings [27].

In summary, reported results of diversity (both alpha and beta) as well as differential abundance (ANCOM-BC) analyses showed one most interesting observation: gut microbiota profiles were significantly different between asymptomatic and severe malaria. Similarly, Mandal et al. [76] reported significantly different stool bacteria populations between Ugandan children who

had asymptomatic and severe *P. falciparum* infection. Such findings may suggest that malaria severity, unlike the presence or absence of infection, may shape gut bacterial changes in humans and vice versa. Additionally, that is in agreement with the lack of link between *Plasmodium* infection and gut microbiota composition reported in our first study.

Importantly, like in other microbiome studies, current findings are subject to confounding factors such as age, geographic location and more. Age can influence gut bacterial composition analysis results as reported in our first study and previous publications [180]. In our study, we checked for age confounding effect by analyzing malaria severity association with gut microbiota profiles in one sample subset/age-group: preschool. We noticed that only Faith-pd metrics showed significant differences between severe and mild samples , suggesting that, although age could influence the observed differences, the difference in phylogenetic diversity was independent of host age. Consistently, in a study conducted on exclusively preschool children in Uganda, significant gut bacterial differences were associated with malaria severity [76]. Therefore, we can argue that although age might have minimal effects, observed alpha diversity differences are largely associated with malaria severity. Geographic location is another important factor to consider for bias checking as shown by our previous study (chapter 1) and Yatsunenko et al., [61]. In the current cohort, we found no significant gut bacterial differences based on geographic location (Bugesera, Gasabo, Gicumbi and Rulindo districts).

Limitations are inevitable and worth discussing in order to inform future directions in this nascent research field. Firstly, as we did with age and geographic location, we attempted to control for STH coinfection bias but we could not reach enough prevalence: 14/150 (9%) with 5 found among uninfected subjects. That could be improved by replacing formalin-ether based microscopy - the used method with a high-sensitivity method such as Polymerase Chain Reaction (PCR). Secondly, nutriton and immunology data could not be collected in this study to explore more associations for logistic reasons. Thirdly, our cross-sectional study showed important associations between malaria severity and stool bacterial composition, but by its nature, we cannot establish causality. Therefore, future investigations including longitudinal studies are recommended to fill that gap. Similarly, ANCOM-BC analysis revealed differentially abundant taxa between

severe malaria and other severity groups. However, we could not determine which taxa is linked to which function or specific outcome. Therefore, experimental and mechanistic studies should be conducted to unravel specific mechanisms behind malaria severity – gut microbiota interactions. Finally, the used bacterial 16S rRNA gene sequencing method in limited to exploring amplicons which calls for advanced methods such as shotgun sequencing to explore functional aspects.

Concluding, the current study aimed to assess gut microbiota profiles across malaria severity spectrum. For the first time, to our knowledge, we showed significantly different gut bacterial signatures by full malaria severity spectrum (severe, mild and asymptomatic) in Rwanda. Severe malaria patients were shown to exhibit significantly lower alpha diversity and a distinct beta diversity. Furthermore, ANCOM-BC analysis returned the depletion of genera *Bacteroides* and *Lactobacillus* among others in both asymptomatic and mild *malaria groups* relative to SMA group. Surprisingly, we noticed the least number of differentially abundant genera between severe malaria and uninfected individuals. Collectivelly, findings of this investigation highlight the potential of the gut microbiota as a novel target for interventions to defeat severe malaria. To reach that goal, more investigations – both oberservational and experimental – are recommended to underdand malaria-gut microbiota relationships and interactions.

2.6. Acknowledgements

Doreen Muvunyi and Busasa Rosette for the help with blood and stool sample collection. Lambert Ibyishaka for the help with the qualitative data collection. Doreen Muvunyi and Violette Musaniwabo for the help with stool sample analysis. Anathalie Umuhoza and Obed Tuyishime for the help with DNA extraction.

DISCUSSION AND PERSPECTIVES

Malaria remains a major public health threat in endemic settings including Rwanda. In its malaria strategic plan 2020-2024, Rwanda's Ministry of Health encourages research for evidence-based interventions and strategies to defeat the disease [22]. Therefore, our thesis project aimed to evaluate factors shaping the gut microbiota compostion (first study) ahead of assessing malaria severity-gut microbiota composition relationships (second study) in malaria-endemic regions of Rwanda. In our first study, while no link was reported regarding infection status, geographic location and age were significantly associated with gut microbiota composition differences. Our second study's observations revealed significant differences in gut microbiota composition between malaria severity groups with genera such as *Lactobacillus* and *Bacteroides* differentially elevated in severe malaria. In this section, we reflect on our key findings and their implications, challenges and limitations as well as future directions and research opportunities (Figure 1). Below are the highlights:

Key findings

First study

- Geographic location shapes gut microbiota composition: beta diversity analysis revealed a significantly different cluster of samples from the West compared to the South and East provinces;
- That geographic difference was likely due to lower dietary fibre intake reported in the Western province;
- A multifactorial analysis yielded positive correlations between differentially abundant genera (e.g. *Methanobrevibacter* and *Lachnospiraceae*_UCG.007) and fiber intake values;
- Age is another important factor affecting gut bacterial composition: our analysis showed significantly lower alpha diversity within preschool children compared to school children and adults.

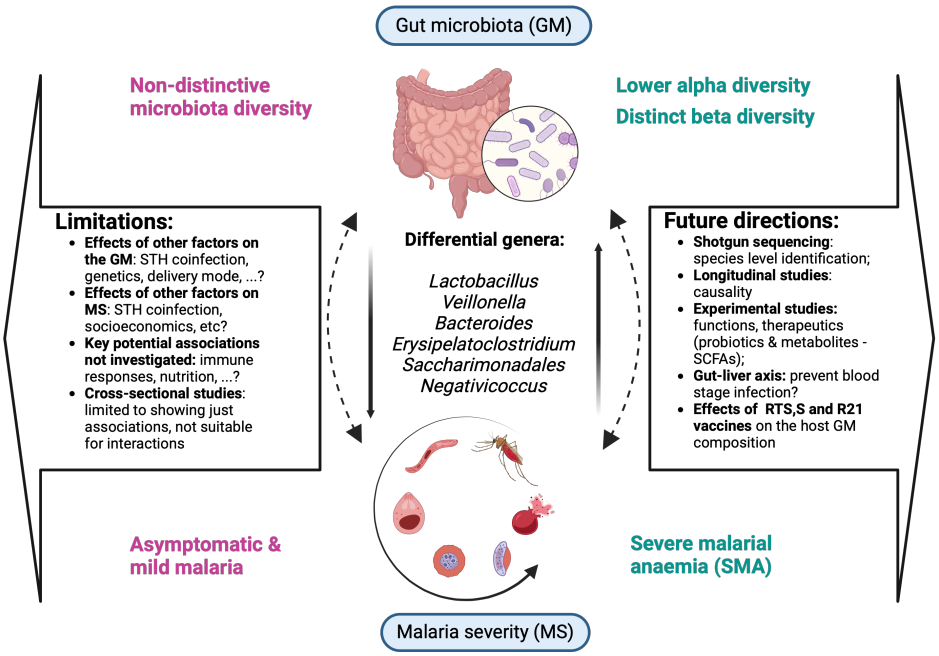

Figure 1. A summary of our research findings, limitations and future directions. While associations between malaria and the gut microbiota composition are dynamic (dashed bidirectional arrows), signficiant gut bacterial differences (lower and distinct diversity versus non-distinctive diversity) were observed between severe malaria (SMA) and other severity groups. Created with Biorender.com

Second study

- Severe malaria samples consistently exhibited significantly lower alpha diversity across all analyzed metrics (observed features, Shannon enthropy, Faith-pd and Pielou evenness);
- The severe malaria sample group was significantly different from other malaria severity groups by beta diversity analysis;
- ANCOM-BC differential abundance analysis results showed that, relative to severe malaria group, top 6 genera were depleted in both asymptomatic and mild malaria groups: *Lactobacillus, Veillonella, Bacteroides, Erysipelatoclostridium, Saccharimonadales and Negativicoccus*;
- Overall weak and non-significant correlations were observed between *Plasmodium* parasitemia and alpha diversity metrics.

Main challenges and limitations

- Thanks to interventions deployed by the government of Rwanda which decreased *Plasmodium* transmission in our study area around our sample collection time, severe and asymptomatic malaria groups were underrepresented in the first study;
- Observed low STH prevalence led to the underrepresentation of STH comparison groups;
- Other factors (e.g. genetics, delivery mode, coinfection, etc) may have had an impact on our findings;
- Both of our studies were cross-sectional, which limits our confidence to establish causality;
- We did not conduct immunological assays to analyse cytokines' associations with malaria severity;
- Socio-economic factors were not assessed, yet they deserve consideration in attempting to understand and design solutions for malaria which is classified among poverty-related disease.

Proposed future directions and research opportunities

- Similar observational studies to check the reproducibility of our findings in other malaria-endemic settings;
- Longitudinal studies on the malaria-gut microbiota interactions to capture a full picture of causality;
- Experimental studies to address both functional and therapeutic aspects of the gut microbiota in malaria context;
- Studies exploring the gut-liver axis given the role of the liver plays in both malaria pathogenesis and gut microbial functions;
- Studies assessing the effects of new WHO-recommended vaccines (RTS,S and R21) on the host gut microbiota composition.

4.1.Key Findings and Their Implications

Our research project reported interesting findings about malaria-gut microbiota composition relationships in Rwanda. The first study's findings revealed significantly different gut microbiota profiles by geographic location and the likelihood of it being explained by nutrional habits such as lower fibre intake in the West province. In our second study, the most interesting finding showed significant gut microbiota profile differences between severe, mild and asymptomatic malaria as well as negative control groups. To our knowledge, this is the first malaria-gut microbiota study that covered the full malaria severity spectrum, as mild infection group was not represented in a similar Uganda-conducted SMA study [76]. Here, we discuss key findings and their implications on public health.

Our first study has shown that geographic variations were associated with a differential beta-diversity of the gut microbiota in the Western province of Rwanda. Additionally, we argued that such differences were likely due to lower dietary fibre intake in the West compared to the South and East provinces of Rwanda. Indeed, together or separately, geographic location and dietary fibre intake have been shown to shape gut bacterial composition. A study which compared the gut microbiota composition of children living in Europe and Burkina Faso reported differences based on dual influence of geographic location and dietary intake [157]. Separately, geographic location has been shown to affect gut microbial communities in South Korea [181], South Africa [149], within Tanzanian Zanzibar archipelago [150] and in Tanzania compared to Botswana [148]. On its own, fibre dietary intake has been shown to affect gut microbiota composition among adults [182]. Particularly, low dietary fiber intake was associated with a gut microbiota favoring lactate fermentation while high fibre intake promoted short-chain fatty acid-producing bacteria among overweight and obese pregnant women [155]. Another study conducted in Spain reported an associations between low fibre intake and obesity as well as other pro-inflammatory diseases among individuals consuming higher levels of saturated fatty acids (SFAs). Previously, diet-induced obesity and insuline resistance have been shown to inversely correlate with higher production of SCFA [62]. Notably, we also did report significantly lower protein intake in the West compared to other provinces. That finding is supported by previous publications showing associations between unique gut bacterial profiles and malnutrition among children [183, 184]. Therefore, the observed

lower fibre and protein intakes in the West province of Rwanda may reflect not only the food insecurity crisis reported by the Rwandan national institute of statistics in that area, but it could also be linked to more pathologies such as malaria [158]. In that case, health interventions in that area should consider addressing that gap as it may be among the causes of ill health in several ways.

Speaking of ill health, imbalanced GM composition has been associated with different pathologies in different ways. In our published short review [77] and that of Valdes et al., [62], we summarized example pathologies that have been associated with gut bacterial imbalance: from cancer to obesity, insuline resistance (type 2 diabetes mellitus), inflammatory bowel disease, mental health, sepsis, autoimmune diseases, atopic eczema and more. For example, in the case of obesity, there is a sense of agreement among researchers that lower diversity and decreased abundance of key gut bacterial such as genera *Christensenella* and *Akkermansia* correlate with that excessive fat pathology [62]. On the other hand, reversing GM imbalance using GM-targeted interventions has been pursued by researchers. As far as we know, the most successful example of that approach is the use of FMT to reverse recurrent *C. difficile* infection, mainly among the elderly [73, 185]. These findings can help to raise our confidence that the emerging malaria-GM research field may yield a similar success story in the prevention or early diagnosis, treatment and control of severe malaria in endemic settings.

Leveraging nutrition habits can directly harness the GM to modulate host immunity. For example, probiotics (e.g. *Bifidobacterium* and *Lactobacillus* species) - likely the most popular interventions, are adminitered in several ways: foods, supplements or drugs [62]. In a different approach, manipulating the gut microbiota through feeding African Americans a rural African diet (fibre-rich prebiotics) for two week resulted in a 2.5 fold increase of butyrate while the synthesis of bile acid reduced significantly thanks to a selective growth of gut bacteria [186]. However, inconsistent and temporal interventions have been shown to lead to short-lived benefits [187]. Since literature is still limited in data about mechanisms linking immune response to malaria with intestinal bacteria and their metabolites, comparable inflammatory conditions may serve as examples. In an inflammatory bowel diseases (IBD) study, mediterranian and vegetarian diets which consist of fibre-rich foods such as fruits and vegetables have been associated with anti-inflammatory effects [188]. Therefore, we can hope that similar diet-based

interventions could help to reverse inflammation and restore gut microbiota diversity in malaria if made long-lasting. Achieved changes in the gut and overall health of the population would hopefully have multiplier effects including improved resistance to several pathologies.

In both studies, we observed a significantly lower alpha diversity within preschool children and severe malaria groups respectively. Both groups belong to the same age-group (below 5) which has been shown to exhibit changes in their gut microbiota composition following the milk to solid food shift [63]. This finding shows that, like geography, age is an important factor to consider in designing, analyzing and reporting results of gut microbiota composition research projects. In fact, it has been well established that gut microbiota diversity increases with age in general [61] and particularly, in malaria context [75]. Interestingly, age has been shown to correlate negatively with *Plasmodium* parasitaemia among individuals aged 1-96 in Gabon [189]. It remains unkown whether the role of age in gut microbial composition and malaria pathogenesis is linked or not. However, immune response development is know to be associated with both age and gut microbiota composition.

Severe malaria anaemia (SMA), affecting children under 5 years of age disproportionately, involves dysregulation of immune responses [190]. In immunologically naïve children, it has been proposed that SMA is characterized by a reduced erythropoietic response characterized by the accumulation of hemozoin (Hz) pigment, leading to inefficient reticulocyte production index (RPI) [27]. On the other hand, evidence revealed an association between SMA and gut microbiota composition in Ugandan children [76]. Further studies are necessary to establish clearer connections. Nevertheless, the ultimate goal of ours and similar studies is to establish links between immune responses to malaria and the gut microbiota. While we did not conduct cytokine analyses in this study, a study which did so in humans showed that infection with *Plasmodium vivax*, without STH coinfection, was associated with both higher relative abundance of genus *Bacteroides* and elevated levels of interleukin 6 (IL-6), IL-8, and IL-10 [86], implying some level of balance between Th1 (IL-8) and Th2 (IL-6 and IL-10) responses [27]. Functionally, bacterial metabolites such as SCFAs enable crosstalk between the gut microbiota and host immunity [191]. In susceptible mice, propionic acid (PA) is a SCFA that has been associated with *P. yoelii* 17XNL hyperparasitemia [192]. As summarised in a

recent review [6], *Bacteroides* have been shown to ferment polysaccharides to PA which in turn is associated with suppressing inflammation. Hence, we can speculate that the elevated levels of *Bacteroides* in SMA is part of the innate immune response to reduced erythropoiesis in children [27]. Immunologically, a recent study conducted in Rwanda to compare mild and severe cases of malaria reported that IL-6, IL-13, TNFα and IFNγ were significantly increased while IL-7, IL-10, IL-17 and IL-27 were decreased in SMA compared to TGF-β1, β1 and IL-9 that were upregulated in mild malaria [193]. These results point towards interesting, still poorly understood, links between malaria severity and both immune responses and gut microbiota composition.

In the second study, supplementing beta diversity analysis which revealed a unique severe malaria cluster, ANCOM-BC differential abundance analysis returned interesting results. Relative to severe malaria group, the analysis showed that the following top 6 genera were depleted in both asymptomatic and mild malaria groups: *Lactobacillus, Veillonella, Bacteroides, Erysipelatoclostridium, Saccharimonadales and Negativicoccus*. Leveraging these gut bacteria may avail much needed gut microbiota-target anti-malaria products (e.g. probiotics). For example, genus *Lactobacillus*-derived probiotics such as the ones produced from *Kimchi*- a Korean traditional food – were shown to modulate the Th1/Th2 balance which is essential in regulating clinical malaria outcomes [194]. Previously, *Lactobacillus* has been shown to play important roles such as immune modulation and preserving gut barrier integrity in the context of pathogen control [18]. In oral health, *Veillonella* spp have been associated with the production of nitrite – recently found to affect health positively – and with limiting the growth of pathogens such as *Streptococcus mutans* [195]. Therefore, on the one hand, it seems these two bacterial genera could most likely promote Th2 immune responses. On the other hand, several species of *Bacteroides* (e.g. *B. caccae*, *B. fragilis* , *B. uniformis*, *B. ovatus*, *B. vulgatus*, etc), except *B. thetaiotaomicron*, have been shown to, in consortium with other gut bacteria, cause susceptibility to severe malaria, suggesting their role in shaping Th1/Th2 response balance in hyperparasitemia murine models [111]. Available literature does not say much about the remaining genera's association with pathologies and inflammation. These data indicate that the identified genera may influence anti-*Plasmodium* inflammatory responses in different ways, but further research is needed to fully understand their individual role, particularly in severe malaria context.

Keeping in mind the state-of-the-art in malaria-gut microbiota research (Figure 3), the available interventional studies have reported interesting mechanistic pathways. On one hand, beneficial bacterial metabolites were shown to regulate the function of innate and adaptive immune cells. For example, butyrate SCFA modulates regulatory T cells and dentritic cells, suggesting its role in controling the host's control of parasitemia [196]. On the other hand, it has been shown that the depletion of beneficial taxa (e.g. *Akkermansia muciniphila*) and the enrichment of pathobionts (e.g. *Clostridium spp*) in a murine model infected with *P. yoelii* resulted in modified T cell function as well as mucosal immunity [3]. Additionally, tryptophane-derived indole, whose production depends largely on the gut microbiota, has been shown to activate the aryl hydrocarbon receptor (AhR), potentially influencing both hepatic function and immune responses involved in *Plasmodium* infection [197]. A better understanding of these pathways will enable scientists to discover GM-targeted interventions to prevent or fight severe malaria.

In conclusion, malaria-gut microbiota research is in its early phase. Our first study reiterated two important factors to consider when designing studies in that field, that is geographic location and age. We also showed the likely role of nutritional habits (i.e low fibre intake) in the observed distinct gut profiles within the West province. Our second study highlighted significant gut bacterial differences between malaria severity groups. Relative to severe malaria, the following genera were significantly depleted in both mild and asymptomatic groups : *Lactobacillus, Veillonella, Bacteroides, Erysipelatoclostridium, Saccharimonadales and Negativicoccus.* Combined, our two studies have generated new insights, particularly in assessing gut bacterial profiles by full malaria severity spectrum. Compared to results published in previously studies, our findings supported a hypothesis that severe malaria may be associated with significant changes in the gut microbiota composition and vice versa. Although, a number of other studies communicated contradictory results, it is clear that available data are still limited to allow researchers to draw any conclusions. Therefore, much more research is need to generate enough evidence. Still, taking context into consideration, despite all efforts made, we acknowledge that the above findings may have been affected by encountered challenges and limitations.

Main conclusions and novelties

- Geographic location is an important factor shaping gut microbiota composition even in as small territories as three malaria-endemic provinces of Rwanda.
- Fiber dietary intake was shown to be an underlying cause that can explain the gut microbiota profile differences observed between regions.
- Differentially abundant bacterial genera (e.g. *Tyzzerella*, etc) correlated positively with nutrional intake values (e.g. fiber).
- Interestingly, *Plasmodium* parasitemia correlated with neither differentially abundant bacterial genera nor other variables such as nutrional intake values, alpha diversity metric and age.
- Age is another factor observed to be associated with differences gut microbiota composition.
- Therefore, both geographic location and age should be considered key confounders in designing gut microbiota studies in malaria endemic settings and beyond.
- While controling for confounders, the second study reported that severe malaria samples were significantly different from other malaria severity samples by both alpha and beta diversity analyses.
- Relative to severe malaria group, top 6 genera were depleted in both asymptomatic and mild malaria groups: *Lactobacillus*, *Veillonella*, *Bacteroides, Erysipelatoclostridium, Saccharimonadales and Negativicoccus*.
- Differentially abundant genera reported can guide future studies aimed at identifying gut bacterial biomarkers of different malaria severity forms (from severe to mild and asymptomatic).
- This project enriched the limited literature on gut microbiota in Africa, especially Rwanda.
  To our knowledge, this is the fist successful attempt to assess GM profiles by full malaria severity spectrum (asymptomatic, mild and severe) in humans.

4.2.Challenges and Limitations

Being nascent, malaria-gut microbiota research field presents both excitement and challenges. For example, as of 2023, only 5 peer-reviewed clinical studies had been published [6]. Addressing the latter requires collective effort and systematic methodologies. Here, we share our first-hand experience combined with evidence from available literature, hoping to ease work for future researchers.

Samples of our first study were not representative of some comparison groups (STH co/infection and malaria severity). Thus, this may have impacted our reported lack of link between the gut microbiota profiles and malaria status as well as its coinfection with STH. Firstly, we think that may be due to the reported overall low STH prevalence as well as a limited number of extreme malaria severity cases (only 2 asymptomatic and 6 severe cases reported). The former may be a result of not setting inclusion criteria to only STH high risk group (school children) which is different from malaria prime high risk group (preschool children). The latter may be a result of the decline in malaria transmission thanks to interventions deployed by the government of Rwanda at the time. Combined, these challenges plus our findings aided in redesiging the project. As a result, we prioritised malaria severity over STH prevalence which had received prime attention in a similar, recent study [86]. Hence, we shifted the study site to the central plateau of Rwanda (Bugesera, Gasabo, Gicumbi and Rulindo districts) where asymptomatic and severe cases were relatively more prevalent at the time. Indeed, following that move, we managed to collected 150 samples representative of all malaria severity comparison groups used in our second study.

Other factors may have had an impact on our findings. Literature tells us that a plethora of factors from genetics, to age, geographic variations, delivery mode, sex, coinfection, antibiotics and more can alter the gut microbiota composition [57, 58, 61, 77, 198, 199]. As much as our miltidimentional study design tried to take into account key factors such as age, geographic variations, sex, antibiotics/anti-parasitic treatments and STH coinfection, other factors may have had an impact on our findings. For example, a study conducted in mice showed that genetics prevailed over geographic location in shaping gut bacterial compostion [200]. Moreover, since majority of SMA group member were preschool children, factors such as mode of delivery and early life nutrition may have played important

roles as shown in literature [201]. Therefore, we acknowledge that and recommend future studies to account for as more factors as possible (Figure 2).

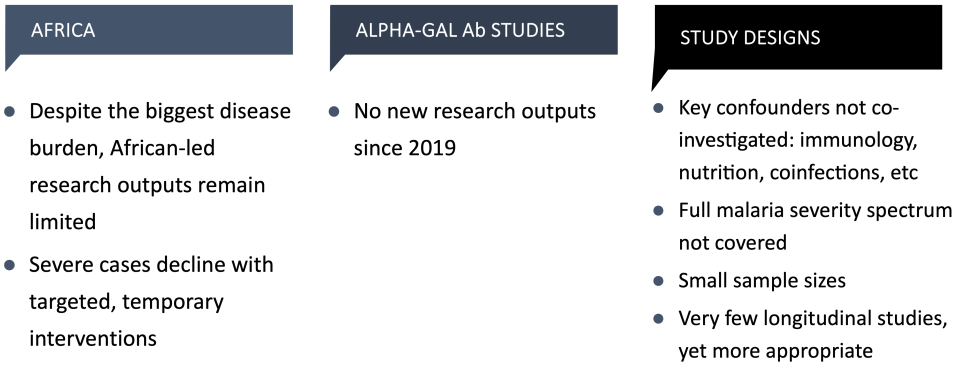

Figure 2. Challenges and limitation summarized in three groups related to Africa, anti-alpha-gal antibodies and study designs. Future studies should aim to overcome many as possible to ensure reproducibility and generalizability of findings.

In our second study, while malaria severity groups were well represented, the STH groups were still underrepresented. Therefore, we did neither analyze nor report the effect of STH infection and coinfection with malaria on gut microbiota composition. Two approaches could improve that: instead of a concentration method, use a high-sentivity screening method such as Polymerase Chain Reaction (PCR) [202] and target exclusively school and preschool children who are the top high risk groups as previously done in Colombia [86].

Both of our studies were cross-sectional, observational studies which limit our confidence to establish causality. Therefore, we recommend further studies to be longitudinal. Futhermore, while the bacterial 16S rRNA gene sequencing provided valuable insights, whole genome shotgun sequencing could have provided deeper bacterial identification down to species level and functional details. Similarly, macronutrient analysis could be supplemented by micronutrient assessment of nutritional intake.

In the introduction of our first study we acknowledged malaria as part of the so-called group of ‘poverty-related diseases’, suggesting the need for solutions beyond conventional healthcare interventions. Hence, in a health economics

context, GM-targeted interventions may be viewed as a game-changer in terms of cost-effectiveness and sustainable public health impact. For example, the WHO estimated that, in 2022 alone, US$ 4.1 billion were spent on traditional malaria control interventions (e.g. insecticide-treated bed nets, antimalarial drugs, and indoor residual spraying) with no considerable gains [9]. Could an alternative intervention such as GM-targeted interventions offer better value for money?

4.3.Future Directions and Research Opportunities

The overall aim of malaria-gut microbiota studies is to generate enough observational evidence presenting potential gut bacterial biomarkers that interventional studies can build on to discover and design new GM-target interventions against severe malaria (Figure 3). While there is still a long way to go, this and other studies contribute to a growing body of literature in the malaria-gut microbiota research field.

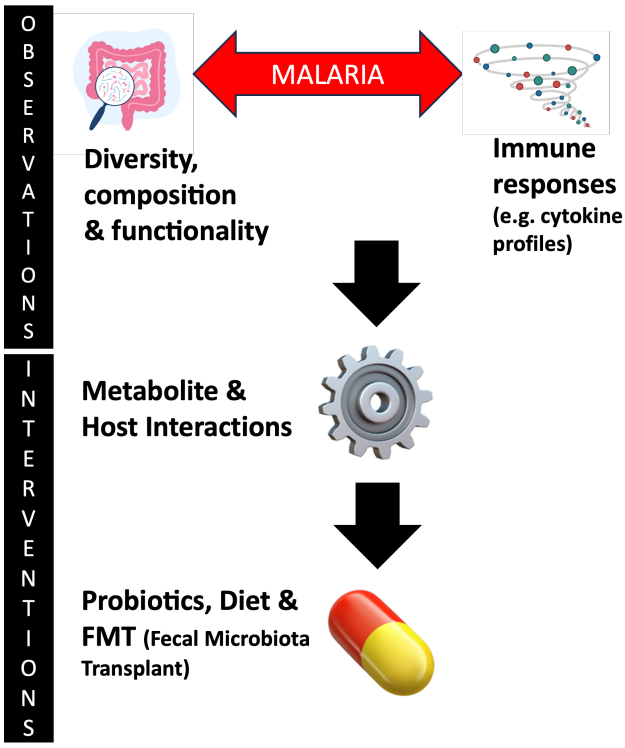

Figure 3. The state-of-the-art for malaria-gut microbiota research. From observational studies, gut bacterial biomarkers identified can be studies in animal models (metabolite – host interactions) and humans (probiotics, diets and FMT) to discover and design new interventions against malaria.

To authors of future studies on this important topic, we recommend:

Firstly, more studies to check the reproducibility of our findings in other endemic areas. Such studies may be observational with larger cohorts and/or more

advanced methodologies such as whole genome shotgun sequencing. Overall, in this new malaria-gut microbiota field of research, study design needs improvement. Previous studies have been criticised for one reason or the other. For example, the two inaugural studies about the role of anti-α-gal antibodies in modulating *Plasmodium* infection outcome were questioned for not demonstrating (1) the source of those antibodies in participants as well as for (2) the association between *E. coli* O86:B7 relative abundance in stool and the protection against *P. falciparum* infection [6]. Another study involving SMA samples (n=40), acknowledged a relative small number (n=7) of asymptomatic samples used to compare [76]. Small sample size (n=10) was acknowledged in another longitudinal study conducted on Kenyan children [88]. One more study involving malaria-STH coinfection did not include severe cases of malaria which leaves the comparison incomplete [86]. Therefore, there is still a long way to go in term of study design and methodology harmonization to achieve more reliable and reproducible results in malaria-gut microbiota research. Most importantly, study designers should keep in mind possible confounders such as geographic location, age and more in order to limit their bias as shown in our first study.

Secondly, longitudinal studies of the malaria-gut microbiota interactions, considering before, during and after malaria infection in order to capture a full picture of causal relationships instead of stopping at observed associations. For such investigation to provide comprehensive, it should include both clinical and animal studies. Starting with clinical investigations, a cohort of children (focusing on one age-group) would be recruited prior to intense *P. falciparum* transmission season and followep up over a minimum of six months using methods described earlier in the publication of Yooseph et al. [75]. Next, germ-free mice would be colonized by fecal material transplant (FMT) from either malaria-susceptible or -resistant children according to methods described earlier [173]. Fingings from such investigations have the potential to shed light on associations between *P. falciparum* infection, malaria severity and the gut microbiota ahead of establishing causality by identifying gut bacterial biomarkers linked to the disease development towards revolutionizing global finght againt severe malaria (Figure 3).

Thirdly, in due time, experimental studies would address both functional and therapeutic aspects of the gut microbiota, providing insights into the specific biochemical pathways impacted by malaria as well as GM-targeted interventions

(i.e. probiotics, prebiotics, and FMT) for a better understanding and control of malaria in endemic settings. For example, evidence has shown that maintaining an adequate dietary intake of vitamin D, iron, fibre, zinc and magnesium may have a beneficial effect on alleviating inflammation in the body, reducing oxidative stress, and improving the condition of the intestinal microbiota through various epigenetic mechanisms [203]. Thus, studying these micronutrionets in malaria animal models would generate new insights on the topic.

Fourthly, studies on the role of the Gut-Liver Axis. Considering that *Plasmodium* parasites travel from the skin through bloodstream to infect liver cells where they stay during the infection window period, investigating the gut-liver axis could make a new research avenue for systemic immunity modulation.

Fifthly, studies on the effects of new WHO-recommended vaccines (RTSS and R21) on the gut microbiota composition. As new interventions to control malaria, these vaccines should be evaluated for compatibility with human microbiota, optimizing their action thanks to considering other factors such as nutrition habits which promote healthy gut directly and balanced immune response indirectly. A recent review which summarized a bidirectional interaction between the GM and drugs/immunotherapy, suggest potential GM changes due to non-antibiotic drugs as well as their potential clinical consequences [204].

Finally, more investment in this research field which promises alternative interventions to defeat malaria. With the current interventions against malaria that have costed more and delivered not as much in endemic settings, a new avenue for innovative solutions to malaria should be supported. In fact, that is in line with the WHO's global technical strategy for malaria 2016-2030 which calls for more investment in research for innovative tools to defeat malaria [1].

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