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Exploration of the Cuticular and Intestinal Microbiota of Pyrethroid-Resistant *Anopheles gambiae* at Tokpa-Zougo in the Commune of Abomey-Calavi in Southern Benin

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Abstract

Background: Mosquito microbiota have recently been identified as one of the multiple mechanisms underlying insecticide resistance. The aim of this study was therefore to assess the cuticular and intestinal microbial diversity of *Anopheles* mosquitoes exposed or not to pyrethroids (deltamethrin and permethrin) at Tokpa-Zoungo in the commune of Abomey-Calavi in southern Benin. **Methodology:** The *Anopheles* larvae collected in Tokpa-zoungo were transported to the Entomological Research Center of Cotonou where insecticide susceptibility tests and molecular identification on the adult mosquitoes aged 2 to 5 days were carried out. The microbiological analyses were carried out in three steps: the preparation of the mother suspensions of the mosquito microbiota, the culture and isolation on the EMB, MSA and PDA agars and the identification and conservation of the isolated microbial strains. **Results:** The species *Anopheles coluzzii* was predominant (62%), with high resistance to pyrethroids. Microbiological analysis revealed that species of the *Enterobacteriales* family (71.11%) were the most represented. Differences were also noted between the microbial species identified in terms of microbiota and resistance phenotype. Yeasts appeared only on the cuticle surface of Kisumu, and wild strains were not exposed to insecticides. Susceptible mosquitoes had

lower intestinal microbial diversity than deltamethrin- and permethrin-resistant mosquitoes and wild strains not exposed to insecticides. **Conclusion:** The study showed a variation in microbial communities carried by *Anopheles coluzzii* according to microbiota type and resistance phenotype. But these differences do not allow us to define a microbial identity specific to a given resistance phenotype. Further research is needed to better understand insecticide-microbiota interactions.

Keywords

Anopheles coluzzii, Pyrethroids Resistance, Intestinal and Cuticular Microbiota, Abomey-Calavi, Benin

1. Introduction

Malaria is an endemic disease, or worse, a public health problem, caused by a protozoan parasite of the genus *Plasmodium*, whose sporozoite forms are inoculated into humans by the infesting bites of the female mosquito of the genus *Anopheles* [1]. It is one of the world's most deadly parasitic diseases, responsible in 2023 for 597,000 deaths out of 263 million cases recorded in 83 countries. This represents around 11 million more cases than in 2022, and almost the same number of deaths. Africa accounts for a large and disproportionate share of the global burden, with 94% of the total number of cases and 95% of associated deaths worldwide. Children under the age of 5 accounted for almost 76% of malaria deaths on the continent [2]. According to annual health statistics from Benin's Ministry of Health, for over 10 years (2010 to date), malaria has been the leading cause of consultation and hospitalization in health services, the leading cause of death, and accounts for around 40% of all sick people in the country each year [3]-[15].

Similarly, the persistence of this pathology not only affects the economic burden and resources of healthcare systems, but also induces significant mortality and morbidity in populations, and even more so in endemic areas [16].

This extensive involvement of mosquitoes in the transmission and spread of pathogens has prompted a great deal of thought, research and decision-making, leading to the introduction of various control methods, mainly targeting adult vectors, the mainstay of which is the use of insecticides through IRS, extra-domiciliary spatial spraying and, above all, the provision of LLINs to populations [17] [18]. Thus, the introduction of various control interventions has prevented 663 million cases of malaria since the year 2000, of which 68% and 10% have been contributed by LLINs and IRS respectively [19]. Unfortunately, progress has virtually ground to a halt since 2015 in several countries where *Plasmodium* transmission has gone from moderate to elevated [20]. There are several underpinnings to this concern, all converging on the phenomenon of vector mosquito resistance to insecticides approved for vector control [21]-[25].

A great deal of research has been carried out into the various mechanisms un-

derlying this phenomenon, with the aim of finding effective solutions. Apart from the behavioral, physiological, metabolic and molecular mechanisms identified [26] [27], the mosquito microbiota has also been the subject of particular interest [28] [29].

Indeed, the intestines, salivary glands, and reproductive organs of mosquitoes are colonized by a wide diversity of microorganisms that influence certain factors that determine their ability to transmit pathogens [30]. Researchers detected *Delftia* (15.46%) in *Anopheles triannulatus s.l.* pupae, and *Asaia* (98.22%) in *Anopheles darlingi*, associated with *Plasmodium* inhibition. There was also a low abundance of the genera *Elizabethkingia, Klebsiella* and *Serratia*, which were recognized for their potential in paratransgenesis [31]. The works of Rashad Abdul-Ghani *et al*., published in 2012 showed that the microbiota plays a role in modulating the vectorial competence of the Anopheles mosquito during Plasmodium infection [32].

Similarly, certain bacteria in the microbiota play an important role in the detoxification of insecticide molecules [28]. Work by Dada *et al*., in 2019 in Guatemala highlighted significant differences in intestinal bacterial composition between *Anopheles albimanus* exposed to pyrethroids: alpha-cypermethrin and permethrin and those not exposed [33]. In 2021 in Côte d'Ivoire, Pelloquin *et al.* showed that an overabundance of *Asaia* and *Serratia* bacteria was linked to deltamethrin sensitivity in *Anopheles coluzzii* from Agboville [34].

In 2017, works by Soltani *et al.* in Iran demonstrated that treatment of the bacterial symbiont of Temephos-resistant *Anopheles stephensis* mosquitoes had the effect of destroying the bacterial composition of the microbiota and significantly reducing the activity of the three main resistance enzymes (esterase, glutathione S-transferase and acetylcholinesterase), thereby restoring the mosquitoes' sensitivity [35]. Gut microbes have also been implicated in pesticide resistance in insect pests [36] [37]. *Enterococcus spp* isolated from the intestines of the diamondback moth *Plutella xylostella* increased the insect's resistance to chlorpyrifos, while *Serratia spp* decreased resistance [38]. These studies demonstrate the existence of a potential interaction between the internal microbes of mosquitoes and their resistance or sensitivity to chemical insecticides.

In Benin, the microbiota of mosquitoes is one of the least explored areas. A recent study revealed the natural presence of the *Wolbachia* endosymbiont and microsporidia in *Anopheles* mosquitoes [39], but to our knowledge, no study has yet looked at the interactions between insecticides and mosquito microbiota.

We therefore hypothesized that the microbial communities present in the gut and on the cuticle surface of pyrethroid-resistant mosquitoes might be different from those of sensitive mosquitoes. This potentially implies interactions between microbes and insecticide resistance mechanisms. The aim of this study is therefore to assess the cuticular and intestinal microbial diversity of *Anopheles* mosquitoes exposed or not to pyrethroids (deltamethrin and permethrin) in the locality of Tokpa-Zoungo in the commune of Abomey-Calavi in southern Benin, in order to propose solutions for better control of resistant malaria-vector mosquitoes. The

specific aim was to determine the sensitivity of *Anopheles* mosquitoes to deltamethrin and permethrin, and to compare the cuticular and intestinal microbial diversity of resistant mosquitoes with that of sensitive mosquitoes not exposed to these same insecticides.

2. Material and Methods

2.1. Study Area

The commune of Abomey-Calavi (6˚27'N; 2˚21'E) is located in the Atlantic department of southern Benin, about 18 km north of the economic capital Cotonou. It has a sub-equatorial climate, with high humidity and alternating dry seasons (early December to March and August to mid-September) and rainy seasons (March to late July and mid-September to early December). It has more than 600,000 inhabitants and is divided into 9 arrondissements, including Abomey-Calavi, where the Tokpa-Zoungo locality, the target area of our study, is located [40]. Moreover, in 2022, according to the annual health statistics of the Ministry of Health, more than 200,000 cases of simple and severe malaria were recorded in the Atlantic department, with an incidence of 14.3% [15].

2.2. Type of Study and Sample Collection Site Selection Criteria

This is an experimental and descriptive study with analytical aims, which took place over the period from May 2024 to December 2024. Mosquito specimens were collected in the Tokpa-Zoungo locality (**Figure 1**). The choice of sample collection site was made taking into account the proximity of the target area to the premises of the University of Abomey-Calavi (UAC) and also the availability of a large number of permanent water collections favorable to the proliferation of mosquitoes of the genus *Anopheles*.

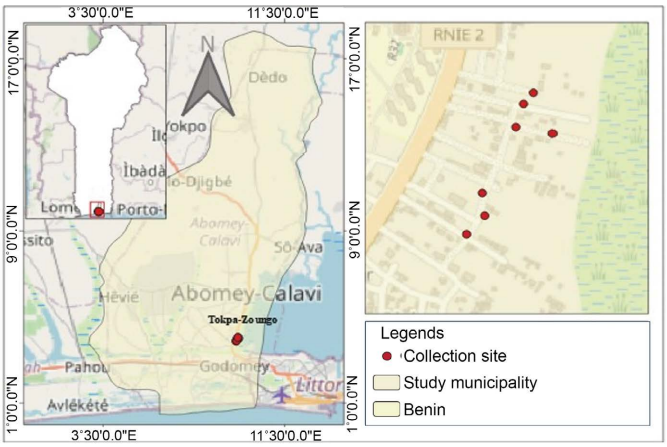

Figure 1. Map of *Anopheles* larvae collection sites in Tokpa-Zoungo. Source: KiN, 20122 & OpenStreet Map. Realization: Constantin ADOHA. Date: May 2024.

2.3. Sampling and Sensitivity Testing to Pyrethroid Insecticides

Anopheles larvae were collected from several breeding sites on the surface of per-

manent water collections found in the Tokpa-Zoungo locality (Figure 2 and Figure 3), then transported to the insectarium of wild strains at the Centre de Recherche Entomologique de Cotonou (CREC), where they were reared under standard conditions (temperature: 27 ± 2˚C; humidity: 70% to 80%) until winged adults were obtained. The resulting adults (Figure 4), aged 2 to 5 days, were fed with a 10% sucrose solution and subjected to WHO tube sensitivity tests (Figure 5) with the pyrethroids deltamethrin (0.5%) and permethrin (0.75%). Mosquitoes of the genus *Anopheles* have been distinguished from other species at the larval stage: *Anopheles* larvae are easily recognized by their horizontal position on the water surface.

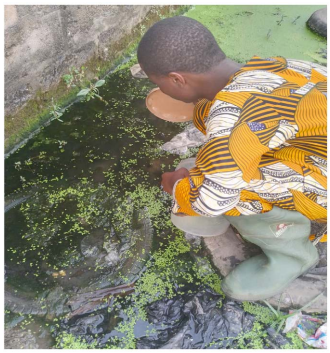

Figure 2. Collection of *Anopheles* larvae at Tokpa-Zoungo.

Figure 3. *Anopheles* larvae collected at Tokpa-Zoungo.

Figure 4. Anopheles mosquitoes aged 2 to 5 days from larvae collected in Tokpa-zoungo.

Figure 5. WHO tube sensitivity testing at the CREC bioassay laboratory.

2.4. Preparation of Stock Suspensions of Mosquito Microbiota

After insecticide susceptibility testing, live mosquitoes (control and insecticide-resistant) were euthanized under cold conditions (15 to 30 minutes at −4˚C). Mosquitoes were then randomly selected and grouped into pools of 5 specimens, taking into account only resistance phenotype and insecticide type. Similarly, sensitive mosquitoes (Kisumu strains) from the CREC insectarium were used as controls in the manipulations. A total of 17 pools of 5 mosquitoes were selected for microbiological analysis.

To obtain the microbiota on the cuticle surface of specimens, mosquitoes belonging to the same pool were aliquoted in 500 µl of physiological water and vortexed at 1000 rpm for 20 to 30 seconds. The obtained supernatant was diluted 1:10 in nutrient broth and incubated for 24 to 48 hours.

To avoid contamination of the specimens' gut microbiota stock suspensions, the mosquitoes were surface sterilized by rinsing: once in a 2% bleach solution for 10 minutes, twice in sterile phosphate-buffered saline (PBS) × 1 for 5 minutes, three times in 70˚ ethanol for 5 minutes, and once with sterile distilled water, as described in the protocol carried out by Muturi *et al*., 2021 in Florida [41]. In a sterile area delimited by the Bunsen burner flame, the abdomen of each mosquito was separated from the head and thorax using a dissection kit. The carcass (head-thorax-legs-wings) of each specimen was preserved under silica gel for molecular identification. Mosquito abdomens were grouped by pool, aliquoted in 500µl of sterile physiological water, then ground manually using small sterile grinding rods. The resulting mixture is left to settle for a few minutes, so that the debris from the grinding settles to the bottom of the tube, then the supernatant is diluted 1:10 in nutrient broth (stock suspension) and incubated for 24 to 48 hours. After incubation, turbidity can be observed in both seeded and unseeded broths. The same process was carried out on specimens of susceptible mosquitoes (kisumu strains) bred at the CREC's insectarium for susceptible strains.

2.5. Culture and Isolation of Microbial Strains

Firstly, a slide-to-slide preparation and a Gram-stained smear were taken directly from the stock suspensions to confirm or rule out the presence or absence of microbes. This helped guide the choice of suitable cultural media. Then, for each

pool of mosquitoes, Eosin Methylen Blue (EMB), Mannitol Salt Agar (MSA) and Potato Dextrose Agar (PDA) were inoculated from the master suspensions, and incubated for 24 to 48 hours at 37˚C. Finally, after incubation and reading of the inoculated agar plates, the fresh state and Gram-stained state of the colonies obtained were determined, and specific culture media were selected for purification and re-isolation of the strains, based on the results obtained on microscopic examination.

2.6. Identification and Preservation of Microbial Strains

The various microbial strains isolated were identified using biochemical tests. *Enterobacteriaceae* were identified using the Api 20E Gallery. The genus *Staphylococcus*, being facultative aero-anaerobic, was identified using the catalase test. In the Gram-stained state, *Bacillus* appear in chains, sometimes developing spores and, above all, producing oxidase. Yeasts take on a dark blue color, appearing oval or spherical with a large waist and grouped in clusters. The filamentation test was used to distinguish yeasts belonging to the *Candida albicans* complex.

On the last day of handling, the microbial strains isolated and identified were preserved using trypticase-soy broth with 20% glycerol for bacteria and yeasts using a 20% glycerol solution following the CDC preservation protocol [42].

2.7. Molecular Identification of Mosquitoes

Species identification was carried out using Hot start Taq PCR following the protocol of Santolamazza *et al*., 2008 [43]. DNA was extracted with CTAB from the carcass (head, thorax, legs and wings) of each mosquito. Two microliters of each DNA sample were diluted in 10.5µl of Mix solution and put through the thermal cycler for amplification. PCR products were mixed with Bromophenol Blue and migrated onto agarose gel. Two primers: F6 and R6 and four controls were used: the negative control; the positive control; the control for *Anopheles gambiae* and the control for *Anopheles coluzzii*.

Table 1 shows the composition of the Mix solution used to carry out the polymerase chain reaction for molecular identification of the various specimens in this study.

Table 1. Composition of the mix solution used for species PCR.

Reagent	Volume for one sample
Master Mix PCR 2 × (Buffer + $MgCl_2$ + dNTPs + Taq polymerase)	6.25 µl
ddH_2O	3.25 µl
F6 primer (5'-TCG CCT TAG ACC TTG CGT TA -3')	0.5 µl
R6 primer (5'-CGC TTC AAG AAT TCG AGA TAC -3')	0.5 µl

2.8. Data Analysis

The geographical coordinates of the larvae collection sites were recorded using the

UTM Geo Map for Android application. The results obtained during the study were collected, recorded and ordered using Excel spreadsheet software (Microsoft office professional plus 2019), then directly processed and commented.

3. Results

3.1. Sensitivity Tests with Deltamethrin (0.5%) and Permethrin (0.75%)

Insecticide sensitivity tests revealed 100% survival of mosquitoes exposed to permethrin (0.75%) and 92.60% to deltamethrin (0.5%). *Anopheles* mosquitoes collected in Tokpa-Zoungo are resistant to permethrin and deltamethrin, and therefore to pyrethroids (Figure 6).

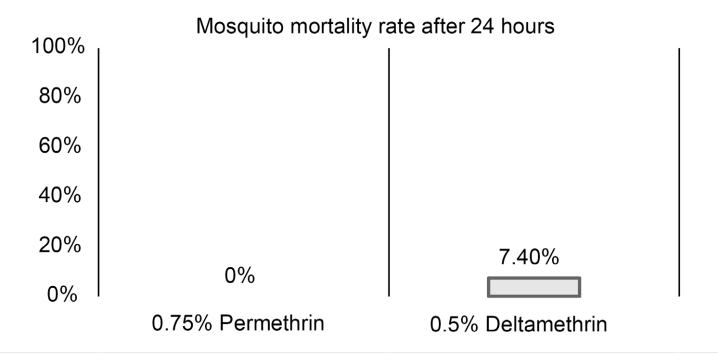

Figure 6. 24-hour mortality rate of mosquitoes exposed to diagnostic doses of permethrin and deltamethrin.

3.2. Molecular Identification of *Anopheles* Species

Anopheles coluzzii was the most represented species (62%) among the insecticide-tested mosquito populations, and 38% of these specimens belonged to other *Anopheles* species (Figure 7).

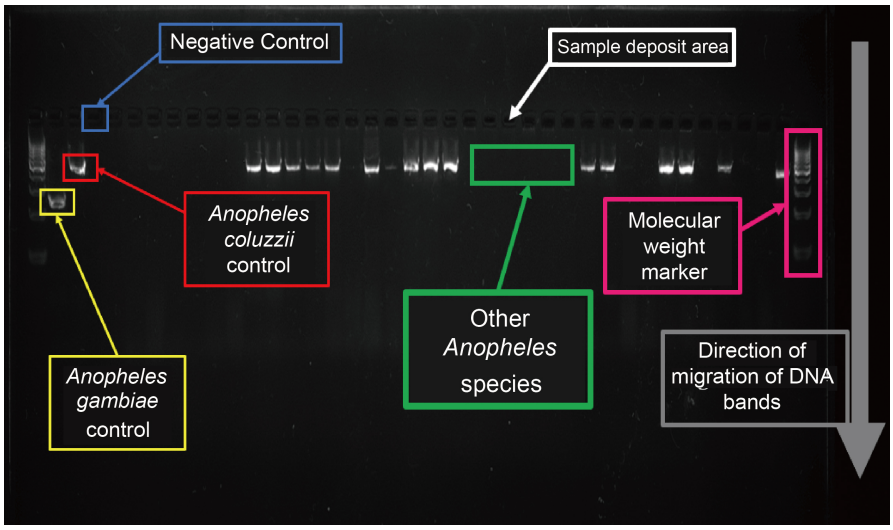

Figure 7. Migration of PCR products visualized by the transilluminator.

3.3. Mosquito Cuticular and Intestinal Microbial Diversity

A total of 45 microbial strains were identified, divided into 5 families: *Enterobacteriales* species were the most abundant (71.11%), followed by yeasts (11.11%), *Micrococcaceae* (6.67%), *non-enterobacteria* (6.67%) and *Bacillaceae* (4.47%). *Enterobacteriales* were the most represented at both cuticular (61%) and intestinal (81%) levels. In addition, *Bacillaceae* (8%) and yeasts (21%) appeared only on the cuticle surface of mosquito specimens, while species of the *Micrococcaceae* family (14%) were present only at the intestinal level (Figure 8).

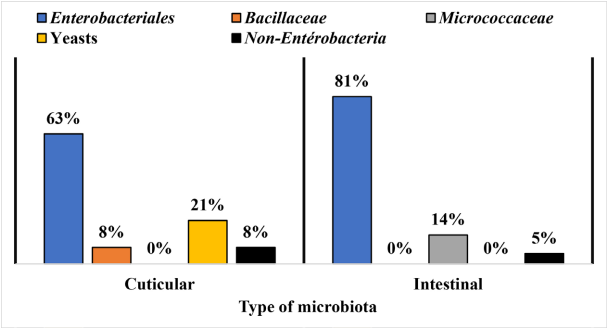

Figure 8. Proportion of strain families isolated from mosquito specimens according to microbiota type.

At species level, 13 microbial taxa were identified, of which 3 were unique to the internal microbial niche, 6 to the cuticle surface and 4 were shared by both. Similarly, *Serratia liquefaciens* was the most represented bacterial species (43.75% of *enterobacteria* identified) both at intestinal (33%) and cuticular (29%) levels, and was present in 55% of the mosquito specimens in the study (Figure 9).

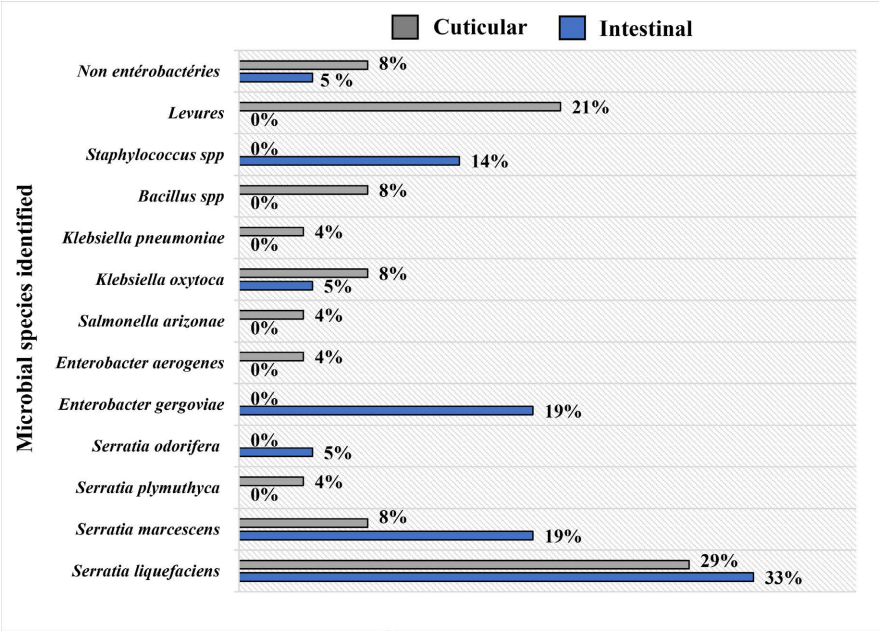

Figure 9. Proportion of microbial species isolated from mosquito specimens as a function of microbiota type.

3.4. Cuticular and Intestinal Microbial Diversity at the Level of Each Resistance Phenotype

Generally speaking, differences were identified between microbial species isolated from mosquito specimens according to the type of microbiota at the level of each resistance phenotype (Figure 10).

In the case of wild strains not exposed to insecticides, of the 5 species identified, *Serratia liquefaciens* and *Serratia marcescens* were the only microbial taxa common to both the gut and cuticular microbiota of the specimens. In the Kisumu strains, of 6 microbial species identified, 4 (*Serratia plymuthyca*, *Salmonela arizonae*, *Enterobacter aerogenes* and yeasts) were specific to the cuticle, one to the internal microbial niche and one was shared. In Permethrin-resistant mosquito specimens, the taxa identified differed according to the type of microbiota. No microbial species were shared between the two niches. In deltamethrin-resistant mosquitoes, of 5 species identified, 2 were unique to the internal microbial niche, one was specific to the cuticular surface and 2 were shared, while no difference was noted in the microbial taxa isolated from strains sensitive to the same insecticide (*Serratia marcescens* was the only species identified).

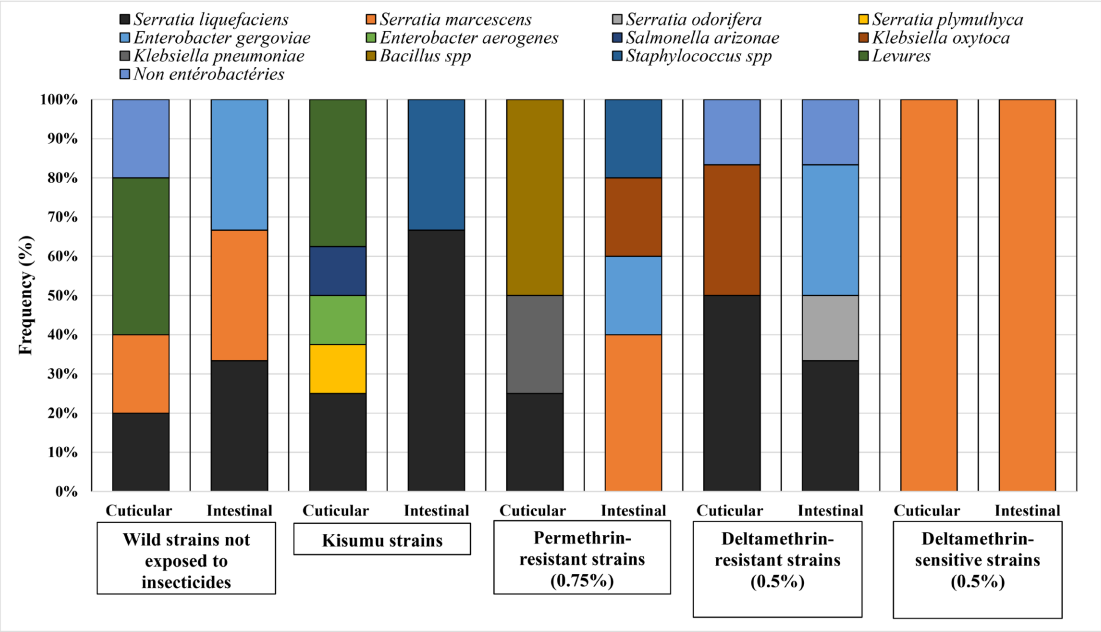

Figure 10. Distribution of identified microbial species according to microbiota type and resistance phenotype.

3.5. Cuticular and Intestinal Microbial Diversity between Resistance Phenotypes

In general, the microbial composition of mosquito specimens varied according to microbiota type between the different resistance phenotypes.

At cuticular level, microbial diversity appeared to be balanced (at least 3 microbial species identified per resistance phenotype, except in deltamethrin-sensitive specimens). Yeasts appeared only in Kisumu strains (75% of specimens) and wild

strains not exposed to insecticides (50% of specimens). *Bacillus spp* appeared only on the cuticle surface of permethrin-resistant mosquitoes (50% of specimens). *Serratia liquefasciens* was the only shared species (Figure 11 and Figure 12).

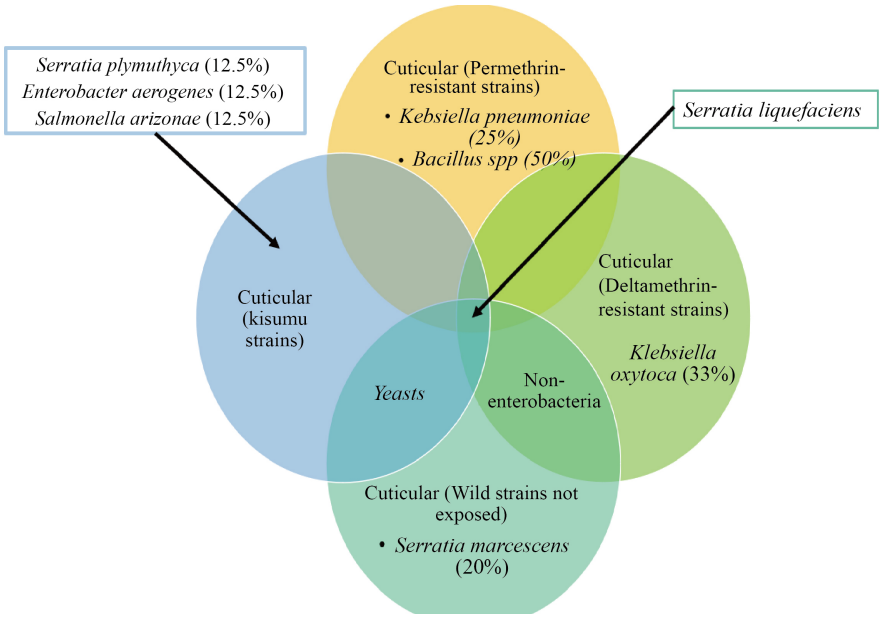

Figure 11. Distribution of microbial species isolated from the cuticular microbiota of mosquito specimens according to resistance phenotype.

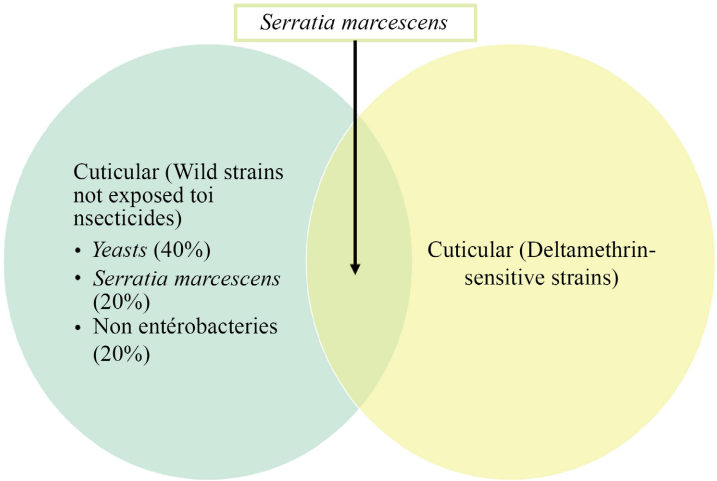

Figure 12. Distribution of microbial species isolated from the cuticular microbiota of wild strains not exposed to insecticides and those sensitive to deltamethrin.

At intestinal level, the sensitive groups (Kisumu strains and deltamethrin-sensitive strains) showed lower microbial diversity compared with deltamethrin- and permethrin-resistant mosquitoes and wild strains not exposed to insecticides. Similarly, numerous differences were noted between the gut microbial communities of deltamethrin-resistant and/or sensitive mosquito specimens, permethrin-resistant specimens, Kisumu strains and wild strains not exposed to insecticides.

Non-enterobacteria species (17%) and *Serratia odorifera* (17%) were identified in deltamethrin-resistant mosquitoes, while permethrin-resistant mosquitoes included *Serratia marcescens* and *Klebsiella oxytoca* (20%). Furthermore, *Serratia liquefasciens* was not identified in Deltamethrin-susceptible and Permethrin-resistant mosquito specimens (Figure 13 and Figure 14).

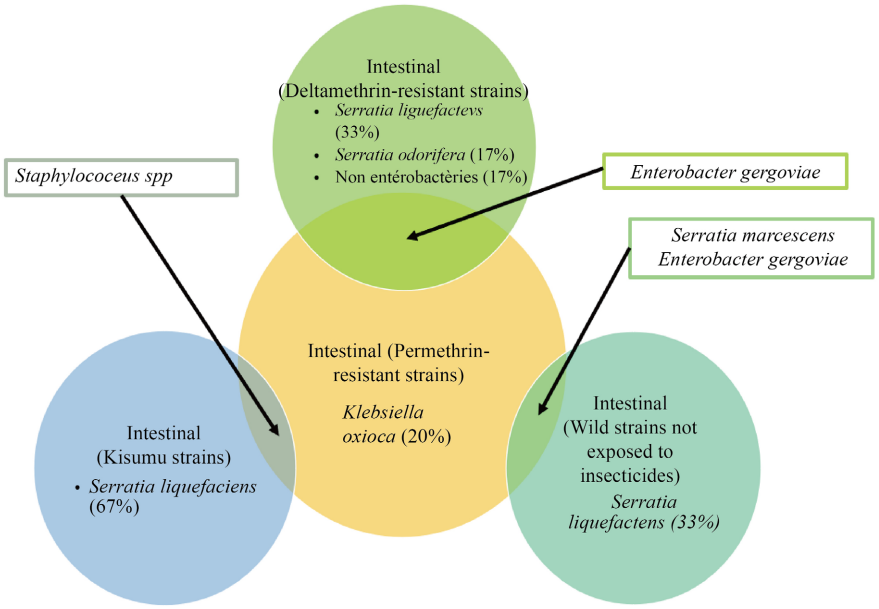

Figure 13. Distribution of microbial species isolated from the gut microbiota of mosquito specimens according to resistance phenotype.

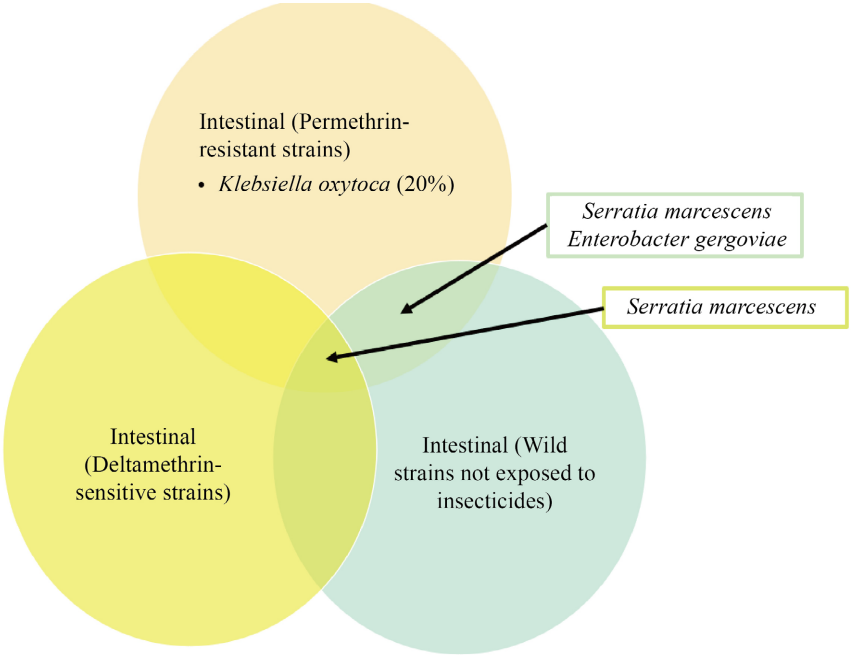

Figure 14. Distribution of microbial species isolated from the gut microbiota of wild strains not exposed to insecticides, permethrin-resistant and deltamethrin-sensitive mosquito specimens.

4. Discussion

Malaria has long been the most deadly parasitic disease in history. The disease is caused by a parasite of the genus Plasmodium, transmitted to humans by the Anopheles mosquito, whose main vector species in Africa is *Anopheles gambiae*. Despite numerous national and international interventions, the incidence and lethality of this pathology are complicated by the growing resistance of mosquitoes to insecticides approved for vector control. Research is therefore crucial to improving strategies for controlling and preventing insecticide resistance, in order to reduce the heavy burden imposed by malaria. To achieve this, it is necessary to have a thorough understanding of the multiple mechanisms underlying the phenomenon of insecticide resistance in vectors.

Beyond the genetic and biochemical mechanisms identified [44], it is plausible that the cuticular and/or intestinal microbiota of mosquitoes may also play an important role in the various processes of insecticide detoxification in their host. In Benin, very few studies have focused on the involvement of microbial communities carried by mosquitoes in their resistance or susceptibility to insecticides. This study provides information on the cuticular and intestinal microbial communities of mosquito specimens collected in the Tokpa-Zoungo locality (Abomey-Calavi commune) and exposed to pyrethroids (deltamethrin and permethrin).

The study revealed a predominance of the *Anopheles coluzzii* species in the Tokpa-Zoungo locality and high resistance to pyrethroids, with a 24-hour mortality rate of 0% for permethrin and 7.40% for deltamethrin. This corroborates the work of Djègbè *et al*., in Abomey-Calavi in 2019, which revealed a predominance of *Anopheles coluzzii* in the various sites surveyed (54% versus 46% for *Anopheles gambiae*). Also, the mortality rate after 24 hours was 52% following exposure to deltamethrin [23]. The very low mortality rate in our study may be due to the small sample size.

Microbiological analysis of mosquitoes in general revealed an abundance of the *Enterobacteriales* family (71.11%) within the cuticular (63%) and intestinal (81%) microbial communities. These results are similar to those of the study conducted by Dada *et al*., in 2018 in Peru, which showed an abundance of the *Enterobacteriales* and *Moraxellaceae* families within bacterial communities carried by *Anopheles albimanus* resistant and susceptible to the insecticide fenitrothion [45]. The genus *Serratia* dominated both the cuticular (29%) and intestinal (33%) surfaces of the mosquitoes, which is at odds with the results of work by Dada *et al*., in 2019 in Guatemala, which revealed a predominance of the genus *Asaia* on the cuticular surface and intestinal surfaces of adult *Anopheles albimanus* [33]. Similarly, work carried out in Peru in 2018 by Dada *et al*., revealed a predominance of the bacterial species *Klebsiella pneumoniae* accounting for 74% and 49% respectively in fenitrothion-resistant and -susceptible *Anopheles albimanus* specimens [45]. In comparison, *Klebsiella pneumoniae* species were in the minority (4%) and found only on the cuticle of permethrin-resistant mosquitoes. Furthermore, at intestinal level, *Serratia liquefaciens* was the most abundant species, found only in Kisumu

strains (67%) and Deltamethrin-resistant specimens (33%). This could suggest that the mosquito microbiota varies according to *Anopheles* species and even insecticide type.

Of 45 microbial strains isolated from mosquito specimens, 13 microbial taxa were identified. Of all the microbial taxa detected, 46% were specific to the cuticle surface (n = 6), 23% to the internal microbial niche (n = 3) and around 31% shared between cuticle and gut. The microbiota on the surface of the mosquito cuticle (n = 10) contains almost twice as many taxa as the internal microbiota (n = 7) in *Anopheles coluzzii*. These results corroborate those of Dada *et al.* in 2019 in Guatemala, who showed that the adult microbiota on the cuticle surface contained almost twice as many taxa (n = 106) as the internal microbiota (n = 62). The works of Dada *et al.*, had shown that of 118 adult microbial taxa detected (n = 118), 47% were specific to the cuticle surface, 10% to the internal microbiota and 43% were shared [33]. In contrast, in larvae, the cuticle surface microbiota comprised only slightly more taxa (n = 194) than the internal microbiota (n = 180), and of all the taxa detected (n = 203), 11% were specific to the cuticle surface, 4% to the internal microbial niche and 85% were shared [33]. This could be explained by the difference in developmental stages.

Variations in microbial communities between insecticide-resistant and insecticide-sensitive mosquito populations have already been observed [41] [45]-[47]. Our study is also in line with this perspective. We note distinct differences in the microbial communities carried by specimens depending on resistance phenotype and insecticide type. This has been the case in numerous studies, including the works of Omoke *et al.* in 2021 in western Kenya, who showed significant differences in bacterial composition in *Anopheles gambiae* sensu stricto as a function of phenotypic resistance status by comparing Bray-Curtis dissimilarity indices using PERMANOVA [48]. These results are also supported by work carried out by Pelloquin *and al.,* in 2021 at Agboville in Côte d'Ivoire. In their study, Pelloquin *et al.*, noted significant differences in the diversity of the microbiota of 2- and 3-day-old deltamethrin-resistant and -sensitive *Anopheles coluzzii* [34]. Similarly, work by Worku *et al.*, in 2025 in Ethiopia showed clear differences in the microbiota composition of deltamethrin-resistant and deltamethrin-sensitive *Anopheles arabiensis* populations. However, a different result was observed in Burkina-Faso. Worku *et al.* also showed that the composition of the microbiota between deltamethrin-resistant and sensitive populations of *Anopheles gambiae*, *Anopheles coluzzi* and *Anopheles arabiensis* did not differ in Burkina-Faso. This is at odds with our results [49].

This suggests that the impact of the microbiota on resistance or susceptibility to pyrethroid insecticides could be contextual, and therefore dependent on specific local factors, such as the environment, the diet of these insects or the interactions of Anopheles mosquitoes with other organisms. Disagreement with our results could therefore be attributed to methodological, environmental or biological differences.

Moreover, at the intestinal level, the sensitive groups (Kisumu strains and deltamethrin-sensitive strains) showed lower microbial diversity than the deltamethrin- and permethrin-resistant groups. This corroborates the work of Pelloquin *et al*., who noted a significant reduction in alpha diversity (Shannon index) in sensitive specimens compared with resistant individuals [34]. On the other hand, at cuticular level, our work showed that although microbial diversity within Kisumu strains was higher than in other mosquito specimens, they appeared to be roughly balanced. Similar results were obtained by Dada *et al*., in 2019 in Guatemala, who found significant differences in Shannon diversity indices as a function of insecticide type and insecticide exposure in the internal microbiota but not on the cuticle surface in adult *Anopheles albimanus* mosquitoes [33]. This suggests that insecticide resistance or susceptibility is associated with differences in gut microbial communities, but not in cuticular microbial communities. However, in our study, the low frequency of occurrence of some of the microbial species that mark the difference between the microbiota of mosquito specimens does not allow us to define a microbial identity specific to a given resistance phenotype.

It should be noted that the low frequency of appearance of the microbial species that mark the difference in the composition of the microbiota of mosquito specimens does not allow us to define a microbial identity specific to a given resistance phenotype.

Serratia marcescens was the only microbial taxon isolated from the cuticular and intestinal microbiota of deltamethrin-sensitive mosquito specimens, but this bacterial species also appears in some insecticide-resistant mosquito populations. The absence of a phylogenetic tree of isolated *Serratia marcescens* strains prevents us, in this context, from establishing a significant link between this bacterial species and insecticide sensitivity. Nevertheless, it is essential to note that the work of Xia *et al*., recently published in 2023, highlighted the presence of the organophosphate-degrading metallo-hydrolase gene MBL in the *Serratia marcescens* strain isolated from the gut of the bean bug *Riptortus pedestris* [50].

This study had the merit of associating yeasts with variations in the mosquito microbiota as a function of resistance phenotype and insecticide exposure. Indeed, the study revealed that yeasts only appeared on the cuticle surface of Kisumu strains (in 75% of specimens) and unexposed wild strains (in 50% of specimens). This difference is very important. Resistant strains, having developed mechanisms to survive these insecticides, could have a different microbiota, less favorable to yeast development. More or less similar results were reported in the work of Dada *et al*., in Guatemala in 2019. These works showed that bacterial taxa not identified to species, *Pantoea agglomerans* (a bacterial species known to degrade insecticides) and *Pseudomonas fragi* were more abundant in adult mosquitoes exposed to insecticides than in those not exposed [33]. This suggests that insecticide exposure modifies the microbial communities carried by mosquitoes.

However, it is important to note that our study still faces a number of limitations, in particular the method used for microbiological analysis, which does not

favor the identification of certain microbial species, such as bacteria of the *Asaia* genus.

In the work of Pelloquin *et al*., in particular, it was noted that an overabundance of bacteria of the genera Asaia and Serratia were associated with deltamethrin sensitivity in *Anopheles coluzzii* [34]. Worku *et al.* found a genus associated with survival, *Sphingomonas* (pANOVA = 0.029), in samples collected in the field in Burkina Faso, which was significantly more abundant in survivors [49]. Similarly, a significant association was noted between mosquito survival and the genera *Escherichia*, *Pantoea* and *Kosakonia* [49].

The abundance of taxa was not taken into account to assess the potential effect of insecticides on these microbial communities. This has been the case in numerous other studies [33] [34] [45]. Nevertheless, this study offers the opportunity to explore resistant mosquitoes with their specific microbiota in their living environment. This approach could lead to the association of resistance mechanisms developed by mosquitoes with the different microbial communities they carry. Some studies could look at how mosquitoes behave after antibiotic treatment. Other researchers could repeat our work, but this time using many more culture media while varying the culture conditions (aerobic and anaerobic), or even better, using 16s RNA metagenomic sequencing. This will enable us to detect a wider range of bacterial strains, and thus bring out many more remarkable differences. Many aspects remain to be clarified in order to better understand insecticide-microbiota interactions and the various mechanisms involved.

5. Conclusion

This study has shown a high level of resistance of *Anopheles coluzzii* to pyrethroids at Tokpa-Zoungo in the commune of Abomey-Calavi. Several mechanisms could be at the root of this phenomenon, notably cuticular and intestinal microbial composition. In fact, microbiological analyses revealed not only an abundance of enterobacteria in *Anopheles coluzzii*, but also distinct differences in the microbial communities carried by the mosquitoes according to resistance phenotype and type of microbiota: yeasts appeared only on the cuticle surface of Kisumu and wild strains not exposed to insecticides. But these differences do not allow us to define a microbial identity specific to a given resistance phenotype. However, several interesting aspects of this theme were not addressed in this study. It would therefore be essential to carry out in-depth research with a view to better understanding insecticide-microbiota interactions, and thus to highlight the possible role of microbial communities carried by mosquitoes in the latter's resistance or sensitivity to insecticides approved for vector control, as well as the various mechanisms involved.

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Authors' Contributions

TFT, FH, BA, RO, SH and MA designed the research; TFT, FH, BA, SH, IJGY, BA and FH conducted data collection; all authors conducted data analysis. TFT, BA, FH, OR, GL and VF coded the data; TFT, FH BA, and led the drafting with substantive input from ZSDK, MA and EAE in the results section; all authors revised the manuscript.

All authors read and approved the final manuscript.

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Ethics Approval

The study was conducted in accordance with the Declaration of Helsinki.

Availability of Data and Materials

Data is contained within the article.

Conflicts of Interest

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

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