Impact assessment of CDC bottles treated with a combination of insecticide and synergist on insecticide-resistant *Anopheles gambiae* populations in Benin

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**Abstract**

Despite the proven efficacy of LLINs and IRS in reducing malaria transmission, vector resistance to insecticides seriously threatens to undermine vector control efforts. The present study aims to assess the impact of CDC bottles treated with a combination of insecticide and DEF or PBO synergists on insecticide-resistant *An. gambiae* populations in Benin. The study was conducted in 13 localities in Benin, following the south-north transect from September 2019 to June 2020. Thus, adult females of *An. gambiae* s.l. reared from field-collected larvae were used to assess the susceptibility of the vectors to permethrin and bendiocarb. Molecular species identification, as well as L1014F Kdr and Ace-1R mutations, was performed using PCR. Also, the expression level of biochemical enzymes was assessed. Mortality rates obtained with permethrin ranged from 34% to 71% in the 13 localities in our study. When mosquitoes from these same populations were pre-exposed to PBO, mortality rates increased significantly and ranged from 52% to 97%. With bendiocarb alone, mortality rates ranged from 93% to 100% while pre-exposure of vectors to the synergist DEF resulted in 100% mortality for all localities. The present study showed that the addition of the synergist PBO or DEF to pyrethroids or carbamates significantly increased their effectiveness. Thus, dual-active LLINs such as PBO-based LLINs or IRS using a combination of bendiocarb and the synergist DEF should be evaluated in the Benin context for possible use as an alternative or rotation of vector control tools in Benin.

**Keywords:** Malaria, DEF, permethrin, PBO, bendiocarb, *An. gambiae*

**1. Introduction**

The widespread resistance of vectors to pyrethroids [1] and carbamates [2, 3] is a major threat to the success of insecticide-based malaria vector control programme. In 2007, N’Guesan et al. [4] reported on the declining efficacy of pyrethroid-treated nets in experimental huts in Ladjji, an area of high pyrethroid resistance in malaria vectors in southern Benin. Studies on insecticide resistance conducted in some regions of Benin [1, 2, 5, 6, 7, 8] have confirmed the presence of this resistance and its evolution over time and space.

Indeed, Benin’s National Malaria Control Programme (NMCP) is based on two malaria vector control tools, namely the nationwide distribution of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS). Since 2011, efforts have been made by Benin’s NMCP, with donor support, to achieve universal coverage of ITNs. As a result, millions of pyrethroid-treated nets have been distributed in the country over the past 10 years. Mass distribution campaigns of LLINs are conducted on a regular basis at the national level approximately every three years. For IRS, it has been implemented since 2008 using bendiocarb in the department of Ouémé in southern Benin, which is characterised by a long period of malaria transmission. This control tool was then transferred to the department of Atacora in the north of Benin where the transmission period is relatively shorter, which facilitates its coverage by a single spray round.
Thus, bendiocarb was used there from 2011 to 2013, but given the emergence of vector resistance to this product and its poor persistence \[^3\], it was replaced by Actellic 50 EC and then Actellic 300 CS (Organophosphorus) respectively between 2014 and 2016. Actellic 300 CS was finally the successful candidate to conduct the two IRS campaigns of 2017 and 2018 in the zone.

While the efficacy of LLINs \[^9\] and IRS \[^10\] in reducing malaria transmission is undisputed, vector resistance to insecticides seriously threatens to undermine vector control efforts \[^4, 11\] In a context where LLINs are truly the major tool for malaria transmission control, the widespread resistance to pyrethroids and the high intensity of this resistance \[^12\] is becoming a permanent concern for the NMCP.

The rapid evolution of phenotypic resistance to all classes of insecticide does not guarantee a long period of use of insecticides currently used in IRS in Benin. It is therefore necessary to find alternatives to anticipate the operational failure of these different insecticides in use. The present study aims to evaluate the impact of CDC bottles treated with a combination of insecticide and DEF or PBO synergists on insecticide-resistant An. gambiae populations in Benin.

### 2. Materials and methods

#### 2.1 Study area

The study areas for this activity are distributed as follows:

- **Cotton production zone (Kandi, N’Dali and Parakou):** this zone is characterised by a high use of pesticides against cotton pests. In this zone, intensive cotton cultivation is practiced, associated with the use of several families of insecticides.
- **Rice perimeter (Malanville):** The Malanville perimeter is a rice perimeter of 70 hectares. Two rice crops are grown each year, one of which is off-season. It is therefore an off-season rice crop. This activity therefore provides permanent breeding grounds.
- **Urban market gardening zone:** zone that has not undergone any IRS treatment but where people use impregnated mosquito nets, spray cans and smoke coils. It is defined by the cities of Cotonou and Porto-Novo.
- **Cereal zone:** the localities of Misséréité, Banté, Ouidah and Allada located respectively in the departments of Ouémé, Collines and Atlantique. Chemicals are therefore used to improve agricultural production.
- **Forest area:** Bohicon located in the Zou department. This is an area where millet and maize are grown.
- **Hill zone:** Dassa and Savè.

![Fig 1: Map of the study area](image-url)
2.2 Mosquito larvae collection
Mosquito larvae were collected from different breeding sites in the 13 study districts from September 2019 to June 2020, using dippers. These larvae kept in different trays labeled by collection site, were taken to the insectary of the Center for Research in Entomology of Cotonou (CREC) for rearing to the adult stage. The adult mosquitoes that emerged were caged, fed with a 10% sweetened juice, and maintained at a temperature of 27 ± 2 °C and relative humidity of 75 ± 5%.

2.3 Resistance testing
The intensity of insecticide resistance was assessed by the CDC bottle bioassay method. Thus, 2-5 day old female An. gambiae s.l. specimens were exposed to 250 ml Wheaton glass bottles coated with pyrethroid insecticides (permethrin) at 21.5 µg of active ingredient (ai) / bottle and Carbamates (bendiocarb at 12 µg of active ingredient (ai)) / bottle over a 30 minute exposure period. For each dose of insecticide, 20-25 mosquitoes were used for each replicated bottle. One bottle coated with acetone only was used as a control. After exposure, mortality was recorded at the 30-minute diagnostic time, according to CDC guidelines [14].

2.4 Identification of the molecular species of the An. gambiae complex species and characterization of the L1014F Kdr and G119S Ace-1* resistance mutation
In each study district, 50 mosquitoes from CDC bottle tests performed with permethrin 21.5 µg of active ingredient (ai) / bottle were analysed by PCR according to the protocol of Santolamazza et al. (2008) [15] to identify sister species of the An. gambiae complex.

The genotypes of the L1014F Kdr and G119S Ace-1R mutations were determined according to the protocols of Martinez et al. (1998) [16] and Weill et al. (2004) [17], respectively. The allelic frequency of these two mutations was assessed in the molecular species identified within the An. gambiae complex in each study district.

2.5 Biochemical analysis
30-50 An. gambiae s.l. females from the study districts, aged 2-5 days but not previously used for any test, were used for biochemical analyses. Prior to these analyses, the mosquito specimens were stored at -80 °C in dry Eppendorf tubes. The level of expression of biochemical enzymes [mixed function oxidases (MFO), non-specific esterases (α and β-esterases), and glutathione S-transferases (GST)] in the populations of An. gambiae s.l. from the four districts surveyed, as well as the Kisumu susceptible strain of An. gambiae s.s., was evaluated using the protocol of Hemingway et al. (1998) [18]. To determine the susceptibility of the vectors, mortality rates obtained at the 30-minute diagnostic time for CDC bottle bioassays were interpreted according to the following WHO criteria [14]:

- <90% mortality at 1x dose: susceptible population.
- [90%; 97%] mortality at dose 1x: suspected resistant population.
- < 90% mortality at 1x dose: resistant population.

Confidence intervals for the mortality rates as well as for the frequencies of the L1014F Kdr and G119S Ace-1R mutations were determined using the exact binomial test. Logistic regression was performed to assess whether there is spatial variation in the distribution of molecular species of the An. gambiae complex between the southern and northern districts.

Comparison of metabolic enzyme activity between An. gambiae s.l. populations collected in the thirteen study districts and the susceptible laboratory strain from Kisumu was performed using the Mann-Whitney U test. The statistical software R, version 3.6.2 was used to perform all statistical analyses.

3. Results
3.1 Effect of permethrin in combination with the synergist PBO on An. gambiae complex populations
Using the bottle test method, the mortality rates recorded were below 50% for the localities of Porto-Novo, Ouidah, Allada, Parakou and Missérété while the vector populations of Bohicon, Cotonou, Kandi, Malanville, Savé, Banté, Dassa and N'Dali showed mortality rates between 50% and 69%. When permethrin was combined with the synergist PBO, only the vector populations of Savé, Kandi, Malanville and N'Dali showed mortality rates of at least 90% resistant to the resistance status of these populations from resistant to resistant-suspect. For the other localities (Bohicon, Porto-Novo, Banté, Allada, Missérété, Cotonou, Ouidah, Dassa and Parakou), the mortality rates were lower than 90% varying between 58% and 85% (Table I).

Table I: Mortality rate of An. gambiae s.l. exposed to doses of permethrin and Bendiocarb combined with the synergist (PBO or DEF) in 13 districts of Benin using the CDC bottle bioassay method.

| CDC test Insecticide | Dose | Cotonou | R | Bohicon | R | Allada | R | Misséréte | R | Dassa | R | Save | R | Parakou | R | Kandi | R | Malanville | R | Banté | R | Ouidah | R | Dali | R |
|----------------------|------|---------|---|---------|---|--------|---|----------|---|-------|---|------|---|--------|---|-------|---|----------|---|------|---|--------|---|------|---|
| Per % (Nbre testé)   | 1x   | 69.1a   | R | 34.2a   | R | 61.4a  | R | 44.4a   | R | 44.3a  | R | 55.3a | R | 58.5a  | R | 43.2a  | R | 71.6a  | R | 65.8a  | R | 68.5a  | R | 49.3a  | R | 64.1a  | R |
|                      | 1.5x | 87.5b  | R | 52.5b   | R | 85.5b  | R | 68.5b   | R | 61.2b  | R | 55.5b | R | 58.5a  | R | 43.2a  | R | 71.6a  | R | 65.8a  | R | 68.5a  | R | 49.3a  | R | 64.1a  | R |
| Bendio % (Nbre testé) | 1x   | 98.8a  | S | 100a    | S | 100a   | S | 100a   | S | 97.5a  | S | 96.4a | S | 97.5a  | S | 97.5a  | S | 97.5a  | S | 97.4a  | S | 100a   | S | 96.4a  | S | 97.5a  | R |
|                      | 1.5x | 100a   | S | 100a    | S | 100a   | S | 100a   | S | 100a   | S | 100a  | S | 100a   | S | 100a   | S | 100a   | S | 100a   | S | 100a   | S | 100a   | S | 100a   | S |

%: Percentage of mortality, R: Resistance, SR: Resistance Suspected, S: Susceptible. Percentages with the same letters for the same insecticide in the same column do not differ significantly.

3.1 Effect of bendiocarb combined with the synergist DEF on An. gambiae complex populations
Only the CDC bottle test method was used to determine the effect of bendiocarb combined with the synergist DEF due to the unavailability of standard DEF impregnated papers. Thus, by exposing the vector populations of the thirteen districts to the diagnostic dose of bendiocarb, only the vector populations of Bohicon, Porto-Novo, Ouidah, Allada and Cotonou showed a total susceptibility (100%). The localities of Kandi, Malanville, Savé, Banté, Dassa, Parakou, N'Dali and Missérété showed mortality rates between 94% and 97% indicating suspected resistance to
bendiocarb in these localities. When mosquitoes from these same populations were pre-exposed to bottles treated with the synergist DEF, perfect sensitivity (100%) was observed in all localities (Table I).

3.2 Frequency of the L1014F kdr and G119S Ace-1 gene
mutations in molecular species identified within the An. gambiae complex

The distribution of An. gambiae s.l shows the presence of three main species of the An. gambiae complex: An. gambiae, An. Coluzzii and An. arabiensis with very high proportions for An. gambiae in almost all localities except in Malanville and Allada where all the Anopheles analysed are Coluzzii. As for the distribution of resistance genes, the frequency of the L1014F allele of the kdr gene is high in all localities. However, when the frequency of the L1014F allele of the kdr gene is analysed between dead and live mosquitoes resulting from exposure to permethrin (pyrethroids), there is no significant difference even though the frequency of the live ones is globally higher than the dead ones (Table II). In contrast to the L1014F allele of the kdr resistance gene, the frequency of the G119S allele of the ace-1 gene is very low in all surveyed localities. However, there was generally no significant difference between the frequency of the ace-1 gene G119S allele in dead and live mosquitoes after exposure to bendiocarb (Table III).

Table 2: Distribution of species of the An. gambiae complex exposed to permethrin and frequency of the resistant kdr allele (L1014F) in the communes of Cotonou, Porto-Novo, Bohicon, Allada, Misséréte, Dassa, Save, Parakou, Kandi, Malanville, Bante, Ouidah and NDali

| Localities     | Species          | Number tested | Genotypes | f (1014F) | df | p-value  |
|---------------|------------------|---------------|-----------|-----------|----|----------|
|               |                  |               | 1014F     | 1014L     | 1014L | 1014F   |
|               |                  |               | 1014F     | 1014L     | 1014L |          |
| Cotonou       | An. colu (Live)  | 14            | 10        | 3         | 1   | 0.82     | 1        | 1        |
|               | An. colu (Dead)  | 26            | 18        | 6         | 2   | 0.8      | 1        | 1        |
|               | An. gamb s.s (Live) | 6         | 4         | 2         | 0   | 0.83     | 1        | 1        |
| Porto-Nov      | An. gamb s.s (Live) | 3         | 1         | 2         | 0   | 0.66     | 1        | 1        |
|               | An. colu (Live)  | 7             | 5         | 1         | 1   | 0.78     | 1        | 0.848    |
|               | An. colu (Dead)  | 27            | 21        | 4         | 2   | 0.85     | 1        | 0.7205   |
| Parakou        | An. gamb s.s (Live) | 12         | 9         | 2         | 1   | 0.83     | 1        | 0.5271   |
|               | An. gamb s.s (Dead) | 28         | 17        | 9         | 2   | 0.76     | 1        | 0.9166   |
| Kandi          | An. colu (Vivant) | 8             | 6         | 0         | 2   | 0.75     | 1        | 0.5901   |
|               | An. gamb s.s (Live) | 2           | 1         | 1         | 0   | 0.75     | 1        | 0.8164   |
| Dassa          | An. gamb s.s (Dead) | 14         | 11        | 1         | 2   | 0.82     | 1        | 0.7365   |
|               | An. Arab (Live)  | 6             | 4         | 2         | 0   | 0.83     | 1        | 0.8593   |
|               | An. Arab (Dead)  | 5             | 3         | 1         | 1   | 0.7      | 1        | 0.7697   |
| Savè           | An. gamb s.s (Live) | 10         | 8         | 1         | 1   | 0.85     | 1        | 0.5721   |
|               | An. garb s.s (Dead) | 28         | 19        | 6         | 3   | 0.78     | 1        | 0.7697   |
|               | An. colu (Live)  | 6             | 6         | 0         | 4   | 0.8      | 1        | 0.5271   |
|               | An. colu (Dead)  | 2             | 1         | 0         | 1   | 0.5      | 1        | 1        |
| Bantè          | An. gamb s.s (Live) | 14         | 10        | 3         | 1   | 0.82     | 1        | 0.9166   |
|               | An. gamb s.s (Dead) | 23         | 15        | 6         | 2   | 0.78     | 1        | 0.7365   |
|               | An. colu (Live)  | 4             | 3         | 1         | 0   | 0.87     | 1        | 0.7697   |
|               | An. colu (Dead)  | 7             | 4         | 2         | 1   | 0.71     | 1        | 0.7697   |
| Bohicon        | An. gamb s.s (Live) | 2           | 1         | 1         | 0   | 0.75     | NA       | NA       |
|               | An. gamb s.s (Dead) | 30         | 13        | 11        | 6   | 0.61     | 1        | 1        |
| N’Dali         | An. gamb s.s (Live) | 9            | 5         | 3         | 1   | 0.72     | 1        | 1        |
|               | An. gamb s.s (Dead) | 15         | 8         | 5         | 2   | 0.7      | 1        | 1        |
| Allada         | An. colu (Live)  | 11            | 4         | 6         | 1   | 0.63     | 1        | 0.8539   |
|               | An. colu (Dead)  | 15            | 8         | 5         | 2   | 0.7      | 1        | 1        |
|               | An. gamb s.s (Live) | 8            | 5         | 2         | 1   | 0.75     | 1        | 0.7555   |
|               | An. gamb s.s (Dead) | 24         | 12        | 8         | 4   | 0.66     | 1        | 1        |
| Malanville     | An. Arab (Live)  | 12            | 6         | 4         | 2   | 0.66     | 1        | 1        |
|               | An. Arab (Dead)  | 6             | 3         | 2         | 1   | 0.66     | 1        | 1        |
| Misséréte      | An. colu (Live)  | 30            | 16        | 12        | 2   | 0.73     | 1        | 0.1764   |
|               | An. colu (Dead)  | 15            | 10        | 2         | 3   | 0.73     | 1        | 0.7019   |
|                | An. colu (Live)  | 20            | 12        | 6         | 2   | 0.75     | 1        | 0.9633   |
|                | An. colu (Dead)  | 18            | 9         | 7         | 2   | 0.69     | 1        | 1        |
|                | An. colu (Live)  | 27            | 13        | 10        | 4   | 0.67     | 1        | 0.7019   |
|                | An. colu (Dead)  | 2             | 1         | 1         | 0   | 0.75     | 1        | 1        |
|                | An. colu (Live)  | 3             | 1         | 2         | 0   | 0.67     | 1        | 1        |

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~ 43 ~
Table 3: Distribution of species of the *An. gambiae* complex exposed to increasing doses (5x and 10x) of carbamates (Bendiocarb) and frequency of the resistant ace-1 allele (G119S) in the communes of Cotonou, Porto-Nov, Bohicon, Allada, Misséré, Dassa, Savè, Parakou, Kandi, Malanville, Bante, Ouidah and N'Dali

| Localities   | Species          | Number tested | Genotypes | f (119S) | df | p-value<sup>*</sup> |
|-------------|------------------|---------------|-----------|----------|----|---------------------|
|             |                  |               | 119S      | 119G     | 119G |                    |
|             |                  |               |           |          |     |                     |
| Cotonou     | *An. colu* (Live)| 3             | 0         | 0        | 3   | 0                   | 1          | 1*           |
|             | *An. colu* (Dead)| 40            | 0         | 2        | 38  | 0.02                |            |
|             | *An. gambiae s.s.* (Live) | 1   | 0         | 0        | 1   | 0                   | 1          | 1*           |
|             | *An. gambiae s.s.* (Dead) | 6  | 0         | 2        | 4   | 0.17                |            |
| Porto-Nov   | *An. gambiae s.s.* (Live) | 5  | 0         | 1        | 2   | 0.17                | 1          | 1            |
|             | *An. colu* (Live) | 7             | 0         | 2        | 3   | 0.14                | 1          | 1            |
|             | *An. colu* (Dead) | 28            | 0         | 8        | 20  | 0.14                |            |
| Parakou     | *An. gambiae s.s.* (Live) | 3  | 0         | 1        | 2   | 0.17                | 1          | 0.8788       |
|             | *An. colu* (Vivant) | 1             | 0         | 0        | 1   | 0                   | 1          | 1*           |
| Kandi       | *An. gambiae s.s.* (Live) | 7  | 0         | 3        | 4   | 0.21                | 1          | 0.0026*      |
|             | *An. colu* (Live) | 3             | 0         | 0        | 3   | 0                   | 1          | 1*           |
|             | *An. Arab* (Live) | 8             | 0         | 1        | 7   | 0.06                |            |
| Dassa       | *An. gambiae s.s.* (Live) | 6  | 0         | 4        | 2   | 0.33                | 1          | 0.0035       |
|             | *An. colu* (Live) | 4             | 0         | 0        | 4   | 0                   | NA         | NA           |
|             | *An. colu* (Dead) | 9             | 0         | 0        | 9   | 0                   |            |
| Savè        | *An. gambiae s.s.* (Live) | 7  | 0         | 2        | 5   | 0.14                | 1          | 0.2274       |
|             | *An. colu* (Live) | 3             | 0         | 0        | 2   | 0.16                | 1          | 0.3643*      |
|             | *An. colu* (Dead) | 15            | 0         | 0        | 15  | 0.2                 |            |
| Bantè       | *An. Arab* (Vivant) | 5             | 0         | 2        | 3   | 0.2                 | 1          | 0.296        |
|             | *An. gambiae s.s.* (Live) | 45 | 0         | 5        | 40  | 0.06                | 1          | 0.8579       |
| Bohicon     | *An. gambiae s.s.* (Live) | 2  | 0         | 1        | 2   | 0.25                | 1          | 0.4932       |
|             | *An. Arab* (Live) | 17            | 0         | 1        | 16  | 0.03                |            |
| N’Dali      | *An. colu* (Dead) | 4             | 0         | 2        | 2   | 0.25                | 1          | 0.5582       |
|             | *An. gambiae s.s.* (Live) | 20 | 0         | 4        | 16  | 0.1                 | 1          | 1            |
| Allada      | *An. Arab* (Live) | 10            | 0         | 1        | 10  | 0.05                |            |
|             | *An. colu* (Live) | 50            | 0         | 4        | 46  | 0.04                | NA         | NA           |
| Ouidah      | *An. colu* (Dead) | 1             | 0         | 0        | 1   | 0                   | 1          | 1*           |
|             | *An. gambiae s.s.* (Live) | 45 | 0         | 4        | 41  | 0.04                | NA         | NA           |
| Malanville  | *An. colu* (Dead) | 10            | 0         | 2        | 8   | 0.1                 | 1          | 0.3718       |
|             | *An. colu* (Live) | 40            | 0         | 2        | 38  | 0.03                |            |
| Misséré     | *An. colu* (Dead) | 2             | 0         | 0        | 2   | 0                   | 1          | 1*           |

3.3 Biochemical analysis

The activities of non-specific esterases (α and β esterases) are higher in the populations: Kandi, Parakou, Porto-Nov, Dassa, Savè, Bohicon and Cotonou than in the Kisumu strain (p<0.05). The activity of mixed function oxidases was higher in the populations of Kandi, Cotonou, Dassa and Savè than in Kisumu (p<0.05). Regarding GST (Glutathione S-transferase), the highest activity was observed in the populations of Kandi, Parakou, Allada, Bohicon, Misséré, Dassa and Cotonou compared to the Kisumu strain (Fig.2).
3.4 Mapping of resistance mechanisms

Figure 3 shows a south to north distribution of the kdr resistance mechanism with a high frequency of the L1014F allele in all localities where larval surveys were carried out. The Ace-1R mutation is only present at very low frequencies (Figure 3). Metabolic resistance is present in Cotonou, Bohicon, Parakou, N’Dali and Malanville while it is absent in Porto-Nov, Misséré, Banté, Allada, Kandi, Dassa and Savè (Figure 3).
Using the CDC bottle test method, mortality rates recorded with permethrin ranged from 40% to 88% suggesting pyrethroid resistance in all 13 localities. These results obtained with the diagnostic dose of permethrin confirm the results of Aïzoun et al. (2013), Aïkpon et al. (2014), Gnanguenon et al. (2015) and Salako et al. (2018) \([1, 7, 19, 20]\). The low mortality rates recorded with the diagnostic dose of pyretrinoids (permethrin) in the centre and north of the country (Dassa, Savè, Bantè, Parakou, N’Dali, Kandi and Malanville) are thought to be due to the very high insecticide selection pressure exerted on mosquitoes in these localities, particularly in N’Dali, Kandi, Parakou and Malanville, which are characterized by high agricultural production (cotton and rice) associated with a high or even abusive use of chemical products to control pests. This is due to the widespread cultivation of cotton in the north of the country, as already reported by other authors \([21, 22]\), but also to insecticide pressures resulting from the massive use of pyrethroid-impregnated bednets \([23, 24]\). Pre-exposure of mosquitoes from these same mosquito populations to PBO resulted in significantly higher permethrin (pyrethroid) mortality rates for all locations. This is consistent with previous work by Aizoun et al. 2013; Salako et al. 2018 and Akoton et al. 2018 \([19, 20, 25]\). However, the significant increase in PBO-induced mortality
rates did not allow a return to the threshold of susceptibility (mortality $\geq 98\%$) in any of the mosquito populations at the different study sites. However, this increase did allow a change in resistance status in the communes of Savè, Ouidah and N’Dali. This partial susceptibility obtained with PBO indicates a combination of resistance mechanisms with moderate to high frequencies in An. gambiae s.l. These results are in line with those of Protoperop et al. in Tanzania [30] and Sovi et al. in Mali [27] who showed that PBO improved the control of malaria transmission compared to standard pyrethroid nets. While more epidemiological studies are needed, this finding suggests that new generation PBO LLINs should be prioritised in areas where the PBO synergist improves pyrethroid efficacy [27]. Also, several types of new generation nets are being evaluated in the country, including pyriproxyfen (PPF)-based LLINs such as Royal Guard (PPF + Alphacypermethrin), Olyset Duo (PPF + Permethrin) or Interceptor G2 (Chlorfenapyr + Alphacypermethrin), and could serve as an alternative or rotation for the control of malaria transmission through LLINs.

Results obtained with CDC bottle tests for bendiocarb show a sensitivity (mortality $\geq 98\%$) observed in Bohicon, Allada, Missérété, Ouidah, Porto-Novo and Cotonou. These mortality rates were between 90% and 97% for the localities of Dassa, Savè, Parakou, Malanville and Banté. Only the vector populations in Kandi and N’Dali showed mortality rates below 90%. These results confirm those of Aizoun et al., 2013, Aïkpon et al. (2014), Agossa et al. (2014) and Salako et al. (2018) [19, 20, 28].

Pre-exposure of mosquitoes from these same populations to the synergist DEF resulted in significantly higher mortality rates due to bendiocarb (carbamates) in all localities with a return to threshold susceptibility (mortality $\geq 98\%$) in all localities where mosquitoes were collected. This suggests that bendiocarb in combination with a synergist (DEF) may be an alternative for IRS in areas where this insecticide is beginning to select for resistant individuals. Also, other products such as Fludora Fusion or SumiShield 50WG should be evaluated in the Benin context to possibly serve as alternatives for future IRS campaigns as proven by some work in other countries in the sub-region [29, 30]. Furthermore, while the kdr and ace-1R genes confer a significant effect on vector resistance, they do not fully explain the observed cases of resistance as many homozygous susceptible individuals survive exposure to pyrethroids and carbamates [31]. This suggests that other alternative resistance mechanisms such as metabolic mechanisms operate in these mosquito populations. In order to explore the metabolic resistance mechanisms involved in the resistance of these populations, a biochemical approach was used. The high activity of non-specific esterases in the communes of Kandi, Parakou and Dassa shows that resistance to bendiocarb is not only linked to the presence of the ace-1R gene. Indeed, esterases can confer resistance to organophosphates and carbamates [32]. This overproduction of esterases is thought to be due to the insecticidal pressure exerted on mosquito larvae in cotton crops to control pests [22, 33]. Resistance to permethrin (pyrethroids) is due not only to the high frequency of the L1014F allele of the Kdr gene but also to the high oxidative activity obtained in mosquitoes from these same populations, especially in the communes of Kandi, Cotonou, Dassa and Savè. Indeed, several authors have already shown the contribution of oxidases secreted by mosquitoes to pyrethroid resistance [34, 35]. This resistance to pyrethroids may also be due to glutathione S-transferase (GST) activity as reported by Vontas et al. [36].

5. Conclusion
Widespread resistance to pyrethroids (permethrin) and carbamates (bendiocarb) and the intensity of this resistance along the south-north transect of the country is becoming an ongoing concern for the NMCP as these classes of insecticides are the most widely used by the main vector control tools in Benin. The present study has shown that the addition of the synergist PBO or DEF to these insecticides has considerably increased their efficacy. Thus, dual-active LLINs such as PBO-based LLINs [Olyset Plus (PBO + Permethrin), VEERALIN (PBO + Alphacypermethrin), Permanet 3.0 (PBO + Deltamethrin)] could be considered as an alternative option in areas where pyrethroid-only LLINs are less effective. Also, the combination of bendiocarb with the synergist DEF or other products such as Fludora Fusion or SumiShield 50WG should be evaluated in the Benin context to possibly serve as an alternative or rotation for future IRS campaigns.

6. Author’s Contributions
MCA, RO, and GGP conceived the study. MCA, HWS, WS, RO, FD, ASS and GGP have participated in the design of the study. Entomologic data was collected by RO, ASS, HWS, AAS and GGP and laboratory analysis was carried out by KZC, EO, RO, PGG and ASS. CDK drafted the manuscript. Statistical data analysis by CJA, BY, SA, AJF, HWS, IA, RO, ASS, LSB and MCA critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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

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