frontiers | Frontiers in Tropical Diseases

TYPE Original Research
PUBLISHED 30 January 2026
DOI 10.3389/fitd.2026.1764297

OPEN ACCESS

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RECEIVED 09 December 2025
REVISED 03 January 2026
ACCEPTED 09 January 2026
PUBLISHED 30 January 2026

CITATION
Machani MG, Zhou G, Oyweri J, Nzioki I, Githure J, Atieli H, Wang C, Zhong D, Lee M-C, Afrane YA and Yan G (2026) Reduced bio-efficacy of aged PermaNet® 3.0 Nets against local pyrethroid-resistant *Anopheles arabiensis* in Western Kenya. *Front. Trop. Dis.* 7:1764297. doi: 10.3389/fitd.2026.1764297

Reduced bio-efficacy of aged PermaNet® 3.0 Nets against local pyrethroid-resistant *Anopheles arabiensis* in Western Kenya

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Background: Long-lasting insecticidal nets (LLINs) are central to malaria control and designed to protect for up to three years; however, increasing pyrethroid resistance undermines their effectiveness. Piperonyl butoxide (PBO) LLINs, designed to enhance pyrethroid efficacy, are recommended in high-resistance areas, yet their long-term operational bio-efficacy remains unclear. This study evaluated the killing efficacy of PBO LLINs over three years of field use compared to standard LLINs and monitored insecticide resistance in local *Anopheles gambiae* s.l. populations.

Methods: During a trial in Muhoroni, western Kenya (2021–2024), standard and PBO LLINs were collected at 6, 18, and 36 months of use. Residual bio-efficacy was assessed using WHO cone bioassays against a susceptible *Anopheles gambiae* s.s. Kisumu strain and field *An. gambiae* s.l. populations. WHO tube and bottle assays determined insecticide resistance, while synergist assay assessed metabolic resistance. Quantitative polymerase chain reaction detected target-site mutations (*kdr*-1014 F/S and *Ace-1* G119S).

Results: Molecular identification confirmed all *An. gambiae* s.l. were *Anopheles arabiensis*, showing increasing pyrethroid resistance, with deltamethrin mortality declining from 96.3% (2021) to 22.7% (2024) while remaining susceptible to pirimiphos-methyl and clothianidin. PBO pre-exposure restored deltamethrin mortality from 22.7% to 98.9%. The frequency of 1014F increased from 0.09 to 0.17 and 1014S from 0.04 to 0.06, with no *Ace-1* mutations detected. Standard LLINs retained >80% efficacy against the susceptible strain for 18 months but were below threshold against field mosquitoes even when new. PBO LLINs were effective at baseline against field populations but declined sharply, with mortality dropping to 24% by 6 months. Overall, both net types exhibited a marked decline in killing efficacy over time against field mosquitoes, with mortality falling < 20% within six months.

Conclusion: *Anopheles arabiensis* showed increasing pyrethroid resistance, driven largely by metabolic mechanisms. Standard LLINs showed suboptimal killing efficacy against field populations, while PBO LLINs achieved high baseline efficacy but declined significantly by six months. These findings indicate that PBO

LLINs can improve protection against resistant vectors but may be insufficient in high-resistance areas, underscoring the need for alternative non-pyrethroid interventions, strategic deployment, and revised LLIN re-distribution cycles aligned with their functional lifespan.

KEYWORDS

anopheles, bio-efficacy, insecticide resistance, PBO LLINs, standard LLINs, Western Kenya

Background

Despite decades of progress, malaria persists across sub-Saharan Africa, where more than 90% of global cases still occur (1). In western Kenya, malaria transmission remains high, even with widespread deployment of LLINs and indoor residual spraying (IRS) (2). This persistent transmission is largely sustained by highly efficient vectors (*Anopheles gambiae*, *Anopheles arabiensis*, and *Anopheles funestus*) which exhibit remarkable ecological adaptability (3). These species have developed physiological resistance that enables them to survive exposure to commonly used insecticide, along with behavioral plasticity that allows them to evade or reduce contact with insecticide-based interventions (4–6). As a result, the protective efficacy of conventional interventions (7) is being progressively eroded in areas of intense transmission.

To safeguard the gains of the past two decades, next-generation LLINs incorporating dual active ingredients or synergists have been developed and recommended as a strategic response to sustain efficacy against insecticide-resistant mosquito populations (1). Some of these new LLINs are now being scaled up in malaria-endemic regions in Africa (8). For instance, LLINs incorporating the synergist piperonyl butoxide (PBO) have been recommended for use in areas with high metabolic insecticide resistance to restore pyrethroid effectiveness (9). In settings where IRS has been withdrawn, PBO LLINs are considered a superior alternative to standard LLINs, improving vector control outcomes and contributing to the sustained reduction of malaria transmission (10). PBO functions by inhibiting cytochrome P450 monooxygenases (key enzymes involved in pyrethroid detoxification), thereby restoring the susceptibility of resistant mosquito populations and enhancing the overall efficacy of pyrethroid-based nets (11). Evidence from experimental hut trials and cluster-randomized community studies demonstrates that PBO LLINs provide superior protection compared to standard LLINs, supporting their endorsement by the WHO and their distribution in several African countries (10, 12). Nevertheless, most evidence has been derived under controlled trial conditions (13–15), with limited evaluation of PBO LLINs performance under routine household use in high-transmission, rural settings.

Long-lasting insecticidal nets are distributed every three years through mass campaigns, as their durability and insecticidal effectiveness are expected to decline after this period (16). While standard LLINs have been shown to retain insecticidal activity for at least 20 washes under laboratory testing and they are expected to last up to three years under field use, however, empirical evidence indicates that both bio-efficacy and fabric integrity often decline faster than anticipated under operational use (17, 18). For PBO LLINs, in particular, questions remain about how long their added synergistic benefit is retained under operational use. Despite large-scale deployments, few studies have systematically monitored the residual bio-efficacy of PBO LLINs over time against wild mosquito populations. Such evidence is critical for informing National Malaria Control Programs (NMCPs) to optimize LLIN replacement strategies and sustain community-level protection. This study therefore assessed the field performance of PBO LLINs at multiple time points post-distribution in rural western Kenya, providing operational evidence on their durability and effectiveness against locally dominant malaria vectors.

Methods

Study site

The study was conducted in Muhoroni sub-County, Kisumu County, Kenya, located in the Kano Plain, at an elevation of 1,120–1,140 meters above sea level. The area is characterized by extensive sugarcane plantations and rice fields, which are often flooded during the rainy season, creating suitable habitats for mosquito breeding. Muhoroni experiences perennial malaria transmission, with *Anopheles arabiensis* as the dominant vector and *Plasmodium falciparum* accounting for 95.2% of infections (19), and standard LLINs serving as the main malaria control strategy. The study monitored standard LLINs distributed through Ministry of Health mass campaigns and PBO LLINs provided by the ICEMR program as part of a cluster-randomized trial evaluating optimal combinations of vector control interventions for malaria control between March 2021 and August 2024 (20). Standard LLINs were distributed by community health promoters and health facility staff during routine mass campaigns led by the Ministry of Health. In clusters assigned to PBO LLINs, participating household received WHO-certified PermaNet® 3.0 (PBO polyester) nets to replace standard LLINs, ensuring adequate coverage of one net per two people or one net per sleeping space.

10.3389/fitd.2026.1764297

Mosquito used for the study

Anopheles gambiae s.l. larvae were collected from at least four clusters per intervention arm and pooled in the laboratory. Larvae were reared to adulthood under controlled conditions (27 ± 1 °C, 70 ± 10%, 12:12 h light: dark cycle) and fed on a mixture of fish food and yeast until pupation. Emerging adults were maintained on 10% sugar solution. Similarly, the susceptible *An. gambiae* s.s. Kisumu strain used as control was raised and maintained under the same conditions.

Sampling of long-lasting insecticidal nets

Nets were randomly retrieved from clusters assigned to either PBO LLINs or standard LLINs, with cluster selection and randomization procedures detailed elsewhere (20). Nets of different operational ages were included: 0, 6, 18, and 36 months post-distribution. At each time point, 13 nets were randomly collected from households within each study arm (PBO and standard) and replaced with equivalent new nets. The standard LLINs included Permanent® 2.0 (polyester, deltamethrin-coated fibers at 55 mg/m²), Yorkool® (polyester 100-denier, deltamethrin 55 mg/m²), and Dawa Tana (polyester 100-denier, deltamethrin 2.0 g/kg ± 25%, corresponding to 80 mg/m²). The PBO LLIN was Permanent® 3.0, with a polyethylene roof incorporating deltamethrin (2.8 g/kg ± 25%) and PBO (4.0 g/kg ± 25%) into the fibers, and polyester lateral sides coated with deltamethrin (2.8 g/kg ± 25%). Community Health Promoters (CHPs) supported the trial by sensitizing households on the importance of retaining nets and subsequent utilization.

Phenotypic resistance monitoring

Phenotypic resistance was assessed using the WHO standard tube test (21) with insecticides commonly used for LLINs and IRS. *Anopheles gambiae* s.l. females, 3–5 days old, reared from field-collected larvae, were tested against deltamethrin (0.05%) annually from 2021 (pre-trial) to 2024. In 2024, additional bioassays were conducted using permethrin (0.75%), PBO 4% + deltamethrin (0.05%), clothianidin (X1), and pirimiphos-methyl (0.25%). For each insecticide, four replicates of 20–25 unfed females were exposed to insecticide-impregnated papers for one hour following WHO protocols (Supplementary Table S1). For synergist assays, mosquitoes were pre-exposed to PBO-impregnated papers for one hour prior to exposure to deltamethrin to assess metabolic resistance. *Anopheles gambiae* Kisumu strain was included as a reference susceptible control in all bioassays. Mortality was recorded 24 h post-exposure, and resistance status was classified according to WHO criteria (21).

Net bio-efficacy assessment

Bio-efficacy of sampled nets, including both standard LLINs and PBO LLINs, was evaluated using cone test to determine the ability of insecticides on the roof and sides to induce mosquito mortality. Five pieces (25 × 25 cm each) were cut from standardized positions (positions 1–5) on each net, as specified by the WHO Pesticide Evaluation Scheme (16). Adult female *Anopheles gambiae* s.l., 3–5 days old, reared from field-collected larvae, and susceptible *Anopheles gambiae* s.s. Kisumu strain were used for the assays. For each net piece, a WHO cone was fitted, and five non-blood-fed females were introduced and exposed for 3 minutes. Following exposure, mosquitoes were carefully transferred to separate plastic cups and maintained under standard conditions. Mortality was recorded 24 h post-exposure. The bio-efficacy of each net was determined as the average mortality across the five net pieces. Mosquitoes exposed to untreated nets served as negative control.

Molecular analysis

A sub-sample of surviving and susceptible *An. gambiae* s.l. mosquitoes were randomly selected for species identification after exposure to the WHO tube bioassay. Genomic DNA was extracted from individual mosquitoes following previous methods (22). Molecular identification of sibling species of respective *An. gambiae* s.l. were conducted based on PCR methods described by Scott et al. (23).

Data analysis

Mosquitoes were considered susceptible if mortality was ≥98% and resistant if mortality was <90%. Mortality rates between 90% and 97% indicated possible resistance, requiring confirmation through additional tests (21). If the mortality in controls was < 10%, the results were adjusted by Abbott's formula. If the mortality in controls were > 10%, the results were considered invalid and were discarded. The allele frequencies for resistant genotypes were calculated using the Hardy-Weinberg equilibrium equation. Mosquito populations were checked to determine whether they were in Hardy-Weinberg equilibrium using χ^2 test. For bio-efficacy, mortality rate was first transformed using arcsine transformation. Data was analyzed using analysis of variance (ANOVA) with repeated measures and Tukey-Kramer HSD test used to compare mean mortalities among different time points. Data analysis was performed using the open-source R programming language software R version 4.2.3.

Results

Phenotypic resistance profile

All 600 randomly selected *An. gambiae* s.l. mosquitoes (survivors and susceptible) were confirmed by PCR as *Anopheles arabiensis*, confirming its predominance in the study area throughout the monitoring period. In early 2021, prior to the distribution of PBO nets, mortality of *An. arabiensis* exposed to 0.05% deltamethrin was 96.3 ± 4.3% (mean ± SD, n = 12 replicate tests) (Figure 1A). However, resistance increased sharply following

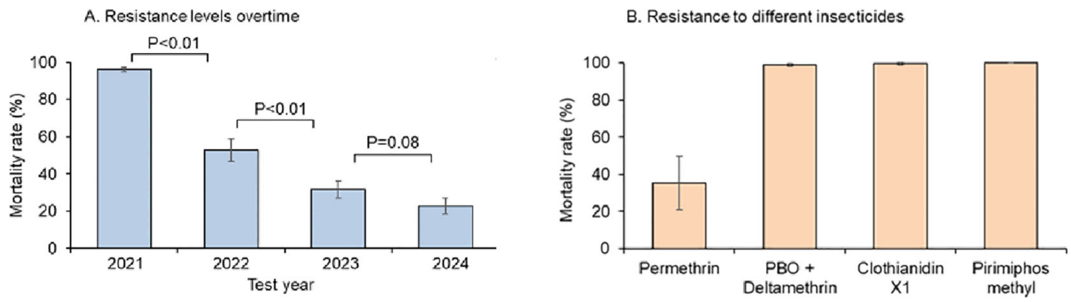

FIGURE 1
(A) Field collected *Anopheles gambiae* s.l. female mortality against deltamethrin from 2021 to 2024; and **(B)** Mosquito mortality against different insecticides used for bed nets and indoor residual spraying.

subsequent years of LLIN use: mortality declined to 53.0 ± 6.0% in 2022 (n = 13), followed by 31.5 ± 4.5% in 2023 (n = 12) and 22.7 ± 4.2% in 2024 (n = 15). No significant difference in mortality was observed between 2023 and 2024 (p = 0.08, Figure 1A). Remarkably, pre-exposure to the synergist PBO in 2024, restored deltamethrin susceptibility with mortality increasing to 98.9 ± 0.4%, confirming role of oxidase mediated resistance mechanism (Figure 1B).

Resistance to other pyrethroid was evident in 2024, with *An. arabiensis* showing reduced susceptibility to permethrin with mortality of 35.2 ± 7.3% against the WHO standard diagnostic concentration (Figure 1B). In contrast, exposure to non-pyrethroid insecticides used for indoor residual spraying showed full susceptibility: mortality was 100% against clothianidin (neonicotinoid) and 99.7 ± 0.3% against pirimiphos-methyl (organophosphate) (Figure 1B).

Knock-down and *Ace*-1 allele frequencies

A total of 350 *Anopheles arabiensis* specimens were successfully genotyped for the three target mutations in 2022 and 2024. Distinct temporal trends in the frequency of *kdr* mutations were observed between the two intervention periods. In 2022, the frequency of the 1014S allele was low (allelic frequency = 0.04), and the 1014F allele frequency was similarly low (0.09). In 2024, the 1014S allele showed a slight increase (0.06), while the 1014F allele exhibited a substantial rise to 0.17 (Table 1). Overall, both loci demonstrated an increase in resistant allele frequencies between 2022 and 2024, with a more pronounced change at the 1014F locus.

Hardy-Weinberg equilibrium analysis showed that none of the *An. arabiensis* populations tested for 1014S were in equilibrium in either year (P < 0.001), likely reflecting heterozygote deficiency. In contrast, the increased heterozygosity observed at the 1014F locus, suggesting active selection and ongoing spread of the resistance allele (Table 2). No *Ace*-1 (G119S) mutations associated with altered acetylcholinesterase were detected in any of the mosquitoes screened.

Bio-efficacy of Standard and PBO LLINs

According to World Health organization Pesticide Evaluation Scheme (WHOPES) guidelines, LLIN bio-efficacy is defined by (i) ≥80% mortality after 24 hours and (ii) ≥95% knockdown (KD) after 60 minutes of exposure (16). For standard LLINs, the mean knockdown rate against the *An. gambiae* s.s. susceptible strain remained high during the first 18 months post-distribution (>95%) but declined below the WHOPES threshold (≥95% knockdown) by 36 months (86.3 ± 2.0%) (Table 3). When tested against field mosquitoes, the nets failed to achieve optimal knockdown efficacy even when new (Mean knockdown rate: 50.2 ± 2.4%), with a progressive reduction to 6.7 ± 1.5% at 36 months post-distribution. Piperonyl butoxide LLINs, knockdown rates for susceptible mosquitoes remained optimal (100%) for up to 18 months but declined (mean knockdown rate: 84.3 ± 5.5%) at 36 months post-distribution. For field mosquitoes, baseline knockdown rate was higher (95.1 ± 4.0%) but decreased markedly to 45 ± 7.8% at 6 months and 25 ± 7.5% at 36 months (Table 1).

In terms of mortality, standard LLINs maintained optimal operational efficacy (>80% mortality) for up to 18 months (91.6%) but dropped below the threshold by 36 months (74.7%) when tested with susceptible mosquitoes (Figure 2A, top-left panel). However, efficacy against field mosquitoes was consistently below the optimal level, with <1-month post-distribution mortality at 64.4%, declining sharply to 13% at 6 months and 10% at 36 months (Figure 2C, bottom-left panel). Similarly, PBO LLINs induced >99% mortality in susceptible mosquitoes up to 18 months post-distribution, followed by a decline to 68.1% at 36 months (Figure 2B, top-right panel). Against field populations, initial efficacy (100%) decreased substantially to 24% at 6 months, 18% at 18 months, and 15% at 36 months post-distribution, well below the 80% threshold (Figure 2D, bottom-left panel). Overall, both net types exhibited a significant decline in killing efficacy over time, with average mosquito mortality dropping below 20% within six months of field use (P < 0.001) (Figures 2C, D, bottom panel).

Discussion

Evaluating the bio-efficacy of long-lasting insecticidal nets (LLINs) under field conditions is critical to ensure that these frontline interventions provide the expected level of protection against malaria vectors in operational settings. In this study, both standard and PBO LLINs demonstrated reduced killing efficacy

TABLE 1 Allele frequency of *Vgsc* (kdr) and *Ace*-1 genes in *An. arabiensis* from Muhoroni western Kenya.

Year	N	*Vgsc* (Locus-1014)							Ace-1 (Locus-119)
		L1014	L1014S	X^2	P-value	L1014F	X^2	P-value	G119S
2022	165	0.87	0.04	118.7	<0.0001	0.09	0.37	0.54	0
2024	185	0.77	0.06	146.8	<0.0001	0.17	0.13	0.72	0

N, number of mosquitoes genotyped; L1014, (Leucine) wild-type allele frequency; L1014S, leucine to serine substitution knockdown resistance (kdr) allele; L1014F, leucine to phenylalanine substitution knockdown resistance (kdr) allele.

against local *An. arabiensis* shortly after deployment (< 6 months), highlighting the challenge of sustaining the operational efficacy of pyrethroid insecticide-treated nets. This diminished effectiveness coincided with an increase in pyrethroid resistance in *An. arabiensis*, dominant malaria vector in the region, underscoring the challenges posed by evolving insecticide resistance in undermining frontline interventions and the need for continuous monitoring coupled with adaptive vector control strategies.

Within the first month of distribution, standard LLINs failed to achieve optimal performance (killing efficacy) against field-collected mosquitoes, highlighting gap between expected protective efficacy and operational outcomes. This diminished killing efficacy is consistent with previous studies reporting a rapid decline in standard LLINs performance, largely attributed to widespread pyrethroid insecticide resistance (24–26). Although PBO LLINs are designed to overcome metabolic resistance by inhibiting cytochrome P450s, their performance in this study was marginally better than that of standard LLINs, with mortality dropping below operational thresholds (< 80%) at six months post-distribution. This rapid decline is concerning, given that LLIN replacement cycles typically occur every three years, meaning households may be left inadequately protected for much of the intended lifespan of the nets. Previous studies have also shown reduced field performance of PBO LLINs over time, with evidence of PBO-synergist decay before the predicted 3-year life span (27, 28). Household practices, including frequent washing, the type detergents used, and outdoor drying, although not evaluated in this study, may further accelerate this decline, as these conditions are not adequately simulated in laboratory evaluations (29–31). Notably, when susceptible mosquitoes were exposed, both net types maintained >80% killing efficacy for up to 18 months, indicating they can provide effective protection for up to 2 years in the absence of resistance. These findings suggest that, under high-resistance settings, neither standard nor PBO LLINs sustain the long-term community-level killing effect required to interrupt malaria transmission.

The increase of pyrethroid resistance observed in *An. arabiensis*, highlights how rapidly vectors adapt under sustained insecticide pressure, rendering standard LLINs ineffective within short operational time-frames. Widespread LLIN deployment has been linked to the selection of pyrethroid resistance (6, 32, 33), additional selection pressures from intensive pyrethroid use in agriculture are also likely contributors (34–36). In the current study area, sugarcane farming is widespread, and agricultural insecticide use (though not evaluated) may have exacerbated resistance selection. The restoration of susceptibility with PBO pre-exposure, coupled with low kdr mutation frequencies, indicates that oxidase-mediated metabolic mechanisms as the primary driver of resistance in *An. arabiensis*. While this mechanistic evidence highlights the potential of PBO synergist to overcome pyrethroid resistance under controlled conditions, its translation into operational field efficacy remains uncertain. In practice, PBO LLINs in this study provided only transient gains in mosquito mortality, failing to maintain superior performance (killing effect) compared to standard LLINs beyond the six months post-distribution. This highlights the limitations of current PBO LLIN formulations in maintaining efficacy under operational conditions against metabolically resistant vectors.

Long-lasting insecticidal nets act through two complementary mechanisms: the physical barrier that prevents mosquito bites and the insecticidal effect that kills or repels vectors (37). In this study, however, the diminished killing efficacy of the two LLINs observed under field conditions highlights a growing gap between expected and actual protection. While the barrier effect remains important, it can be undermined by poor sleeping practices or physical damage (i.e. holes), which allow mosquitoes to reach sleepers (38, 39). Insecticides are designed to offset this vulnerability by killing mosquitoes that make contact with the net, yet increasing pyrethroid resistance in malaria vectors reduces this killing effect, enabling resistant mosquitoes to withstand sub-lethal exposure and continue biting. Previous studies have shown resistant mosquitoes

TABLE 2 Genotype frequency of L1014F/S in *Anopheles arabiensis* from Muhoroni, Western Kenya.

Year	Sample size (N)	Locus-1014					
		Sensitive wild type	Heterozygote			Homozygote	
		LL	LS	LF	SF	SS	FF
2022	165	77.0%	1.2%	17.6%	0.0	3.6%	0.6%
2024	185	61.6%	1.1%	28.6%	0.5%	5.4%	2.7%

TABLE 3 Mean knockdown rates of field mosquitoes exposed to Standard and PBO LLINs.

Mean knockdown rates -KD60 min (± standard error)				
Net operation Age (Months)	Susceptible strain		Wild population	
	Standard LLINs	PBO LLINs	Standard LLINs	PBO LLINs
0	98 ± 0.5	100	50.2 ± 2.4	95.1 ± 4
6	97 ± 0.6	100	13.9 ± 1.3	45 ± 7.8
18	95 ± 0.9	100	10.6 ± 1.9	35 ± 8.8
36	86.3 ± 2.0	84.3 ± 5.5	6.7 ± 1.5	25 ± 7.5

persisting on treated or holed nets (39, 40), raising concerns about the long-term sustainability of net efficacy. At the same time, evidence suggests that sub-lethal exposure may still induce delayed mortality or altered biting behavior (41, 42), which may partially preserve protective benefits. However, such effects are unlikely to fully offset the loss of killing efficacy under intense resistance.

The present study did not include tunnel assays to assess behavioral outcomes, representing a limitation in fully characterizing the functional protection of nets under resistance pressure and also chemical analysis to confirm if the mortality observed was due to chemical loss or it's the increased resistance observed. Nonetheless, the combined evidence points to the need for urgent innovation in malaria vector control. Specifically, policy should prioritize the deployment of next-generation LLINs with dual-active or non-pyrethroid insecticides and improve the durability and retention of insecticide and synergist compounds within nets. In addition, complementary interventions (e.g., larval source management and spatial repellents) should be integrated into control programs to sustain progress toward malaria elimination. Importantly, NMCP should also revisit LLIN re-distribution policies to prevent long periods of reduced protection and to mitigate the inadvertent selection of highly resistant mosquito population.

Conclusion

Anopheles arabiensis showed increasing pyrethroid resistance, primarily driven by metabolic mechanisms. Standard LLINs had suboptimal killing efficacy against field vector populations even when new, and although PBO LLINs performed well at baseline, their effectiveness declined sharply within six months. These findings demonstrate that PBO LLINs can improve protection against resistant vectors but may be insufficient in high-resistance areas. This underscores the need for alternative nets with dual-active or non-pyrethroid insecticides, strategic deployment of PBO LLINs, and revised net re-distribution cycles aligned with their lifespan to maintain effective malaria control.

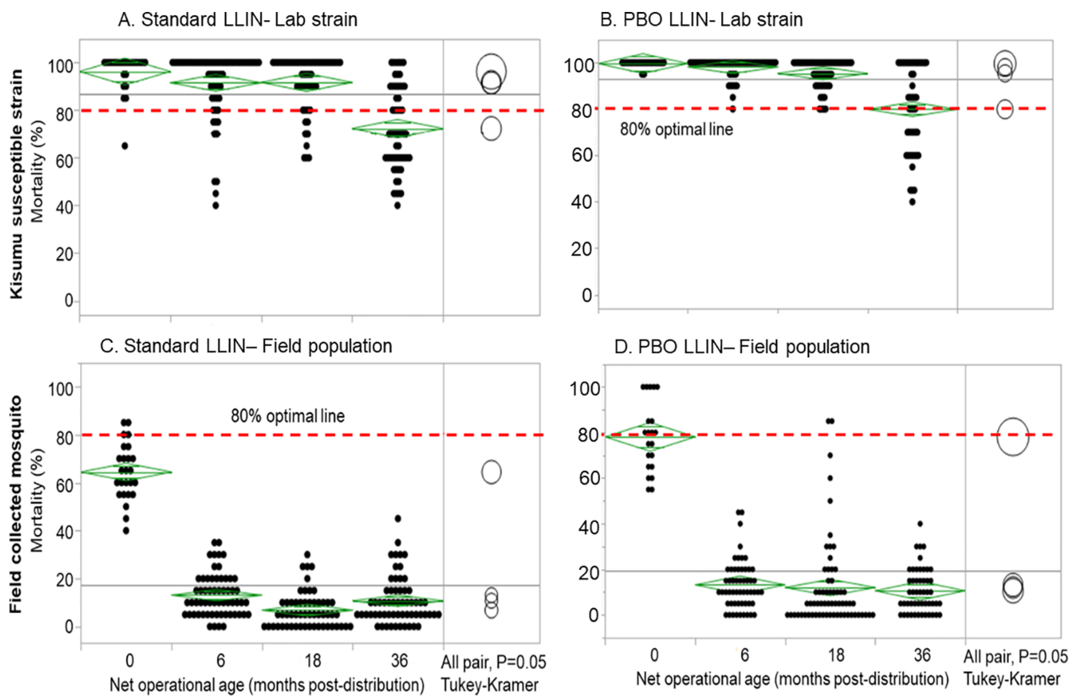

FIGURE 2
Mosquito mortality by net operational age for standard LLINs and PBO nets, tested against Kisumu susceptible **(A, B)** and field-collected mosquitoes **(C, D)**. Green diamonds indicate the mean mortality with 95% confidence intervals and overlap marks. Red lines indicate the 80% WHO threshold for net bio-efficacy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The study was approved by the Maseno University Ethics Review Committee (MUERC Protocol No. 00456) and the University of California, Irvine Institutional Review Board (UCI IRB) and received authorization from the Ministry of Health, Kenya. Written informed consent was sought from household heads before data were collected from the households. All experiments and methods were carried out in accordance with the relevant guidelines and regulations of MUERC and UCI-IRB.

Author contributions

MM: Conceptualization, Methodology, Visualization, Investigation, Supervision, Formal analysis, Writing – original draft, Data curation, Writing – review & editing. GZ: Methodology, Writing – review & editing, Conceptualization, Formal analysis, Visualization. JO: Data curation, Writing – review & editing. IN: Writing – review & editing, Data curation. JG: Project administration, Conceptualization, Writing – review & editing, Supervision. HA: Writing – review & editing, Project administration. CW: Visualization, Validation, Writing – review & editing. DZ: Validation, Writing – review & editing. M-CL: Validation, Writing – review & editing. YA: Funding acquisition, Writing – review & editing, Validation, Conceptualization. GY: Validation, Investigation, Conceptualization, Writing – review & editing, Funding acquisition.

Funding

The author(s) declared that financial support was received for this work and/or its publication. This study was supported by grants from the National Institute of Health (R01 AI123074, U19 AI129326, R01 AI050243, D43 TW001505). There was no additional external funding received for this study.

Acknowledgments

The authors wish to thank the volunteers for their participation in this study and the leadership of Kisumu County for allowing us to conduct the study in the area. The ICEMR- Kenya field assistants for providing technical support.

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2026.1764297/full#supplementary-material

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