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The Entomological Efficacy of Piperonyl Butoxide (PBO) Combined with a Pyrethroid in Insecticide-Treated Nets for Malaria Prevention: A Village-Based Cohort Study Prior to Large-Scale Deployment of New Generation Mosquito Nets in Burkina Faso

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Abstract

The efficacy of insecticide-treated nets (ITNs) is increasingly compromised by the prevalence of malaria vectors resistant to pyrethroids. In response to this issue, a new generation of ITNs has been developed that incorporate synergistic components, such as piperonyl butoxide (PBO). The purpose of this study is to provide entomological evidence for the efficacy of a PBO-based ITN brand at the village level, serving as a basis for decision-making before large-scale net deployment. During the high malaria transmission period, ITNs were distributed in each group and vector sampling was conducted biweekly in selected households. Bionomic data were collected to assess the resistance of wild *An. gambiae* populations to various chemical insecticides. There was a significant disparity in total *An. gambiae* sl. collected between the ITN arms, the intervention arm (ITN arms), and the control arm (P = 0.003). *An. coluzzi* was identified as the predominant species in the study area, as confirmed by PCR analysis. Analysis of the blood-feeding inhibition rate revealed that 100% permethrin + PBO ITN exhibited significantly greater inhibition than 66.81% permethrin only ITN.

According to the log-time probit regression analysis, permethrin exhibited a knockdown time of 256 min without synergists, while it decreased to 139 min (P = 0.001) when pre-exposed to PBO. The evidence from this trial supports the use of PBO ITNs over standard ITNs for pyrethroids to combat pyrethroid resistance and improve protection against malaria for both individuals and communities, particularly in areas with high pyrethroid resistance.

Keywords

Pyrethroid Resistance, Permethrin + PBO ITNs, Permethrin ITN, Bionomic Data, Insecticide Susceptibility Data, Burkina Faso

1. Introduction

In the realm of malaria vector control, insecticide-treated nets (ITNs) and indoor residual spraying (IRS) are frontline tools. As a result of renewed commitment and increased funding for the control and elimination of malaria, vector control has increased significantly since 2000 [1]. There is clear evidence that high coverage and use of ITN reduce malaria mortality and morbidity rates and improve pregnancy outcomes in a variety of transmission settings [2]. Indeed, by 2013, ITN ownership increased in Africa: 49% of households owned at least one ITN and 44% of children slept under one [3]. Increased funding contributed to the rapid rise in the number of ITNs procured and distributed within countries. The number of ITNs delivered by manufacturers in sub-Saharan Africa increased from 11 million in 2005 to 392 million in 2013. In sub-Saharan Africa, the number of households that own at least one ITN increased from <2% in 2000 to 55% (95% CI 50% - 58%) in 2015 [4]. This high coverage of ITN has decreased the malaria cases in sub-Saharan areas. Indeed, between 2000 and 2015, the incidence of *Plasmodium falciparum* infection in Africa was estimated to be reduced by 50%, and ITN and IRS contributed to 81% of this decline [5]. Through this finding, the World Health Organization (WHO) recommends the use of ITNs and IRSs as components of a national integrated vector management (IVM) plan. IVM is defined as a rational decision-making process to optimise the use of resources, promoting the use of a variety of interventions, alone or in combination, selected on the basis of local evidence [6]. Although there is clear evidence of ITNs effectiveness in the past, nowadays, a decline in the field has been increasingly observed, which could be due to vector resistance to insecticides [7].

Pyrethroid-resistant malaria vectors are now a major threat to the effectiveness of ITNs [7]. To address this threat, a new generation of insecticide-treated nets (ITNs) has been developed using pyrethroids combined with a synergist, such as piperonyl butoxide (PBO) [8] [9]. In fact, synergists have been used commercially for more than 50 years and have contributed significantly to improving the efficacy of insecticides [10] [11]. PBO acts synergistically by block-

ing oxidases (cytochrome P450) that commonly detoxify pyrethroid insecticides within the mosquito's body to restore the efficacy of pyrethroids [12]-[14]. Compared to standard pyrethroids ITNs, pyrethroid-PBO ITNs were found to reduce the incidence of malaria cases for up to 21 months of use in areas with a high level of pyrethroid resistance in mosquitoes [13] [15]. Furthermore, pyrethroid + PBO ITNs have also been shown to increase mosquito mortality in experimental hut studies when they are unwashed or after 20 washes [14]. This can be attributed to their enzyme-inhibiting action, which restores the susceptibility of insects to chemicals that would otherwise require higher levels of the toxicant for their control [16]. Synergists are also useful for laboratory investigations of resistance mechanisms because of their ability to inhibit specific metabolic pathways [16]. Furthermore, PBO is an insecticide synergist that inhibits the action of resistance-associated metabolic enzymes of the cytochrome P450 family [8] [17]. Inhibition of P450 enzymes by PBO leads to the availability of pyrethroids on the Internet, which induces excitation, repellency and mortality. The role of these enzymes in the detoxification of insecticides, including pyrethroids, and in causing resistance is well documented [18]-[20]. The addition of PBO to pyrethroid nets as a strategy to overcome resistance, especially in areas where this resistance is driven by the overexpression of P450 enzymes known to metabolise pyrethroids, has been demonstrated in a variety of experimental hut trials in Africa, in Benin [21], Tanzania [22], Ivory Coast [23] and Burkina Faso [24].

In Burkina Faso, a mapping of vector susceptibility to insecticides including new insecticides (PBO + pyrethroids, neonicotinoids, pyrroles) was carried out in 2019 to provide evidence prior to deployment in large-scale new generations nets [25]. Based on these findings, vector control tools that use the new insecticides mentioned above were recommended for use in Burkina Faso, where resistance to pyrethroids was more prevalent. However, no research was conducted on the performance of these new vector control tools in real condition of use prior to large-scale deployment. The aim of this study was to establish entomological evidence supporting the efficacy of a specific brand of PBO ITNs at the village level. This rapid investigation aimed to determine and compare the entomological parameters of pyrethroid-only ITN and pyrethroid + PBO ITN in malaria-endemic areas to provide entomological data for decision-making before large-scale deployment of nets.

2. Methods

2.1. Study Area

The "Vallée du Kou," which is situated at 4˚24'59'' west longitude and 11˚24' north latitude, lies 30 km north of Bobo-Dioulasso, Burkina Faso. It is a rice-growing area that was built in 1970 and covers an area of 1200 ha. It is made up of seven rice villages surrounded by a wooded savannah. Rice cultivation provided by a system of irrigation of the plains creates many breeding sites suitable for *Culicidae,* of which *Anopheles gambiae* has high density throughout the

year but mainly in the rainy season. The two species *An. gambiae* and *An. coluzzii* coexist within the same geographical area, displaying varying frequencies in accordance with the seasons. Throughout the year, *An. coluzzii* predominates, but there is an increase in the frequency of *An. gambiae* by approximately 20% - 45% towards the end of the rainy season (September-November). The *kdr* mutation, which provides resistance to pyrethroids, is present in both species, with highly variable allele frequencies ranging between 0.8 and 0.9. Furthermore, the ace-1^R mutation, which confers resistance to carbamates and OP, is found in species with different allele frequencies of 0.4 and 0.03, respectively, within *An. gambiae* and *An. coluzzii,* indicating multi-resistance to *kdr* and ace-1^R in this area [26]. However, low activity of these oxidases and monooxygenases (cytochrome P450s) was detected in *An. gambiae* populations in this area in 2012 [27]. *Anopheles funestus* and *Anopheles arabiensis*, other vectors of malaria, are found with low relative density (3% - 5%). *Culex quinquefasciatus* is also observed at frequencies of less than 10% [26]. The study was carried out in VK1, corresponding to village number 1 of Vallée du Kou in Bama (**Figure 1**). The village was divided into three parts corresponding to the group where each treatment arm was assigned.

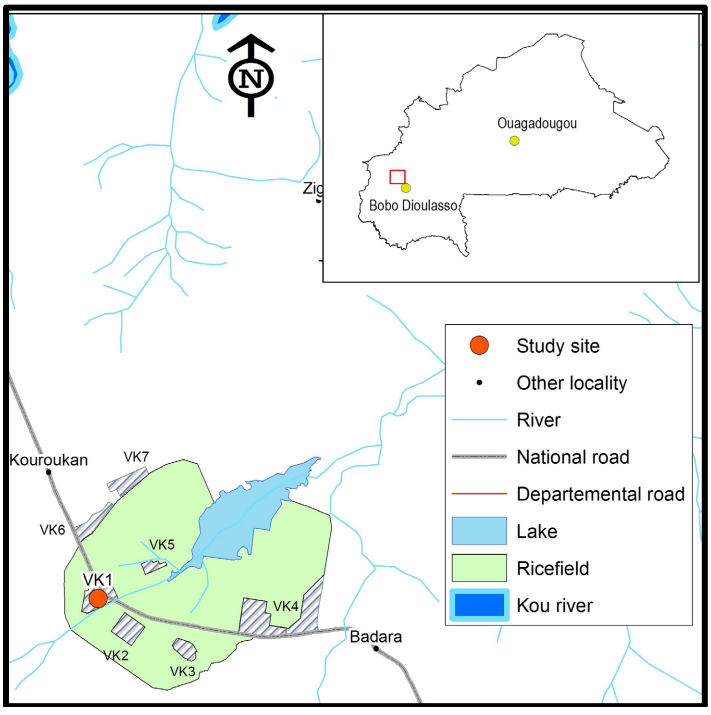

Figure 1. Study area.

2.2. ITN Study and Treatment Allocation

Olyset® Net is a long-lasting insecticidal mosquito net (LN) produced by Sumitomo Chemical, Japan. This product is constructed from high-density monofilament polyethylene yarn (weighing 50 g/m^2) and contains technical permethrin 2% (w/w) as an active ingredient (AI), equal to approximately 1000 mg of AI/m^2

or 20 g AI/kg. The insecticide is integrated into the filaments and diffuses through the threads of the net. Olyset®Net has a wide mesh (4 mm × 4 mm). The Olyset®Net received the full WHOPES recommendation in October 2001 [28] for use in the prevention and control of malaria, before the current WHO guidelines for the testing and evaluation of ITNs were published [29]. Olyset®Plus is a long-lasting insecticidal mosquito net (LN) manufactured by Sumitomo Chemical, Japan. This product is composed of 150 deniers high density monofilament polyethylene yarn (weighing 40 g/m^2) and contains technical permethrin (40:60 cis: trans isomer ratio) at 2% (w/w) as the active ingredient (AI), equivalent to 20 g AI/kg (approximately 800 mg AI/m^2), together with piperonyl butoxide (PBO) at 1% (w/w) as a synergist, corresponding to 10 g PBO/kg (approximately 400 mg PBO/m^2) [30]. Permethrin and the synergist are incorporated into the filaments and migrate through them by diffusion [6]. Permethrin and piperonyl butoxide are integrated into the filaments and diffuse to the surface.

The study site (VK1) was divided into three groups or arms corresponding to "Bolosso", "Di", and "Massawé", as shown in **Figure 2**. The arms had the same characteristics (all located during rice growth); therefore, there was no bias in selection. ITNs were randomly distributed as follows: the Di group received Olyset® Net (permethrin only ITNs), the Bolosso group received Olyset® Plus (permethrin + PBO ITNs), and the Massawé group was the control group where no study ITNs were distributed, but people used common vector control tools for their protection. After the distribution of ITNs in each ITN group, 10 households in each group were randomly selected for vector sampling two days a week a month during the high malaria transmission period. Along with vector sampling, ten other ITNs were randomly selected in each group for assessment of the physical conditions of the ITNs followed by bioassays in the laboratory.

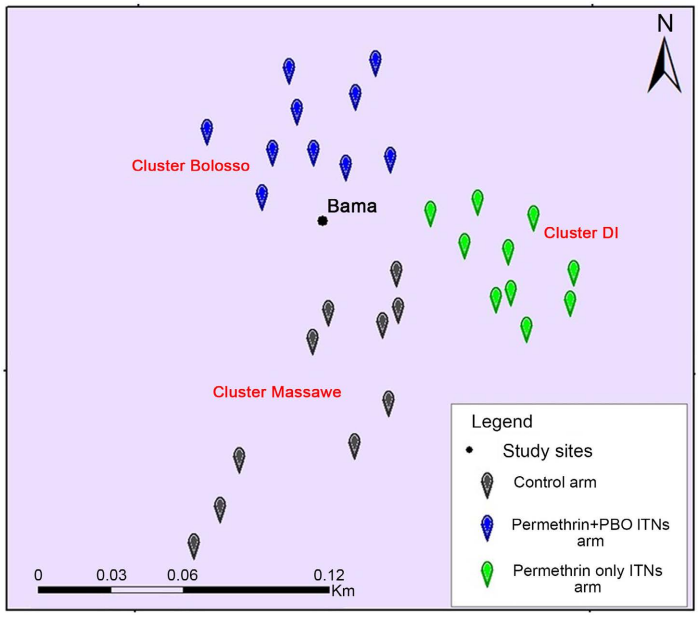

Figure 2. Allocation of treatment in clusters (Bolosso, Di and Massawé) in Bama, Vallée du Kou in Burkina Faso.

2.3. Monthly Entomological Mosquito Collection

Monitoring of the density of indoor resting, species composition and longevity of vector species was conducted in arms (Di, Bolosso, and Massawé) of the VK1 village after the distribution of ITNs. Monitoring occurred during the rainy season (characterised by high malaria transmission) from June to November 2019. To sample the resting insects at home, hand catches were carried out with mouth aspirators. Ten randomly selected ITNs from each group were subjected to laboratory vector sampling. Mosquito collection was carried out two days a week (Monday and Friday). Three teams visited the village groups, one team per group for a total of 128 days of surveys corresponding to 480 collection houses surveyed during the study period in the village of VK1 (40 collection houses per group per month). Adult mosquitoes were collected indoors between 06:00 a.m. and 09:00 a.m. Subsequently, the collected resting mosquitoes were identified by species and counted. A subsample of collected mosquitoes was individually preserved in Eppendorf tubes with silica gel for subsequent molecular analysis. Furthermore, baseline entomology data were collected by manually capturing mosquitoes using a mouth aspirator during the dry season in May 2019, covering approximately 15 houses per treatment arm, before ITN distribution. The ANOVA test did not reveal any differences in the indoor baseline resting density for the three arms located in the VK1 village when analysed according to the treatment allocation.

2.4. ITN Efficacy Evaluation

2.4.1. WHO Cone Bioassay

Ten randomly selected ITNs from the permethrin + PBO ITN arm and the permethrin only ITN arm were used in the bioassays each month. Bio-efficacy was initially evaluated using the reference Kisumu susceptible laboratory strain of *An. gambiae* s.s. according to a standard WHO exposure cone [29]. Subsequent bioassays were conducted using a local strain of *An. gambiae* sl. from the study site, which had been collected in the larval stage and reared until the adult stage. For all types of nets, four side panels and the roof panel of each net were tested [31]. The evaluation involved conducting one cone test per side panel, with five (2 - 3-day-old non-blood-fed) female mosquitoes used per cone, totalising 25 mosquitoes of each strain tested on each net. In total, 500 mosquitoes (250 *An. gambiae* Kisumu strains and 250 local populations of *An. gambiae sl.)* were used per village in each round of the bioassay. The mosquitoes were exposed to each netting sample in five standard WHO cones fixed with a plastic manifold for 3 minutes and then kept for 24 hours in paper cups with cotton wool soaked in a 10% sugar solution; Dead status was recorded after 60 minutes and mortality after 24 hours. Mosquitoes concurrently exposed to an untreated net were used as a control. All bioassays were performed at 25˚C ± 2˚C and 75% ± 10% relative humidity. If the ITNs did not meet the efficacy criteria of a 95% knockdown rate after 60 min or mortality of <80% after 24 h in cone bioassays, they were sub-

jected to tunnel tests using a guinea pig as bait with the expected result of nets achieving >80% kill and >90% blood feeding [31]. A tunnel assay was employed to determine the mortality and success of blood feeding of host-seeking mosquitoes within an experimental chamber. This study was designed to provide further insight into the toxicity of washed and unwashed nets used in the huts. Pieces of nets cut from the unwashed and washed nets of the different treatments were tested, and pyrethroid resistant mosquitoes collected in the field were used. The assay was conducted in the laboratory by releasing 100 females of *An. gambiae* sl. without blood at 7:00 p.m. in the largest compartment of the glass tunnel, while a guinea pig was placed in the smaller confined compartment as a positive attractant. The two compartments were separated by a cardboard frame containing a net piece (25 cm × 25 cm) punctured with nine holes, each 1 cm in diameter. The next day at 08:00 a.m., dead, living, blood-fed, and non-blood-fed mosquitoes were removed from the compartments. Living mosquitoes were observed for 24 hours to assess delayed mortality. Treated nets, washed and unwashed, were compared with untreated control. The efficacy of each ITN was measured in terms of 1) inhibition of blood feeding (the reduction in blood feeding compared to that of the control tunnel) and 2) mortality (the proportion of mosquitoes collected dead after contact and that died 24 hours after being removed from the tunnel). Tunnel design can be found in the WHO Guidelines for Long-lasting Net Laboratory and Field-testing [31].

2.4.2. Physical Integrity

To assess the physical condition of the ITN during their regular use, 30 cohort PBO ITN arms and a permethrin only ITN arm were tracked in the same groups each month for four months. All available nets in households were inspected for their presence and physical integrity. The nets were removed from the sleeping places, individually hung on a rectangular frame outside the house, and examined for the presence of holes was examined on the side and roof panels. The holes were counted for each net, their diameter was measured, and they were classified into the following categories according to the WHO criteria for hole sizes [31]: Size A1: 0.5 - 2 cm diameter, Size A2: 2 - 10 cm diameter, Size A3: 10 - 25 cm diameter, Size A4: >25 cm diameter.

The parameters to assess the integrity of the nets included the proportions of Permethrin + PBO ITNs and permethrin only ITNs with any size of holes or tears and the proportionate hole index (pHI) for each net, which was calculated as follows [31]:

The integrity of each type of ITN was determined by two indicators:

• The proportion of nets with any hole was calculated as follows:

(Total number of ITNs with holes/total number of ITNs in all households) × 100.

• The proportionate hole index (pHI), according to WHO guidelines (WHO, 2017a), was calculated using the following formula: PHI = 1 × No. of Size-1 + 23 × No. of Size-2 + 196 × No. of Size-3 + 576 × No. of Size-4. This index provides

an estimate of the area occupied by the holes in each type of ITN.

Based on the calculated PHI, each net was categorised as follows:

- Under good conditions (pHI < 64),
- To be repaired (64 < pHI < 642), or
- To be replaced (pHI > 642)

The sampled nets were also inspected for any holes or tears that the households carried out. Subsequently, the nets were returned to their original sleeping place within the same household.

2.5. Owner Acceptance and Adverse Events of ITNs

Data collection for owner acceptance and adverse events of ITNs was carried out using a single method. Monthly house-to-house surveys were conducted to assess net usage and physical condition of the nets, with 10 randomly selected nets per arm (Permethrin + PBO ITN and Permethrin only ITN).

The interviews were conducted with all self-identified heads of households using the net master list. Questionnaires were used to gauge people's perceptions of the benefits and/or side effects experienced during the use of nets.

One month after net distribution, a survey was conducted to document adverse events reported by net owners and capture their general experiences of net use. A pre-tested questionnaire adapted from WHO guidelines [31] was administered to the heads or an adult person of the households after providing informed consent.

2.6. Insecticide Resistance Monitoring

The susceptibility of wild *An. gambiae* populations to various chemical groups of insecticides was monitored using the World Health Organization (WHO) protocol [32]. Batches of 25 adult females 3 to 5 days old, not fed blood, were exposed to filter papers impregnated with 0.75% permethrin, a pyrethroid insecticide. The number of mosquitoes that were knocked down was recorded every 5 minutes during the exposure period. After exposure, mosquitoes received a glucose solution to feed and mortality was recorded 24 hours after exposure. Control tests using untreated papers were conducted simultaneously. To perform synergistic tests, Whatman No. 1 filter paper impregnated with synergist piperonyl butoxide (PBO) was used. Approximately 20 - 25 mosquitoes from *An. gambiae* sl. were exposed to four (4) replicates of 4% PBO for 1 h to suppress oxidase enzymes. Subsequently, the mosquitoes were immediately transferred and exposed to 0.75% permethrin for an additional hour. Two control tubes were used in parallel during the test. The knockdown rates were recorded as before for 60 minutes, after which the mosquitoes were transferred to observation tubes and held for 24 hours to record mortality. In addition to the diagnostic concentration (DC) of permethrin, 0.75%, 5× and 10× DC bioassays for permethrin were performed using the standard WHO bioassay method described previously to assess resistance intensity. All exposures lasted for 1 hour and the final mortality was recorded after a 24-hour holding period, during which a 10%

glucose solution was provided to the surviving mosquitoes. Subsequently, living and deceased specimens of WHO bioassays were used to determine the genomic and allelic frequencies of *kdr* mutations. The detection of mutations involved in insecticide resistance was also performed by PCR using the Martinez-Torres *et al.* [33] for the *kdr* L1014F mutation from samples that were previously used to identify molecular species and forms [34].

2.7. Laboratory Mosquito Analysis

The laboratory conducted the analysis on subsamples of *An. gambiae* s.l. collected by hand catches with an aspirator in the mouth. The purpose was to determine the circumsporozoite rates of *P. falciparum* by ELISA [35]. All collected Anopheles spp were numbered by the house and their status (*i.e.* dead/alive, blood fed/unfed) was recorded. Live mosquitoes from indoor resting catch collections were transferred to paper cups, provided glucose solution (10%), and kept for 24 h in the laboratory to measure delayed mortality. Additionally, all freshly fed and half-grain *An. gambiae* sl. underwent an ELISA analysis to identify the origin of the mammalian host blood meal using antigen to detect human or animal hosts [36]. Furthermore, a subsample of *An. gambiae* sl. collected while resting indoors was analysed for species identification using the method of Santolamazza *et al.* [34].

2.8. Data Analysis

2.8.1. Indoor Resting Density

Hand catch data were used to calculate the mean density of vectors in a room per village arm using the following formula: a number of vectors collected/total number of rooms surveyed [37]. Comparison of the number of *An. gambiae* sl. was collected among the different treatments and groups using the negative binomial regression Z test.

2.8.2. Mortality

The mortality rate of WHO cone bioassays was calculated as the total number of mosquitoes unable to fly (dead) after 3 minutes of exposure divided by the total number of mosquitoes tested, multiplied by 100. When control mortality fell between 5% and 20%, observed mortality was corrected using Abbott's formula. The experiments were repeated when control mortality exceeded 20%. The chi-square test was used to compare mortality.

In the evaluation of the efficacy of each treatment arm in the WHO tunnel assays, the following entomological results were used:

1) Blood feeding refers to the percentage of mosquitoes blood-fed relative to the total collected.

2) Blood-feeding inhibition: This measures the proportional reduction in the percentage of blood feeding in the treated huts compared to the control hut, calculated as $(Pt - Pc)/Pt \times 100$, where Pt represents the proportion of mosquitoes fed blood in the treatment hut, and Pc represents the proportion of mos-

quitoes fed blood in the control hut.

3) Mortality: This indicates the percentage of dead mosquitoes found in a hut in the morning (immediate mortality) or after being caught alive and dead while holding (delayed mortality) in treatment huts out of collected mosquitoes, corrected for control mortality.

The WHO susceptibility data were interpreted as follows [32]:

• If the mortality rate of local *An. gambiae* populations in the permethrin-PBO group was <98%, but the difference between the mortality rates of the permethrin-PBO group and the permethrin only group was less than 10%; No metabolic resistance was detected.

• If the mortality rate of local *An. gambiae* populations in the Permethrin-PBO group was <98%, but the difference between the Permethrin-PBO mortality rates and Permethrin alone was greater than 10%. Metabolic resistance was partially implicated (which implies that a monooxygenase-based resistance mechanism only partially represents the manifestation of the resistant phenotype), indicating the probable presence of other resistance mechanisms in the population under examination.

• If the mortality rate of the local populations of *An. gambiae* was greater than 98% of the in the permethrin-PBO group and the difference between the mortality rates of permethrin-PBO and permethrin alone was greater than 10%. Metabolic resistance was fully involved (which implies that a monooxygenase-based resistance mechanism fully accounts for the expression of the resistant phenotype in the population tested). KDt50 (time required to kill 50% of the test mosquitoes) was estimated by probit analysis [38] and by comparing the KDt50 of field-collected mosquitoes exposed to 0.75% permethrin and the combination of permethrin + PBO.

The following interpretation parameters were used as a guide [32] to determine resistance intensity data.

• At a mortality rate ranging from 98% to 100% of the 5× dose, further tests at the 10× dose 10 are deemed unnecessary, indicating a low resistance intensity.

• A mortality rate of less than 98% of the 5× dose suggests a moderate intensity of resistance, necessitating further testing at the 10× dose.

• A mortality rate in the range of 98% - 100% at the 10× dose confirms a moderate level of resistance intensity. A mortality rate of less than 98% of the 10× dose of 10 indicates a high resistance intensity.

2.8.3. Sporozoite Rate

The sporozoite rate (with a 95% confidence interval) of *An. gambiae* sl was determined using the following formula: (total number of vectors positive for *P. falciparum* sporozoites/number of vectors tested) * 100. The chi-square test was used to compare sporozoite rates.

2.8.4. Human Blood Meal Indices

The human blood index (HBI) of *An. gambiae* sl. was determined, accompanied by a 95% confidence interval, using the following formula: (Total number of

vectors tested positive for human blood meal/Number of vectors tested) * 100. Subsequently, the Chi-square test was employed to compare the HBI.

2.8.5. Entomological Inoculation Rate

The entomological inoculation rate (EIR) may be indirectly estimated from samples collected from indoor resting mosquitoes. The entomological inoculation rate (EIR) can be directly estimated from samples obtained by the human landing catch method and indirectly from samples obtained from indoor resting mosquitoes [37]. In the present study, samples from indoor resting collections were used to indirectly calculate the EIR using the formula EIR = (M * SR * HBI)/N, where M is the mean number of blood-fed mosquitoes per house and N is the mean number of human occupants per house per night [39]. There were 160 total houses surveyed per arm for the study period. The EIR was estimated and compared between the arms of allocation treatments and the control arm.

All entomological parameters of the ITN group were compared between the ITN arm and the control arm at a significance threshold of P = 0.05.

3. Results

3.1. Household Characteristics and ITN Use

Forty households from each arm were surveyed to obtain details about ITN owners, including the gender and educational status of the head of the household, the size of the family, wealth status, the presence of open eaves in the house, the type of bed, the usage status of the nets, the hygienic condition of the LN, and the presence of rodents or cats in the household during the four-month follow-up period. A total of 457 people slept the last night before both surveys in net-owned households, 248 of whom were older than 15, 159 between 5 and 15 years old, and 50 people under 5 years of age. The mean number of human occupants per house per night was approximately 6 people per room per arm. Furthermore, a total of 244 sleeping places were used, with an average of approximately 1 to 8 places per household (Table 1). The mosquito nets were all found inside the rooms (bedrooms), which are loosely sleeping places (70 ITNs, including 36 Permethrin + PBO ITNs and 34 permethrin only ITNs). All owners suggested that they had no adverse effects from using the net. However, 1 owner of the permethrin only ITN, who used it alone, reported that he had a running nose during the first three nights of ITN use.

Table 1. Characteristics of households.

	N	%
Total ITNs surveyed		
	80	80
Population size by ITNs		
Permethrin + PBO ITNs	40	50
permethrin only ITNs	40	50

Continued

Gender of head of households		
Male	44	55
Female	36	45
Education Head of HH		
No	45	56.25
Primary	17	21.25
Secondary or more	5	6.25
Others (religious school)	13	16.25
Socio-economic condition of HH		
Use of electricity	63/80	80
WC shared pit	80/80	100
Own pit latrine	9/60	22.5
Water well protected	80/80	100
No. of people sleeping under the nets the night previous surveys		
Permethrin + PBO ITNs	234	54.73
permethrin only ITNs	223	45.27
Age distribution of people sleeping under the net the previous night the surveys (N = 457)		
Less than 5 years	50	10.94
5 - 15 years	159	34.79
More than 15 years	248	54.27
Mean number of human occupants per house per night		
	6	6
Most common type of sleeping place		
Wooden bed with mattress	45	56.25
Wooden bed with bench	10	12.5
Wooden bed without mattress	4	5
Foam mattress only	4	5
Wall structure		
Mud bricks	18	45
Cement bricks	10	25
Painting	11	27.5
Smoothed with clay mud	1	2.5
Soil structure		
Sand or soil	22	55
Cement	18	45
Roof structure		
Corrugated iron	40	100
Appreciation of ITNs by owners		
Color Permethrin + PBO ITNs	80/80	100
Permethrin only ITNs	80/80	100
Mesh size Permethrin + PBO ITNs	71/80	88.75
Permethrin only ITNs	66/80	82.5

To evaluate the ITN conditions, the median proportionate hole index (pHI) of the holed nets and the interquartile range (IQR) were used. Physical examination of the nets after four months of field use revealed that most of the permethrin only ITNs (87.5%; 35/40 with pHI = 19.67 (IQR: 5.52 - 24.775)) and permethrin only ITNs (82.5%; 33/40 with pHI = 53.95 (IQR 28 - 66.25)) were in good condition, showing no holes. Two Permethrin + PBO ITNs and three permethrin only ITNs were classified as "need replacement," indicating excessive tearing (Table 2). Only five nets of each brand had holes ranging in size from 1 to 4. The total number of holes (in all categories) was 24 for permethrin + PBO ITNs and 29 for permethrin only ITNs. There were more holes of size-2 and 3 in the permethrin only ITNs than in the permethrin + PBO ITNs. Permethrin + PBO ITNs had more holes than permethrin only ITNs. Most holes were found in the bottoms of both ITNs due to abrasion. For the permethrin only ITNs, some holes were found in the upper region at the horizontal level of the ITNs, as previously described. The holes in these ITNs were caused by children (for 2 ITNs) and sharp objects (for 2 ITNs). The size 2 holes at the bottom of the mosquito net were the most numerous and were observed in 3 ITNs of each size. The physical conditions of the ITNs are summarised in Table 2.

Table 2. ITNs physical condition.

	permethrin + PBO ITNs (N = 40)	permethrin only ITNs (N = 40)	P-Value
Number of holes			
Total number of nets with no holes	35	33	0.89
Total number of nets with hole	5	7	
Category of holes	26	29	
Size-1 hole	15	8	0.005
Size-2 hole	8	16	
Size-3 hole	3	3	
Size-4 hole	0	2	
Location of holes			
Roof	0	0	
Upper	5	1	
Lower	19	28	
Seams	0	0	
Number of ITNs categorised according to conditions			
pHI < 64 ("good")	35	33	0.23
pHI < 642 ("serviceable")	3	4	
pHI > 642 ("need replacement")	2	3	
Median pHI (IQR)	19.67 (IQR: 5.52 - 24.775)	53.95 (IQR 28 - 66.25)	0.001

3.2. Composition of Vector Species

Between June and December 2022, a total of 5953 Culicine mosquitoes were collected in all groups. The most prevalent species collected was *An. gambiae* s.l. (4500; 75.59%), followed by *Culex* spp. (1413; 23.13%) and *Mansonia* spp. (2; 0.02%) (Table 3). Significant differences in the total number of *An. gambiae sl* were collected between the ITN groups and between the intervention (ITN groups) and the control arms (P = 0.003). PCR analysis using the method of Santolamazza *et al.* (2008) revealed that *A. coluzzii* was the predominant species within the *An. gambiae* complex, followed by *An. gambiae*. In particular, no *A. arabiensis* species were detected during this period.

Table 3. Species compositions of the three arms collected after the net distribution.

Treatment N (%)	Control	permethrin only ITNs	permethrin + PBO ITNs	Total per specie	P-value
***An. gambiae* s.l.**	1799	1,404	1297	4500 (75.59)	0.003
Culex spp.	615	412	386	1413 (23.73)	0.041
Mansonia spp.	0	20	20	40 (0.68)	0.2
Total number per arm	**2414**	**1836**	**1703**	**5953**	

3.3. Effect of ITN Treatment Types on Indoor Resting Density, Sporozoite Rates, Blood Meal Hosts, and Delayed Mortality of *An. gambiae* sl.

During the study period, the indoor resting density of *An. gambiae* sl were found to be not significantly different between the permethrin and permethrin + PBO treatment groups, with 10.96 ± 0.9 and 10.13 ± 0.7 *An. gambiae* sl captured per room/day, respectively (P = 0.21) (Table 4). When analysed, the indoor resting density of the intervention group (permethrin alone and permethrin + PBO) and the control group, a statistical difference was observed (P = 0.03). The sporozoite rate was found to be similar between the permethrin only group and the permethrin + PBO ITN groups, with rates of 4.17% (95% CI 0.6 - 7.7) and 2.5% (95% CI 0.3 - 5.3), respectively (P = 0.47) (Table 4). A similar trend was observed when comparing the intervention groups and the control group (P = 0.39). Furthermore, the human blood index was slightly lower in the permethrin + PBO ITN group at 7.5% (95% CI 3.8 - 11.2) compared to the permethrin only ITN group at 9% (95% CI 5 - 13); however, no significant differences were observed (P = 0.58). In fact, the rate of entomological inoculation was not calculated for the groups due to the low number of infected mosquitoes found per group. Furthermore, the mortality of *An. gambiae* female caught in control houses did not exceed 10%. However, when caught immediately, a low proportion of female *An. gambiae* was found dead in the permethrin + PBO ITN arm (mean 69.37%; 95% CI: 59.78 - 80) compared to the permethrin only ITN arm (mean 61.11%; 95% CI: 53.28 - 70.01) but there was no significant difference (P = 0.312). However, after 24 h of holding in the lab insectary, there was a significant mortality rate of *An. gambiae* sl in the permethrin + PBO ITN arm (mean

90.31; 95% CI: 80.1 - 100) than in the permethrin only ITN arm (mean 72.13; 95%CI: 68.30 - 77.83 with P = 0.018) (Figure 3).

Table 4. Indoor resting density, sporozoite rates and human blood meal indices of *Anopheles gambiae* s.l. collected from three arms.

ITNs treatment arms	Total resting indoors	Collection day	*An. gambiae* s.l. per room per day (±SE)	P-value
Indoor resting density (IRD)of An. gambiae s.l.				
Control*	1799	128	14.05 ± 1.8	
Permethrin only*	1404	128	10.96 ± 0.9	***0.03***
Permethrin + PBO*	1297	128	10.13 ± 0.7	
ITNs treatment arms	**Number tested**	**Number positive sporozoïte**	**% sporozoïte positive [95% CI]**	**P-value**
An. gambiae s.l. sporozoite rate (SR)				
Control	120	8	6.67 [2.2 - 11.1]	
Permethrin only	120	5	4.17 [0.6 - 7.7]	***0.47***
Permethrin + PBO	120	3	2.5 [0.3 - 5.3]	
ITNs treatment arms	**Number tested**	**Number positive Human**	**% HBI [95% CI]**	**P-Value**
Human bloodmeal index (HBI)				
Control arm	200	21	10.5 [6.3 - 14.7]	
Permethrin only ITNs arm	200	18	9 [5 - 13]	
Permethrin + PBO ITNs arm	200	15	7.5 [3.8 - 11.2]	***0.58***

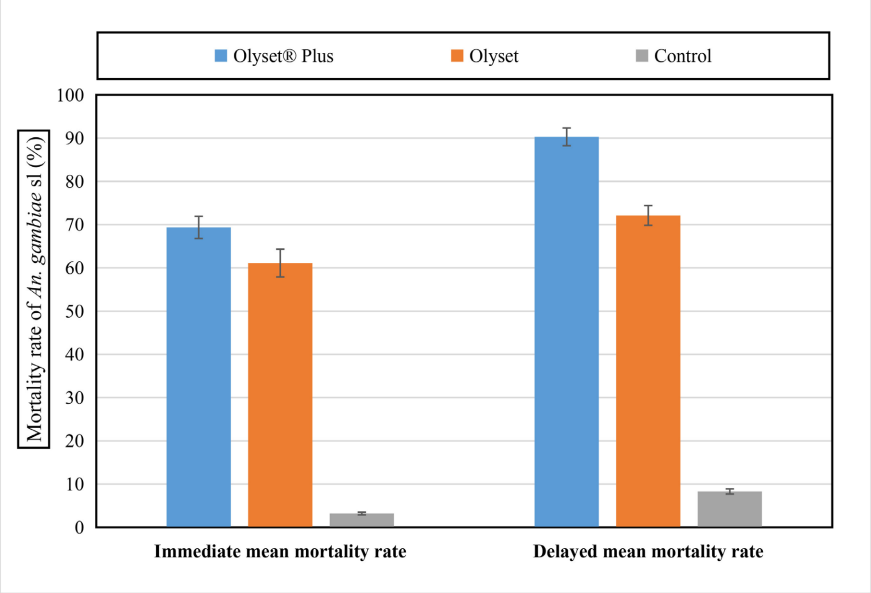

Figure 3. Allocation of treatment in clusters (Bolosso, Di and Massawé) in Bama, Vallée du Kou in Burkina Faso.

3.4. Biological Efficacy of ITNs

Bioassays carried out with the Kisumu susceptible strain of *An. gambiae* resulted in 100% mortality and knockdown rates at the start of the study (**Figure 4**). In general, the mean knockdown between both observed ITNs was not significantly different (P = 0.7681) with *An. gambiae sl.* When mean mortality rates compared both ITNs, a significant difference was observed (P = 0.0076). Additionally, significant mortality rates were observed in the wild *An. gambiae* population during the five months between both ITNs, as indicated by the results of the WHO cone bioassays. The comparison of the mortality rate for permethrin only ITN 89.8% (95% CI 87.4 - 90.9) and 93.4% (95% CI 91.5 - 25.5) for Permethrin + PBO ITN was significant between both ITNs, again showing the role of PBO in improving the efficacy of pyrethroids ITNs (**Figure 4**). Otherwise, after the months of assays, the mortality rate of *An. gambiae sl.* has decreased in the first month after distribution ranging from 2.88% (95% CI: 0.96 - 4.12) for permethrin only ITNs to 26.05% (95% CI: 17.89 - 32.45) for Permethrin + PBO ITNs. This could be explained by abrasion because the ITNs found were very dirty and the WHO protocol does not recommend washing the nets prior to testing (these nets were therefore tested dirty, which might underestimate the performance compared to if tested washed). These ITNs from the first month 1 were tested by tunnel tests to determine blood feeding inhibition rates. With the exception of this month, the mean mortality rate of *An. gambiae* sl. exceeded 80%, indicating that both ITNs provided personal protection against the wild population of vectors from the study site.

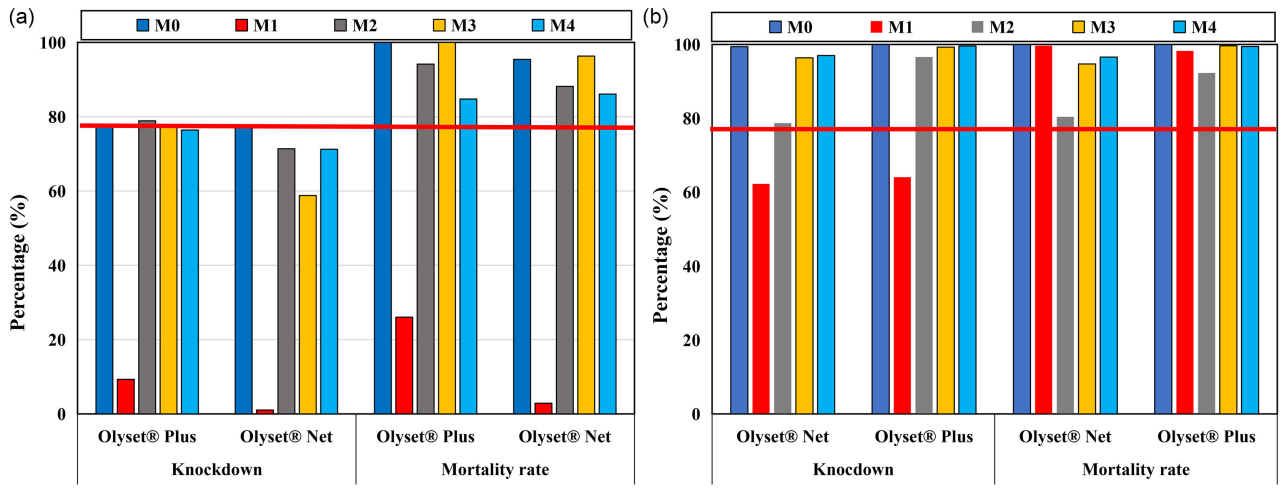

Figure 4. Average knockdown rate (KD_{60}) and mortality rate of (a) susceptible and (b) wild populations of *An. gambiae* sl based on 3-minute exposure to permethrin + PBO ITNs and permethrin only ITNs in WHO cone bioassays at baseline and the following months post-intervention.

Furthermore, the efficacy of permethrin + PBO and permethrin only ITNs, which initially failed to meet the WHO threshold in cone bioassays after the first distribution of ITN, was assessed using the WHO tunnel test with a guinea pig.

These ITNs were evaluated with a wild population of *An. gambiae* from the study sites. In tunnel tests, penetration rates were 57.14% and 35.9%, respectively, representing the proportion of *An. gambiae* sl. mosquitoes that successfully traversed untreated ITNs and permethrin only ITNs (Olyset® Net) to obtain a blood meal. No mosquitoes were able to penetrate permethrin + PBO ITNs (Olyset® Plus). Furthermore, the mean mortality rate of *An gambiae sl.* The percentage of *An. gambiae sl.* exposed to permethrin + PBO ITN was 100%, which was significantly higher (P = 0.0028) than that exposed to permethrin only ITN (Olyset® Net), which was 76.91% (95% CI: 66.58 - 81.29). Furthermore, the blood-feeding inhibition rate (BFI), derived from the blood-feeding rate, was significantly higher for 100% of permethrin + PBO ITN than for 66.81% of permethrin only ITNs. These results demonstrated that permethrin + PBO ITNs provided greater personal protection to permethrin only ITNs. The permethrin + PBO ITN samples successfully met the WHO tunnel test criteria, since both the mortality rate and the inhibition of blood feeding exceeded 80% in contrast to those of the permethrin only ITNs.

3.5. *Anopheles gambiae* sl. Susceptibility Status and Evidence for Metabolic Resistance

The susceptibility assays of *Anopheles gambiae* sl. carried out using a diagnostic dose of the insecticide permethrin revealed a high level of resistance in the population of *An. gambiae* at the study sites, with a mean reduction of 3.9 (95% CI: –4.92 - 12.72) and a mean mortality rate of 2.6 (95% CI: 2.26 - 7.47) (Table 5). Furthermore, wild populations of *An. gambiae* showed high resistance intensity according to the results of the WHO bioassay intensity test at 10x the diagnostic dose of permethrin (Figure 5). However, preexposure to wild populations of *An. gambiae* followed by exposure to permethrin resulted in greater mortality in the permethrin + PBO group (mean mortality rate = 36.25 with 95% CI: 6.43 - 66.06) than in the permethrin only group (mean mortality rate = 3.90 with 95% CI: –4.92 - 12.72) (Table 6), and this difference was statistically significant (P = 0.036). Similar results were observed for the mean reduction in both insecticide groups (P = 0.0049) (Table 6).

Table 5. Mean penetration rate, blood-feeding rates and corrected mortality rates of pyrethroid-resistant *Anopheles gambiae* s.l. exposed to ITN samples using WHO tunnel tests.

Mosquito population	ITNs tested	Number Tested	Penetration rate [95% CI]	Blood-feeding rate [95% CI]	Mortality rate [95% CI]
An.gambiae s.l.	Untreated ITNs	10	57.14% [49.11 - 68.24]	79.6% [71.48 - 88.25]	2.04 [1.03 - 3.29]
	Permethrin + PBO ITNs	10	0%	0%	100%
	Permethrin only ITNs	10	35.90% [28.33 - 39.26]	26.42% [23.45 - 31.08]	76.91% [66.58 - 81.29]

Table 6. Mean knockdown, mortality rate and knockdown times of wild-caught *Anopheles gambiae* s.l. local populations exposed to diagnostic doses of 0.75% permethrin and 4% PBO + 0.75% Permethrin from WHO tube bioassays.

insecticides tested	Mortality rate (%)		Knockdown (Kd) 60 mn (%)		Knockdown Time (KDt) 50 (mn)	
	Mean [95% CI]	P-value	Mean [95% CI]	P-value	Mean (±SE)	P-value
0.75% Permethrin (N = 85)	2.61 [−2.26 - 7.47]	***0.036***	3.90 [−4.92 - 8.72]	***0.0049***	256±47	***0.001***
4% PBO + 0.75% Permethrin (N = 102)	36.25 [6.43 - 66.07]		21.04 [11.03 - 31.04]		139±21	

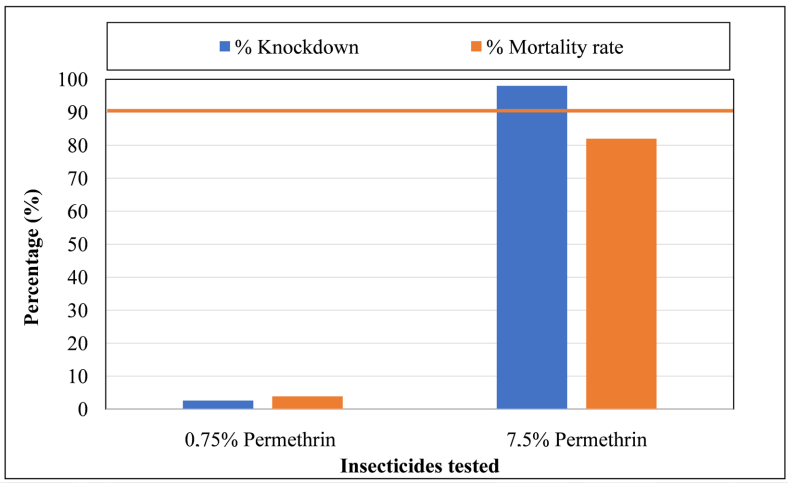

Figure 5. Mortality of *An. gambiae* sl after 24 h post-exposure to 0.75 permethrin (diagnostic dose) and 7.5% permethrin (10x diagnostic dose) in WHO tube bioassays.

In terms of logarithmic time probit regression analysis, the KDt50 values for permethrin were greater than 256 min without synergists (Table 6), while preexposure to PBO led to a significant decrease in the KDt50 values to 139 min (P = 0.001). However, the KDt95 value was much longer, so it was not taken into account. Furthermore, while the increase in mortality in the permethrin + PBO group did not reach 98%, the difference between each mortality exceeded 10%. This suggests that metabolic resistance is partially involved, indicating that a monooxygenase-based resistance mechanism only partially reflects the expression of the resistant phenotype and that other resistance mechanisms are likely to be present in the tested population. Indeed, the detection of allelic frequencies of *kdr* mutations confirmed a high level of resistance to pyrethroids, ranging from 0.8 to 0.9, in the study area (Table 7).

Table 7. Distribution and frequency of the L1014F knockdown resistance (*kdr*) alleles of *An. gambiae* complex members of the study area.

	Genotype and frequency of the *kdr* alleles (%)					
Species	**N**	**SS**	**RS**	**RR**	**F(R)**	**[CI 95%]**
An. arabiensis	0	-	-	-	-	-
An. coluzzii	44	3	3	38	0.897	[0.88 - 0.94]
An. gambiae	5	1	0	4	0.80	[0.53 - 0.85]
An.coluzzii/An. gambiae	1	0	0	1	-	-

N: number of mosquitoes analysed; F(R): frequency of the 1014F resistant *kdr* allele.

4. Discussion

Anopheles gambiae sl. was the predominant species present in the study area, and *An. coluzzii* accounted for more than 90% of the *An. gambiae* complex. Several studies on species composition carried out in this area reported similar results [26] [27]. Indeed, this species generally colonises rice-growing areas [40] [41]. The resistance of *An. gambiae* s.l. to permethrin was notably high, characterised by a high resistance intensity and elevated allele frequencies of the *kdr* gene in the study area. These findings suggest that the combination of PBO and pyrethroids should have a substantial impact on resistant malaria vectors. The susceptibility of *An. gambiae* sl. to permethrin did not fully restore the significant increase in mortality of wild populations of *An. gambiae*, an important role was observed in oxidase-based resistance, although other mechanisms are likely involved. The hypothesis that ITNs containing PBO would result in a greater proportion of malaria vectors being killed than pyrethroid-based ITNs alone has been confirmed, as evidenced by the reduced number of mosquitoes collected in the permethrin + PBO ITN arm compared to the permethrin only ITN arm. Indeed, pyrethroid + PBO ITNs are predicted to have a substantial impact on vectorial capacity by reducing the number of mosquitoes that survive the intrinsic parasite incubation period (monitored by parity and sporozoite rate) and reducing the human bite rate (monitored by a collection of pyrethrum spray catches) [42]. Furthermore, in this study, indoor resting densities were used as a proxy for human biting rates due to the difficulties associated with conducting large-scale human landing catches. The use of indoor resting density as a proxy for biting rate is described by the WHO and has been used in several studies to determine the impact of interventions [43]. This is considered an appropriate proxy for endophilic species, where few blood-fed mosquitoes are likely to emerge before the morning residual fauna is collected [44] (WHO, 2013). However, a limitation of the study is the absence of data collected on mosquitoes actively searching for a host, as well as the lack of data collected using outdoor sampling methods. The resting densities were similar to those of the intervention arms, which could indicate that mosquitoes were looking for host-seeking humans, could have fed goats outdoors and rested indoors. Furthermore, no evidence indicated a difference in sporozoite counts between the permethrin + PBO combination group and the permethrin alone group. In certain regions of Burkina Faso, it is common for people to sleep outdoors without a mosquito net during the dry season to escape the hot and humid indoor conditions. Monitoring host preference for blood feeding was primarily aimed at determining whether the use of combined pyrethroids ITNs diverted vectors to feed on non-human hosts. The human blood feeding index of *An. gambiae* sl was surprisingly low in all cases, ranging from 7.5% to 9%. These results suggest that many mosquitoes feed on other hosts, such as goats, sheep, and cattle. Additionally, several mosquito control tools can be used to reduce the feeding of mosquitoes on humans. Furthermore, the relatively low human blood indices in both cases can be

attributed to the high coverage of ITN and proximity to livestock, resulting in opportunistic feeding patterns. A small, similar difference is unlikely to signify a significant change in feeding behaviour. Furthermore, the sporozoite rates for nets treated (permethrin alone and permethrin + PBO) with permethrin, at 2.5% and 4.17% (mono-treated and combined), were lower than those for untreated nets (6.67%). This corroborates claims that treated nets, in addition to being a physical barrier, are responsible for the high mortality of mosquitoes in houses [45].

During insecticide susceptibility assays, a high level of resistance was observed for permethrin, which poses a potential challenge to the effectiveness of permethrin and other pyrethroid-based control tools in this region. However, the addition of PBO increased the mortality of resistant populations of *An. gambiae* sl. (N'Guessan *et al.*, 2010) as confirmed by increased delayed mortality of primary vectors observed. This finding showed the effectiveness of permethrin + PBO ITNs in real conditions of use in this study, highlighting the need to switch to new generation mosquito nets in Burkina Faso. Until now, many studies have demonstrated this statement by experimental hut studies. In fact, a report from Benin demonstrated that permethrin + PBO ITN offered superior control of *An. gambiae* sl. compared to permethrin ITN [46]. Additional experimental trials conducted in India and Tanzania as part of the WHOPES evaluation process targeting susceptible *Anopheles* did not provide evidence indicating an improvement in combined ITNs over mono-treated ITNs. A single published village-scale study conducted in Nigeria revealed that a 12-month trial at the village level had a more pronounced impact on vector resting density, sporozoite rates, and parity in a village with deltamethrin + PBO ITNs than in a village with deltamethrin-only ITNs. This was also observed in an area with pyrethroid-resistant *An. gambiae* sl., attributed to both Vgsc-1014F and MFO [47]. Our study aimed to provide scientific evidence on the efficacy of the combination of permethrin + PBO ITN before its widespread deployment by the National Malaria Control Program. In this study, the biological efficacy of the permethrin + PBO combination was demonstrated [24]. Even if susceptibility assay data showed a high resistance intensity of malaria vectors from the study area, it is not clear whether this state could compromise the effectiveness of vector control tool-based permethrin. Several studies are needed in different ecological areas to confirm these findings. This trial represents one of the initial village-level evaluations of the field performance of PBO ITNs in Burkina Faso. In addition, this is the first trial to provide evidence suggesting that the inclusion of synergist PBO in long-lasting insecticidal nets offers enhanced community protection compared to standard pyrethroid-only nets, particularly against malaria transmission by pyrethroid-resistant vector populations.

5. Conclusion

Preexposure to PBO followed by permethrin improved mortality, but did not

fully restore vector susceptibility to permethrin. However, it did provide personal protection, as demonstrated in WHO cone bioassays. Furthermore, ITNs containing PBO may have a greater impact in areas where mixed-function oxidases play a significant role in pyrethroid resistance. This trial provides evidence to increase the coverage of PBO ITNs over standard pyrethroids ITNs to address the growing challenge of pyrethroid resistance and to improve personal and community protection from malaria, particularly in areas of intense resistance to pyrethroids.

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Authors' Contributions

AD designed the study. ASH and SM contributed to the implementation of the study. SM, AYO, RB, and GBM performed laboratories and fieldwork. ASH, KB, DDS and SM analysed the data. ASH interpreted the results and wrote the manuscript. KB, MN, TC, RB, LPT, GAO, RKD, and AD oversaw data collection and reviewed the manuscript. All authors read and approved the final manuscript.

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Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

The trial protocol was reviewed and approved under N˚2019-15 /MS/SG/INSP/DG/CEI by the institutional human ethical committee of the Muraz Centre, Bobo-Dioulasso, for the participation of human volunteers in this study. Informed consent was obtained from each human volunteer who caught mosquitoes prior to their participation and heads of households who were allowed to enter their homes. Throughout the course of the study, they were regularly examined for signs of fever by a standby nurse; Any sleepers who tested positive for malaria were withdrawn from the study and treated properly.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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