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Consistent non-inferiority of second-in-class pyrethroid–PBO nets across diverse experimental hut and ambient chamber assays in Côte d'Ivoire and Tanzania

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Article

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Abstract

Pyrethroid–piperonyl butoxide (PBO) insecticide-treated nets are recommended by the World Health Organization for malaria control where metabolic pyrethroid resistance is present. Multiple pyrethroid–PBO nets are WHO-prequalified, but evidence that second-in-class products perform comparably to the first-in-class Olyset® Plus remains limited. We evaluated the non-inferiority of PermaNet® 3.0, Tsara Boost® and Veeralin® relative to Olyset® Plus against *Anopheles gambiae* sensu lato using West African-type experimental huts in Côte d'Ivoire, New-Ifakara-type experimental huts in Tanzania, and the Ifakara-Ambient Chamber Test in Tanzania. The primary endpoint was 24-h mosquito mortality with blood-feeding as a secondary endpoint. Evaluations included unwashed, 20-times-washed and pooled comparisons. Absolute mortality with Olyset® Plus pooled across wash conditions differed markedly among assays: 15.9% (95% CI 13.4–18.5) in Côte d'Ivoire, 38.2% (34.0–42.3) in the Tanzanian hut trial and 89.6% (87.0–92.2) in the chamber test. Despite this heterogeneity, on the primary endpoint, all three second-in-class nets were non-inferior to Olyset® Plus in every assay and wash comparison. Pyrethroid–PBO nets also caused greater mortality than the pyrethroid-only Olyset® Net in every assay ($P < 0.0001$). Thus, although assay design, mosquito population and resistance intensity strongly influenced absolute mortality, they did not alter the comparative product-selection decision. These findings provide proof of concept that concurrent comparison with an established reference product can support consistent evaluation across diverse testing environments, reducing reliance on fixed absolute thresholds and providing evidence to inform procurement of second-in-class products.

Background

Effective vector control relies heavily on insecticide-treated nets (ITNs), which remain the cornerstone of malaria prevention in the sub-Sahara Africa [1]. The massive scale-up of ITNs averted over 1.2 billion malaria cases between 2000–2024. Since 2018, ITNs have been preventing over 75 million cases annually [2]. However, the efficacy (ability to kill or prevent bites) of standard pyrethroid-only ITNs is increasingly compromised by the widespread evolution and intensity of pyrethroid resistance in *Anopheles* vectors, presenting a worsening situation that requires urgent development of new insecticides and tools [3, 4].

Resistance to pyrethroids, historically the only class of insecticide used in ITNs, is widespread across all sub-Saharan African countries [5, 6]. This resistance is largely driven by metabolic resistance mediated by upregulation of enzymes such as cytochrome P450s that detoxify insecticides before they reach their target [7, 8]. Although the use of pyrethroid-based vector control interventions is protective to the user, it reduces the community impact and benefit to non-users [9–12], because insecticide resistance reduces the proportion of malaria vectors that are killed [13].

This situation motivated the World Health Organization (WHO) to develop a Global Plan for Insecticide Resistance Management (GPIRM) [14]. Among the strategies proposed, the development of combination ITNs containing unrelated insecticides or pyrethroid plus a synergist piperonyl butoxide (PBO) gained attention to address insecticide resistance [15–17]. PBO is a known inhibitor of cytochrome P450-mediated resistance to pyrethroids. The first-in-class pyrethroid-PBO net (Olyset® Plus, Sumitomo Chemical Co. Ltd) received WHO policy recommendation as a new class in 2017 based on epidemiological data from a cluster randomised trial in Tanzania [11]. A second cluster randomised trial in Uganda with PermaNet® 3.0 and Olyset® Plus showed that pyrethroid-PBO ITNs had greater epidemiological (reduction in malaria prevalence) efficacy than standard

ITNs [18]. Adding PBO to pyrethroids has proven to be an effective strategy for managing pyrethroid resistance [13] .

The WHO policy recommendation specified that the pyrethroid-PBO net class includes all WHO-prequalified ITNs that contain both a pyrethroid and PBO [19]. In addition to Olyset® Plus, other pyrethroid-PBO nets have been prequalified by WHO including PermaNet® 3.0, Tsara Boost®, and Veeralin® [20]. These products differ from Olyset® Plus in their design and specifications, including the location of PBO (all net panels or only the top panel), PBO loading dose, type of pyrethroid, and pyrethroid loading dose.

Given that all pyrethroid-PBO nets are eligible for procurement by Global Fund grantees, assessing whether all current prequalified products are equally efficacious against metabolically resistant *Anopheles* mosquitoes is vital to ensure maximum impact. The Vector Control Product Evaluation Centre (VCPEC) of Côte d'Ivoire, undertook this multi-site study in collaboration with the Ifakara Health Institute (IHI) of Tanzania, with distinct vector populations and resistance profiles. The study aimed to generate evidence on the relative entomological efficacy of four WHO-Prequalified pyrethroid-PBO nets against pyrethroid-resistant Anopheles gambiae using three different assay systems: West African-type experimental huts (WAEH), New-Ifakara-type experimental huts (NIEH) and the Ifakara-Ambient Chambers Test (IACT). Non-inferiority was assessed using 24-hour mosquito mortality (primary endpoint) and blood-feeding success (secondary endpoint).

Results

Insecticide resistance in malaria vectors in M'Bé and Lupiro

In Côte d'Ivoire, mortality of wild *Anopheles gambiae* sensu lato from M'Bé was below 9% across all three tested pyrethroids (alphacypermethrin, deltamethrin and permethrin), indicating high pyrethroid resistance levels. In Tanzania, wild *An. arabiensis* from Lupiro and lab-reared populations exhibited mortality below 50%, confirming resistance. Pre-exposure to the synergist PBO enhanced pyrethroid-induced mortality in all populations, confirming the presence of the metabolic resistance mechanism (Supplementary Table S1).

Environmental conditions

Environmental conditions in the net storage, semi-field indoors, mosquito holding rooms, insectaries and laboratory testing rooms remained generally stable across the three studies. Median temperatures ranged between 25–28°C and relative humidity between 50–89% (Supplementary Table S2).

Experimental hut trial and IACT mosquito density

A total of 16,569 mosquitoes were collected in Côte d'Ivoire (median: 6 per hut-night; IQR: 3–13), of which 83.1% were *An. gambiae* s.l.; 16.9% were other Culicidae (other *Anopheles* spp., *Mansonia* spp., *Aedes* spp. and *Culex* spp.) while 16,652 wild *Anopheles* were collected in the Tanzania hut trial (median: 15 per hut-night; IQR: 9–24) and PCR confirmed that 99.9% were *An. arabiensis*. In the Ifakara ambient chamber test (IACT) evaluation, 9,494 *An. arabiensis* mosquitoes were tested (median: 15; IQR: 15–15). The distribution of mosquito

catches by treatment arm is shown in the Supplementary Figure S1. The studies were sufficiently powered to detect non-inferiority (Supplementary Table S3).

Mortality

The three evaluations produced markedly different levels of 24-hour mosquito mortality (Fig. 1). Mortality was lowest in the Côte d'Ivoire experimental huts, where mortality across treated nets ranged from 10.5% to 27.0% across wash conditions, consistent with the high intensity of pyrethroid resistance at M'Bé. Among the PBO nets, mortality was highest when nets were unwashed (23.1–27.0%) and generally declined after 20 washes (10.5–19.0%). Mortality was intermediate in the Tanzanian experimental huts, where mortality among the candidate PBO nets ranged from 36.3% to 55.7% across wash conditions. Mortality was generally higher when nets were unwashed (42.3–55.7%) and declined after 20 washes (36.3–40.7%), although the reduction was small for Tsara Boost®, which had similar mortality before and after washing.

Mortality was highest in the Tanzanian IACT, where mortality among the candidate PBO nets ranged from 94.8% to 99.5% across wash conditions. Mortality with the unwashed nets was close to 100% for all three products (98.7–99.5%), resulting in a ceiling effect that limited differentiation between products. Mortality remained high after 20 washes (94.8–96.9%), indicating that all three candidate nets retained substantial insecticidal activity after repeated washing.

Despite these large differences in absolute mortality, PBO nets induced significantly greater mortality than the pyrethroid-only Olyset® Net (Supplementary Table S4a, 4b, 4c) in all three evaluations ($P < 0.0001$).

Non-inferiority analysis on 24-hour mortality

The non-inferiority analysis for the mortality endpoint is summarised in Fig. 2, with the pooled analysis by study in Table 1 and disaggregated by wash status in Supplementary Table S11.

Table 1

Non-inferiority analysis of pyrethroid-PBO second-in-class ITNs compared to Olyset® Plus on 24-hour mortality with pooled data for unwashed and 20x washed nets of each net type. Lower bound of the confidence interval of Odds ratio must fall above Delta to be deemed non-inferior. Lower bound of the confidence interval of OR must be above both Delta and 1 to be superior. N = Number of mosquitoes collected, n dead = Number of mosquitoes dead, OR = Odds Ratio estimated by GLMM adjusting for volunteer and day as fixed effects, hut as fixed effect in M'Bé huts or random effect in Tanzania huts; hut not included for I-ACT; CI = Confidence interval. Delta represents the non-inferiority margin.

Location	Net type	N	n dead	% M24 (95% CI)	Delta	OR (95% CI)	Conclusion
M'Bé huts (Côte d'Ivoire)	Olyset® Plus	2382	365	15.9 (13.4–18.5)	-	1 (ref)	Reference
	PermaNet® 3.0	2543	390	18.5 (15.3–21.7)	0.52	1.00 (0.85–1.18)	Non-inferior
	Tsara® Boost	1601	262	17.1 (13.9–20.2)	0.52	1.15 (0.96–1.38)	Non-inferior
	Veeralin®	2558	462	22.8 (19.5–26.2)	0.52	1.25 (1.07–1.47)	Non-inferior and superior
Ifakara huts (Tanzania)	Olyset® Plus	3418	1395	38.2 (34.0–42.3)	-	1 (ref)	Reference
	PermaNet® 3.0	2779	1406	44.9 (40.1–49.7)	0.73	1.36 (1.21–1.51)	Non-inferior and superior
	Tsara® Boost	2163	982	41.5 (36.9–46.0)	0.73	1.20 (1.06–1.35)	Non-inferior and superior
	Veeralin®	3490	1805	47.5 (43.2–51.8)	0.73	1.40 (1.26–1.55)	Non-inferior and superior
I-ACT (Tanzania)	Olyset® Plus	1584	1419	89.6 (87.0–92.2)	-	1 (ref)	Reference
	PermaNet® 3.0	1584	1539	97.2 (95.9–98.4)	0.54	2.00 (1.61–2.48)	Non-inferior and superior
	Tsara® Boost	1581	1547	97.9 (96.8–98.9)	0.54	2.17 (1.75–2.71)	Non-inferior and superior
	Veeralin®	1586	1545	97.4 (96.3–98.5)	0.54	2.07 (1.67–2.57)	Non-inferior and superior

Pooled analysis. The three assays produced markedly different absolute levels of 24-hour mortality: mortality with Olyset® Plus was 15.9% in the M'Bé huts, 38.2% in the Ifakara huts and 89.6% in the IACT. Nevertheless,

the relative product comparisons were broadly consistent (Table 1). All three candidate PBO nets were non-inferior to Olyset® Plus in every assay, indicating that the same product-selection decision would have been reached despite the large differences in absolute mortality. Evidence of superiority was setting-dependent. In Côte d'Ivoire, only Veeralin® was superior to Olyset® Plus (OR 1.25, 95% CI 1.07–1.47), whereas PermaNet® 3.0 and Tsara® Boost were non-inferior but not superior because their confidence intervals included 1. In the Ifakara huts and IACT, all three candidate nets were superior to Olyset® Plus. Thus, the assays agreed on the principal non-inferiority conclusion, but not completely on whether the candidates provided additional mortality beyond the comparator.

Analysis by wash status. The wash-stratified results show greater variation among assays and products, although every candidate remained non-inferior to Olyset® Plus under both wash conditions (Supplementary Table S5a, 5b, 5c). Among unwashed nets, Tsara® Boost was superior in Côte d'Ivoire, PermaNet® 3.0 and Veeralin® were superior in the Ifakara huts, and none was demonstrably superior in the IACT. The absence of superiority in the unwashed IACT should not be interpreted as poorer candidate performance: mortality with unwashed Olyset® Plus was already 96.9%, leaving little room for improvement and producing a ceiling effect.

After 20 washes, Veeralin® was superior in Côte d'Ivoire, Tsara® Boost and Veeralin® were superior in the Ifakara huts, and all three candidates were superior in the IACT. The washed PermaNet® 3.0 result in the Ifakara huts is borderline (OR 1.16, 95% CI 1.00–1.34) and the lower confidence limit is 1. Overall, washing exposed more differences between products than the pooled analysis, but the three assays still agreed that all candidate nets retained acceptable efficacy relative to Olyset® Plus.

Blood-feeding

Blood-feeding outcomes varied substantially among evaluation settings (Fig. 3) and were less consistent than the mortality results. In the Côte d'Ivoire experimental huts, blood-feeding proportions were comparatively high across treated arms (16.1–33.9%), consistent with the high-intensity, multi-mechanism pyrethroid resistance present at M'Bé. Blood-feeding proportions were considerably lower in the Tanzanian experimental huts (1.2–13.1%) and IACT (2.8% – 9.6%).

Despite these large differences in absolute blood-feeding, PBO nets induced significantly greater blood-feeding inhibition than the pyrethroid-only Olyset® Net in M'Bé experimental huts and IACT ($P < 0.0001$; Supplementary Table S6a, S6c). In the Ifakara experimental huts, only Olyset Plus® was consistently superior to Olyset® across all analysis conditions (pooled, unwashed, and washed; $P < 0.05$; Supplementary Table S6b).

Non-inferiority analysis on blood-feeding outcome

Pooled analysis. The assays produced different absolute levels of blood-feeding. With Olyset® Plus, blood feeding was substantially higher in the M'Bé huts in Côte d'Ivoire (20.2%) than in the Ifakara huts (5.2%) or IACT (5.6%) in Tanzania. The comparative conclusions also differed between settings (Table 2). In Côte d'Ivoire, all three candidate nets were inconclusive for non-inferiority to Olyset® Plus because the upper limits of their confidence intervals exceeded the non-inferiority margin of 1.48. By contrast, all three candidates were non-inferior in both Tanzanian assays. None of the pooled comparisons demonstrated superior protection against blood feeding: superiority would require an odds ratio and its entire confidence interval to be below 1. Although

Veeralin® was associated with significantly more blood feeding than Olyset® Plus in the IACT (OR 1.36, 95% CI 1.02–1.83), the increase remained within the prespecified non-inferiority margin of 2.34. Thus, unlike the mortality endpoint, the pooled blood-feeding analysis did not produce the same product-selection decision across all three assays: the candidate nets met the efficacy criterion in both Tanzanian assays but were inconclusive in Côte d'Ivoire.

Table 2
Non-inferiority analysis of pyrethroid-PBO second-in-class ITNs compared to Olyset® Plus on mosquito blood feeding in experimental huts and I-ACT with pooled data for unwashed and 20x washed nets of each net type. Upper bound of the confidence interval of OR must lie below Delta to be deemed non-inferior. N = Number of mosquitoes collected, n fed = Number of mosquitoes blood fed, OR = Odds Ratio estimated by GLMM adjusting for volunteer and day as fixed effects, hut as fixed effect in M'Bé huts or random effect in Tanzania huts; hut not included for I-ACT; CI = Confidence interval. Delta represents the non-inferiority margin.

Location	Net type	N	n fed	% fed (95% CI)	Delta	OR (95% CI)	Conclusion
M'Bé huts (Côte d'Ivoire)	Olyset® Plus	2382	566	20.2 (17.3–23.0)	-	1 (ref)	Reference
	PermaNet® 3.0	2543	803	30.8(27.1–33.3)	1.48	1.51 (1.31–1.72)	Inconclusive
	Tsara® Boost	1601	508	29.5(25.7–33.3)	1.48	1.49 (1.28–1.73)	Inconclusive
	Veeralin®	2558	740	27.2(23.8–30.6)	1.48	1.35 (1.17–1.54)	Inconclusive
Ifakara huts (Tanzania)	Olyset® Plus	3418	115	5.2 (3.2–7.3)	-	1 (ref)	Reference
	PermaNet® 3.0	2779	109	5.2 (3.5–6.9)	2.59	1.17 (0.89–1.54)	Non-inferior
	Tsara® Boost	2163	124	6.2 (4.2–8.1)	2.59	1.63 (1.25–2.13)	Non-inferior
	Veeralin®	3490	214	8.1 (5.9–10.2)	2.59	1.97 (1.55–2.51)	Non-inferior
IACT (Tanzania)	Olyset® Plus	1584	87	5.6 (3.6–7.5)	-	1 (ref)	Reference
	PermaNet® 3.0	1584	106	6.7 (5.0-8.5)	2.34	1.21 (0.90–1.62)	Non-inferior
	Tsara® Boost	1581	113	7.1 (5.3-9.0)	2.34	1.32 (0.98–1.77)	Non-inferior
	Veeralin®	1586	116	7.3 (5.5–9.1)	2.34	1.36 (1.02–1.83)	Non-inferior

Analysis by wash status. The wash-stratified analysis revealed further differences among assays and products (Supplementary Table S7a, 7b, 7c). Among unwashed nets, all three candidates did not demonstrate non-inferiority to Olyset® Plus in Côte d'Ivoire. In the Ifakara huts, only PermaNet® 3.0 was non-inferior. In the IACT, all three unwashed candidates were not demonstrated to be non-inferior in the I-ACT because their upper confidence limits exceeded the relatively wide non-inferiority margin of 3.59. This result appears to reflect the exceptionally low blood-feeding proportion with unwashed Olyset® Plus in the IACT (2.8%), which made it difficult for the candidate nets to match the comparator. After 20 washes, Veeralin® was superior to Olyset® Plus in Côte d'Ivoire (OR 0.81, 95% CI 0.67–0.97), whereas PermaNet® 3.0 and Tsara® Boost did not demonstrate non-inferiority. In the Ifakara huts, PermaNet® 3.0 and Tsara® Boost were non-inferior, while Veeralin® was narrowly inferior because its upper confidence limit of 2.20 exceeded the margin of 2.16. All three washed candidates were non-inferior in the IACT, although none was superior. Overall, the wash-stratified blood-feeding results were less consistent across assays than the mortality results, showing that relative performance for blood-feeding protection depended on both the assay setting and wash condition which heavily influenced the proportion of blood fed mosquitoes and the precision of the estimates.

Quality assurance bioefficacy of tested net and chemical analysis results

WHO cone bioassay results showed that bioefficacy against susceptible mosquito strains met the WHO threshold (≥ 80% mortality) across all test conditions in Côte d'Ivoire, but in Tanzania this threshold was met only for unwashed nets before and after the hut trial, declining below 80% for some products after 20 washes. Bioefficacy against pyrethroid-resistant strains was substantially lower at both sites: most products fell below the 80% mortality threshold against resistant mosquitoes, particularly after washing, with a small number of PBO nets (e.g., Olyset® Plus, PermaNet® 3.0 roof) approaching or exceeding threshold only when unwashed. Detailed bioefficacy results are provided in Supplementary Figure S3.

Chemical analysis confirmed that initial concentrations of pyrethroid and PBO complied with target doses for all products. Washing was associated with varying degrees of active ingredient loss; Veeralin® showed the greatest PBO retention (72–82%) after 20 washes, followed by PermaNet® 3.0 roof (53–70%), Tsara® Boost (53–64%) and Olyset® Plus (51–63%). Summarised chemical content data before washing, after 20 washes, and after the hut trial are provided in Supplementary Table S8a, S8b and S8c.

Discussion

This multi-site study provides the first comprehensive non-inferiority comparison of four WHO-prequalified PBO ITNs evaluated simultaneously across experimental huts in two African countries as well as in the IACT. The principal finding is that all three second-in-class pyrethroid-PBO nets (PermaNet® 3.0, Tsara Boost®, and Veeralin®) met the WHO non-inferiority criterion on the primary 24-hour mortality endpoint relative to the first-in-class Olyset® Plus, across all three studies when unwashed and after 20 washes. These results provide data to support the WHO policy framework that groups all pyrethroid-PBO nets within a single intervention class for procurement and deployment [21, 22].

The consistency of the non-inferiority finding across sites with markedly different vector species, resistance profiles, and assay designs strengthens the generalisability of these results. At the M'Bé site in Côte d'Ivoire,

where *An. coluzzii* exhibits extremely high pyrethroid resistance driven by multiple mechanisms including kdr, elevated P450s, esterases, and GSTs [23, 24], overall mortality with PBO nets was low (< 22%). In contrast, the Tanzanian sites, where *An. arabiensis* carries predominantly CYP450-mediated metabolic resistance [25], yielded substantially higher mortality (33–48% in Ifakara huts, 82–99% in the IACT). Despite these differences in absolute efficacy, the relative performance of the four PBO products remained consistent, demonstrating that non-inferiority conclusions were robust to variation in baseline mortality levels and resistance intensity. Conducting non-inferiority focuses on a useful question 'is the candidate net not unacceptably worse than the first-in-class for which we have evidence of public health benefit'. It allows meaningful comparison to another net, doing the same thing in each site and avoids the need to specify how much better than a reference (e.g. untreated, pyrethroid-only) because the absolute performance of products vary by site and assay design [13]. A potential limitation of serial non-inferiority evaluations is bio-creep [26], whereby a product that has been declared non-inferior (despite being slightly less effective than its comparator) is subsequently used as the active comparator for the next generation of products. Repetition of this process could progressively lower the efficacy standard, ultimately allowing products with materially reduced activity to satisfy the non-inferiority criterion. Bio-creep is most likely when slightly less effective products repeatedly become the reference for subsequent evaluations or when poor comparator performance makes the non-inferiority criterion easier to satisfy. For ITNs, this risk can be minimised by consistently conducting comparative efficacy assessments to a clearly specified first-in-class reference product whose efficacy in a location has been established against an appropriate negative control, rather than adopting each newly prequalified second-in-class product as the comparator. The reference product's performance must be monitored in every evaluation to confirm assay sensitivity and ensure that it provides the expected biological effect under the study conditions (the consistency assumption). ITNs that have not been kept under optimal conditions, or that have been stored for long periods may become less efficacious so using old or improperly stored active comparators should be avoided. Further, a new comparator should replace the original benchmark only when robust evidence demonstrates that it provides an equal or greater, well-characterised effect. This preserves a stable efficacy standard while allowing the reference framework to evolve when genuinely improved products become available.

Critically, despite this marked variation in absolute point estimates between study sites, the direction of effect was consistent across all three studies: all three candidate products demonstrated non-inferiority to Olyset® Plus on the primary mortality endpoint in every setting, and no product emerged as definitively inferior in a site-specific pattern. This concordance across settings with profoundly different resistance mechanisms, vector species, and experimental conditions validates comparative efficacy evaluations as a reproducible method for within-class product comparisons [27, 28]. Irrespective of where the study was conducted, the evaluation delivers the same conclusion about relative product performance, which is a critical property for a test used to support procurement decisions across diverse malaria-endemic settings.

The superior performance of all PBO nets over the standard pyrethroid-only Olyset® Net confirmed the continued added value of PBO as a synergist against metabolically resistant mosquito populations. This aligns with findings from cluster randomised trials in Tanzania and Uganda that demonstrated the epidemiological superiority of PBO nets over conventional long lasting insecticidal nets (LLINs) [11, 18], and with a Cochrane systematic review concluding that PBO nets reduce malaria prevalence and kill more mosquitoes compared to standard pyrethroid nets [13]. However, the low absolute mortality observed in Côte d'Ivoire raises concern

about the operational efficacy of all current PBO-ITN formulations in settings where multiple resistance mechanisms co-exist beyond P450-mediated detoxification [27, 28].

The differential performance by wash condition merits attention. Unwashed PBO nets generally performed better than their washed counterparts, consistent with the known loss of surface-available PBO following repeated washing [29, 30]. Chemical analysis confirmed varying degrees of PBO retention across products, with Veeralin® retaining the highest proportion (72%) and Olyset® Plus and PermaNet® 3.0 the lowest (53–54%). Importantly, however, even after 20 washes, all candidate products maintained non-inferiority to Olyset® Plus on the mortality endpoint. This is reassuring for programmatic use, as nets in the field undergo regular washing throughout their intended three-year lifespan.

In the unwashed condition, the IACT results showed ceiling-effect mortality (> 96%) for all PBO nets. This did not affect the principal product selection decision: as in the West African and Ifakara hut trials, all three candidate nets were non-inferior to Olyset® Plus. However, the high mortality limited the scope for distinguishing superiority over Olyset ® Plus. The controlled conditions of the IACT, standardised mosquito numbers, laboratory-reared strains, and consistent environmental parameters, likely maximised insecticide exposure through repeated contact with the ITN and reduced sources of experimental variability, resulting in high and relatively precise mortality estimates. While the IACT has been validated as a useful screening tool [31] and is particularly useful for evaluating the bioefficacy of ITNs in durability trials where ITN bioefficacy tends to be lower than brand new nets [32], these findings suggest that ceiling effects should be considered when interpreting differences between highly effective products.

The blood-feeding results presented a more complex picture. There was no consistent pattern across products, testing environments or wash conditions. Low numbers of blood-fed mosquitoes, particularly in the Tanzanian assays, limited the precision of comparisons and contributed to several inconclusive non-inferiority results. These findings should therefore be interpreted as insufficient evidence to establish non-inferiority, rather than evidence of inferior performance.

The 2024 WHO guidelines for non-inferiority assessment prioritise the mortality endpoint [22], recognising that mortality has the most direct relationship with vectorial capacity reduction [33] and that blood-feeding results are very sensitive to hut design [34], and resistance profile [35]. The consistent non-inferiority demonstrated across all studies on the mortality endpoint therefore provides the critical evidence base for policy and procurement.

Despite variability in blood-feeding non-inferiority outcomes, all insecticide-treated nets provided significant personal protection to the sleeping occupant, reducing blood-feeding proportions substantially compared to the untreated control across sites. In the Tanzanian huts, the absolute blood-feeding proportions in all PBO arms were uniformly low (3.5–9.6%), demonstrating a high degree of personal protection in these settings. In Côte d'Ivoire, where mosquito pressure is high and killing is attenuated by resistance, all ITNs still significantly reduced blood-feeding relative to the untreated control, confirming their protective utility even in this challenging environment. These findings reinforce the public health value of all PBO-class nets for the individual user, complementing the population-level implications of the mortality non-inferiority results.

The present study—the first simultaneous multi-platform entomological evaluation of all four currently prequalified PBO nets, fulfils the evidence requirements across two countries and three testing platforms, providing a robust foundation for procurement and deployment decisions [22]. Continued entomological monitoring under operational conditions will be essential to track product durability as resistance evolves. The variability in PBO retention across products also highlights an opportunity for manufacturers to optimise wash-resistant formulations.

Limitations

This study has several limitations. First, the experimental hut designs differed substantially between sites: West African-type huts at M'Bé have a single verandah exit-trap and 1 cm funnel entry gaps, while Ifakara-type huts are larger in volume with eave baffles controlling mosquito entry and retention. During the Tanzanian trial, preliminary assessments after the first round revealed mosquito retention of only 62%; baffles were subsequently adjusted from a 10 cm to a 5 cm eave gap from round 2 onwards, improving retention to 89%. These structural differences influence absolute mosquito mortality and blood-feeding proportions between sites, complicating direct cross-site comparisons of proportional outcomes; however, the non-inferiority analysis is an internal comparison within each site, largely mitigating this concern. Second, the use of different mosquito species (*An. coluzzii* at M'Bé versus *An. arabiensis* at Lupiro) and distinct resistance mechanisms means that product performance rankings could differ in areas with other *Anopheles* species or resistance profiles, although results from another non-inferiority study of PermaNet® 3.0 was consistent with these findings [36] and a separate IACT evaluation gave the same result [37]. Third, due to the impact of COVID-19, WHO insecticide susceptibility testing at the Tanzanian field site (Lupiro) could not be conducted prior to trial commencement; phenotypic resistance characterisation was deferred to February 2021, approximately two months after trial completion in November–December 2020—and may not precisely represent the resistance intensity during the evaluation period, although the resistance profile at Lupiro has been stable over multiple years of monitoring.

Conclusion

This multi-site study demonstrates that three second-in-class pyrethroid-PBO ITNs—PermaNet® 3.0, Tsara® Boost, and Veeralin®—were non-inferior to the first-in-class Olyset® Plus on the primary endpoint of 24-hour mosquito mortality. This conclusion was consistent for unwashed and 20-times-washed nets, across experimental hut trials in Côte d'Ivoire and Tanzania and semi-field IACT evaluations, despite substantial differences in absolute mortality among the assays. All pyrethroid-PBO nets were superior to the pyrethroid-only Olyset® Net. These findings provide comparative evidence consistent with the WHO classification of prequalified pyrethroid-PBO nets within a single intervention class. More broadly, the results provide proof of concept that a non-inferiority framework using an established first-in-class product as the comparator can produce consistent product-selection decisions across different assay designs, mosquito populations and resistance settings. Subject to appropriate comparator selection, prespecified margins and safeguards against bio-creep, the non-inferiority testing framework proved feasible across diverse entomological settings and assay designs and was suitable for routine evaluation of vector control products entering the same WHO policy class.

Methods

Study areas

The study comprised three distinct components conducted at two sites: experimental hut trial in Côte d'Ivoire (November 2020–April 2021), and experimental hut trial (November–December 2020) and the IACT evaluations (November 2021–February 2022) in Tanzania.

In Côte d'Ivoire, the study was performed at the M'Bé field site (5.209963 W and 7.970241 N, 30 kilometers (km) north of Bouaké), a large rice-irrigated valley producing mostly year-round *An. gambiae* s.l., of which *An. coluzzii* accounts for 98% [24]. Previous characterisation showed 51% mortality to permethrin, 68% to alpha-cypermethrin, and 76% to deltamethrin in susceptibility tests, with L1014F kdr allele at a frequency of 0.8. Upregulation of CYP6P3 cytochrome P450s and elevated esterase, oxidase, and GST activities were also present [23, 24]. Pre-exposure to PBO in WHO tube tests induced a significant increase in mortality to pyrethroids [24]. Suites of the West African-type experimental huts at M'Bé served for the Côte d'Ivoire component.

The Tanzanian part was conducted in Lupiro village (8.385°S, 36.670°E), Ulanga District. The main malaria vector was An. arabiensis (> 99.9% of the An. gambiae complex), with metabolic resistance confirmed by CYP450 upregulation [25]. In WHO susceptibility tests (February 2021), 24-hour mortality to permethrin was 16%, 48% to deltamethrin and 22% to alpha-cypermethrin. Pre-exposure to PBO in WHO tube tests markedly increased mortality to 65% for permethrin, 67% for deltamethrin, and 64% for alpha-cypermethrin, confirming the predominantly CYP450-mediated metabolic basis of the resistance in this population. Twenty-four Ifakara-type experimental huts at the IHI Lupiro site served for the hut trial. Additionally, the IACT facility at Bagamoyo was used for complementary semi-field evaluation with laboratory-reared pyrethroid-resistant *An. arabiensis* (Kingani strain) [38]. The IACT study site is located at the Kingani site of the Bagamoyo branch of Ifakara Health Institute, 70 km North of Dar es Salaam, Tanzania, between latitudes 6° 8' S and 30° 37' E. The district had an average annual rainfall of 800–1000 mm, with average temperatures ranging from 24ºC to 29ºC and a mean annual humidity of 73%. The description of the IACT and the huts used in Côte d'Ivoire and Tanzania has been done elsewhere [28, 31].

Study design and interventions

The description of the four evaluated WHO-prequalified pyrethroid-PBO ITNs is found in Table 3. All nets were sourced by the Global Fund. Nets were tested unwashed and after 20 standardised washes following WHO guidelines [39]. Between successive washes, nets were allowed to rest for product-specific regeneration intervals determined in preliminary testing: 2 days for Olyset® Plus, 5 days for PermaNet® 3.0, 3 days for Tsara® Boost, 5 days for Veeralin®, and 7 days for Olyset® Net.

Table 3
Characteristics of nets tested in the experimental huts and Ifakara-Ambient Chamber Test

Net type	Component	Material	Pyrethroid content	PBO Content	Role	Manufacturer
Olyset® Plus	Whole net	Polyethylene	Permethrin 20 g/kg (incorporate)	10 g/kg	Investigational Item	Sumitomo Chemical, Japan
PermaNet® 3.0	Roof	Polyethylene	Deltamethrin 4g/kg (incorporated)	25 g/kg	Investigational Item	Vestergaard Frandsen, Switzerland
	Sides	Polyester	Deltamethrin 2.8g/kg (coated)	None		
Veeralin®	Whole net	Polyester	Alpha-cypermethrin 6.0 g/kg (incorporated)	2.2 g/kg	Investigational Item	V.K.A. Polymers Pvt Ltd, India
Tsara® Boost	Whole net	Polyethylene	Deltamethrin 3 g/kg (incorporate)	11 g/kg	Investigational Item	NRS Moon Netting FZE UAE
Olyset® Net	Whole net	Polyethylene	Permethrin 20 g/kg (incorporate)	None	Standard Comparator	Sumitomo Chemical, Japan
Safi Net	Whole net	Polyester	None	None	Negative control	A to Z Textile Mills Limited

Quality checks via bioefficacy and chemical analysis

Quality check was performed on 25 cm x 25 cm net samples cut from WHO-specified positions [39]. Net bioefficacy was evaluated via standard WHO cone bioassays using susceptible (*An. gambiae*) and resistant (*An. gambiae* s.l.) strains before washing, after 20 washes (allowing for regeneration time), and post-trial. Mosquitoes were exposed for 3 minutes (100 per net), with 60-minute knockdown and 24-hour mortality recorded. Samples were then foil-wrapped and shipped to CRA-W for chemical analysis (HPLC). Full methodological details are provided in the Supplementary Information (Supplementary File 1).

Experimental hut trial procedures

In Côte d'Ivoire, three nets per treatment arm were rotated among 11 West African-type huts following a Latin square design over 22 weeks (n = 132 observations per arm). In Tanzania, 24 Ifakara-type huts were used in four blocks following a 6×6 Williams design for 36 nights (n = 72 observations per arm). The IACT facility used 18 chambers, over (n = 72 observations per arm) and 15 laboratory-reared resistant *An. arabiensis* per chamber-night. At all sites, nets were holed with six 4 cm × 4 cm holes to simulate wear.

Each hut accommodated one volunteer sleeper per experimental night. Volunteer sleepers were randomly rotated among huts nightly using a Latin square design to correct any bias that could result in specific attractiveness from the sleepers. Sleepers remained in the huts overnight from 6:00 PM to 6:00 AM, while those in the IACT chambers entered at 9:00 PM and remained until 6:00 AM. Mosquitoes were collected on hut

floors, within the nets and in exit traps. They were brought back to the laboratory, then scored as dead/alive, fed/unfed and held for 24 hours with 10% sugar solution for delayed mortality assessment [21, 22, 39, 40].

In the first experimental round in both countries, structured questionnaires adapted from WHO guidelines [39] were administered to all volunteers each morning upon waking to record perceived and observed adverse effects.

Outcome measures

The primary endpoint for the non-inferiority assessment was 24-hour mosquito mortality, following 2018 WHO guidelines [40], which originally required non-inferiority to be demonstrated on both the mortality and blood-feeding endpoints. Updated guidelines require non-inferiority only on the mortality endpoint [22]. Absolute values (not corrected for control) were used for the mortality and blood-feeding endpoints.

Statistical analysis

Data were captured on paper forms and double-entered into Microsoft Excel. After cleaning and verification, data were imported into Stata version 16.1 for analysis. Outcomes and their 95% confidence intervals (CIs) were calculated. Proportional outcomes were analysed using generalised linear mixed models (GLMMs) with a binomial distribution and logit link function [41, 42]. For the Côte d'Ivoire hut data, net type, volunteer, hut, and day were adjusted for as fixed effects. For the Tanzanian hut trial, hut was treated as a random effect to account for collinearity from using two huts per treatment per night, while volunteer and day were fixed effects. For the IACT data, hut was excluded due to lack of variation, and mortality was transformed (subtracted by one) to address the high proportion of mortality and ensure model convergence.

Non-inferiority of the second-in-class PBO products was assessed following WHO guidelines and Piaggio et al [43]. For the mortality endpoint, a product was deemed non-inferior if the lower 95% CI of the odds ratio (OR) relative to Olyset® Plus did not fall below the 7% fixed non-inferiority margin. A product was deemed superior if the OR and its entire 95% CI exceeded 1. Superiority of PBO nets over pyrethroid-only nets was assessed using p-values set at the 5% significance level.

Post-hoc sample size calculations for GLMMs belonging to the binomial family were performed through simulation [44] in R using the dplyr and lme4 packages [41], with 1000 simulations following published guidelines [21].

Ethical considerations

Ethical approval was obtained from the Institute of Ifakara Health Institute, Institutional Review Board (IHI-IRB – 29-2020) and the National Institute for Medical Research (NIMR – NIMR/HQ/R.8a/Vol. IX/3521) in Tanzania, and also approval was obtained from the Ivorian Ministry of Health through the National Comity of Ethical Clearence (N° 073 – 19 /MSHP/CNESVS-kp).

Written informed consent was obtained from all participants after the study information sheet and consent form were thoroughly explained in the local language, with interpreters and impartial witnesses present where required. To minimize risk, only adult males aged 25–40 years were recruited, with women and elderly individuals excluded. All participants were offered a free course of malaria chemoprophylaxis. A standby nurse was available throughout to monitor and manage any fever or adverse events.

All methods were performed in accordance with the relevant guidelines and regulations.

Declarations

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Authors' contributions

LPAA, AA, OGO, SJM and RN conceived the study and designed the experiments. LPAA, AA, SC, JPKK, KS, EM, WN, SN, ABM, and AGL conducted different components of the trial. AA and OGO analysed the data. LPAA and AA drafted the manuscript. AAK and JM assisted with project management and logistics. SJM and RN contributed to the manuscript. All authors read and approved the final manuscript.

Consent for publication

All authors have read and agreed to publish this work.

Availability of data and materials

All data supporting the findings of this study can be obtained from the author upon a reasonable request.

Ethics approval and consent to participate

Ethical approval was obtained from the Institute of Ifakara Health Institute, Institutional Review Board (IHI-IRB – 29-2020) and the National Institute for Medical Research (NIMR - NIMR/HQ/R.8a/Vol. IX/3521) in Tanzania, and also approval was obtained from the Ivorian Ministry of Health through the National Comity of Ethical Clearance (N° 073-19/MSHP/CNESVS-kp) in Côte d'Ivoire.

Written informed consent was obtained from all volunteer sleepers at both sites.

Competing interests

The authors declare that they have no competing interests.

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Figures

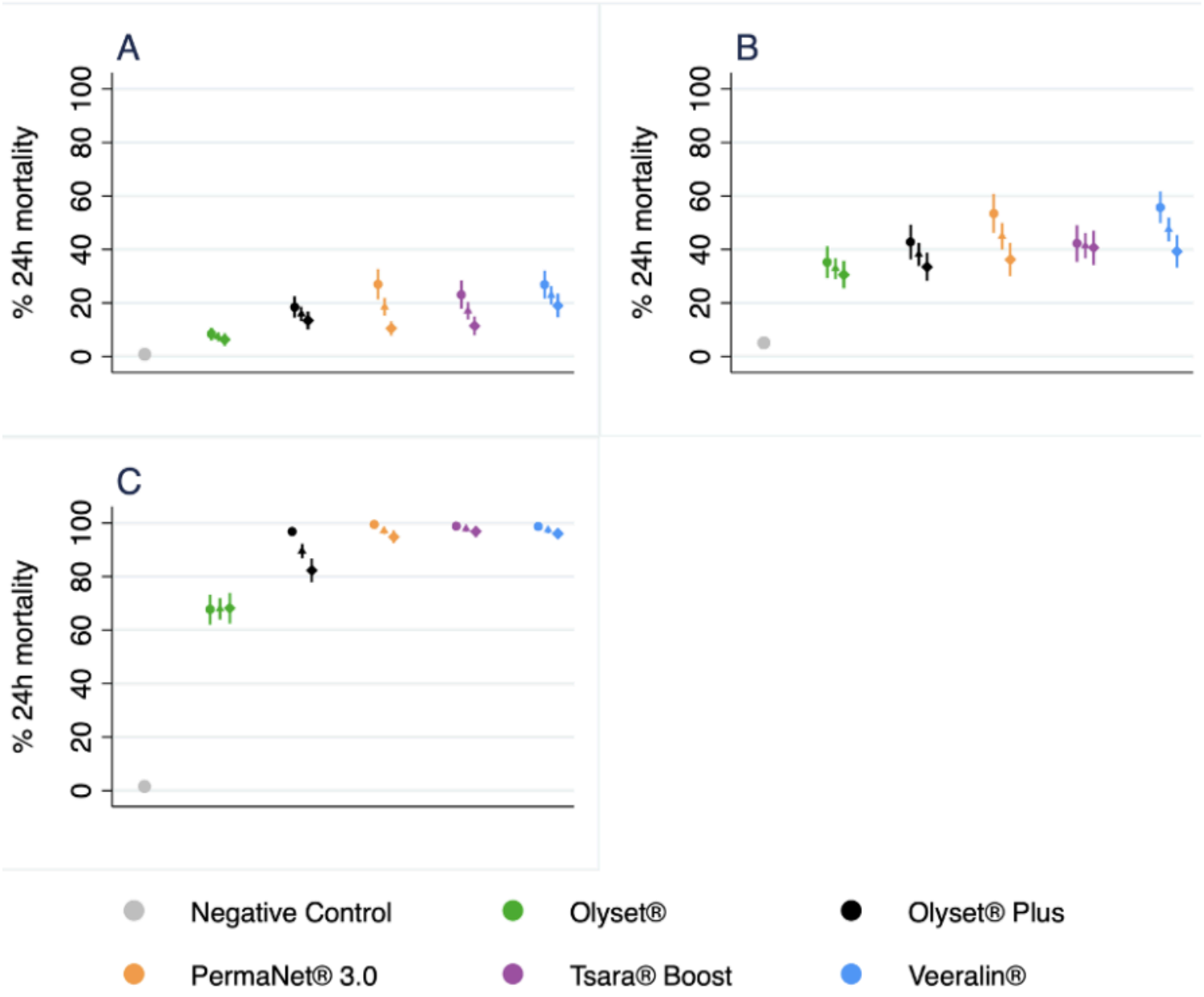

Figure 1

Proportion of female *Anopheles* mosquitoes dying at 24 hours (95% CI) after collection from West African experimental huts in M'Bé, Côte d'Ivoire (**A**), in New-Ifakara experimental huts in Lupiro, Tanzania (**B**), and in I-ACT Tanzania (**C**). Circles represent unwashed nets, diamonds 20×-washed nets, and triangles pooled (unwashed and 20×-washed combined).

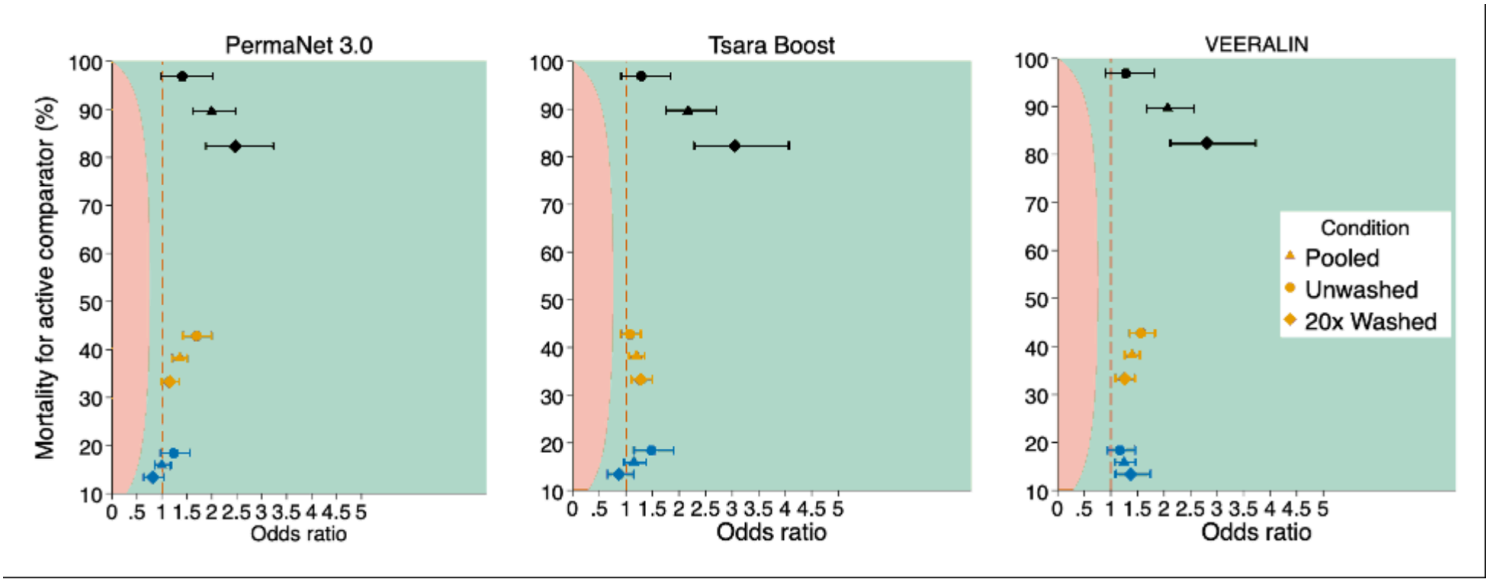

Figure 2

Non-inferiority forest plot of 24-hour mortality (95% CI) of female *Anopheles* mosquitoes across experimental huts in Côte d'Ivoire (blue) and Tanzania (gold), and IACT (black), comparing second-in-class PBO ITNs to Olyset® Plus. The pink semi-circle represents the non-inferiority margin (delta) below which estimates must not fall to be deemed non-inferior. The green area represents non-inferiority. The dashed line is the line of no difference.

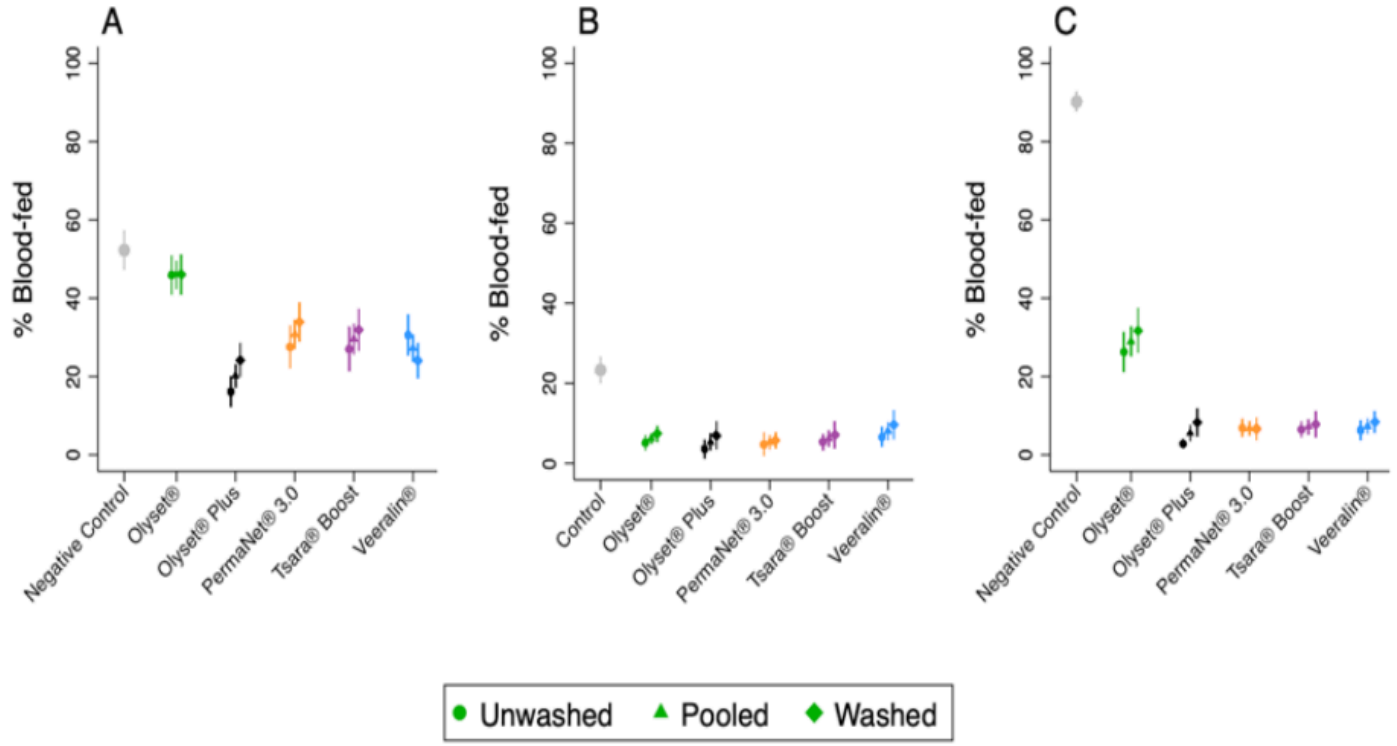

Figure 3

Proportion of blood-fed (%) female *Anopheles* mosquitoes after collection in experimental huts in M'Bé, Côte d'Ivoire (A), in Lupiro, Tanzania (B), and in I-ACT in Tanzania (C).

Supplementary Files

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- SupplementaryFilesAhouaAlouetalScRep.pdf