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Interplay Between Oxytetracycline and the Homozygote *kdr* (L1014F) Resistance Genotype on Fecundity in *Anopheles gambiae* (Diptera: Culicidae) Mosquitoes

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Abstract

The insecticide resistance in *Anopheles gambiae* mosquitoes has remained the major threat for vector control programs but the fitness effects conferred by these mechanisms are poorly understood. To fill this knowledge gap, the present study aimed at testing the hypothesis that antibiotic oxytetracycline could have an interaction with insecticide resistance genotypes and consequently inhibit the fecundity in *An. gambiae*. Four strains of *An. gambiae*: Kisumu (susceptible), KisKdr (*kdr* (L1014F) resistant), AcerKis (*ace-1* (G119S) resistant) and AcerKdrKis (both *kdr* (L1014F) and *ace-1* (G119S) resistant) were used in this study. The different strains were allowed to bloodfeed on a rabbit previously treated with antibiotic oxytetracycline at a concentration of $39{\cdot}10^{-5}$ M. Three days later, ovarian follicles were dissected from individual mosquito ovaries into physiological saline solution (0.9% NaCl) under a stereomicroscope and the eggs were counted. Fecundity was substantially lower in oxytetracycline-exposed KisKdr females when compared to that of the untreated individuals and oxytetracycline-exposed Kisumu females. The exposed AcerKis females displayed an increased fecundity compared to their nontreated counterparts whereas they had reduced fecundity compared to that of oxytetracycline-exposed Kisumu females. There was no substantial difference between the fecundity in the treated and untreated AcerKdrKis females. The oxytetracycline-exposed AcerKdrKis mosquitoes had an increased fecundity compared to that of the exposed Kisumu females. Our data indicate an indirect effect of oxytetracycline in reducing fecundity of *An. gambiae* mosquitoes carrying kdr^R (L1014F) genotype. These findings could be useful for designing new integrated approaches for malaria vector control in endemic countries.

Key words: oxytetracycline, resistance genotype, *Anopheles gambiae*, malaria, vector control

Malaria vector control strategies are based primarily on long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) of insecticides which have contributed to a significant decrease in malaria incidence and *Plasmodium falciparum* infections prevalence (Bhatt et al. 2015). Unfortunately, the widespread insecticide resistance in natural populations of malaria vectors across sub-Saharan Africa has become the primary threat to these control measures (Hemingway et al. 2016, Ranson and Lissenden 2016). The well-characterized resistance mechanisms in the major African malaria vectors *Anopheles gambiae*, are the target-site modification which is due to the point mutations in the targets of insecticides. They are: 1) the knock-down resistance (*kdr*) mutations in voltage-gated channel (*Vgsc*) gene (i.e., the substitution of Alanine by either Phenylalanine or Serine at codon 1014 (L1014F/S) (Martinez-Torres et al. 1998, Ranson et al. 2000) and the Asparagine-to-Tyrosine substitution at position 1575 (N1575Y) (Jones et al. 2012)) conferring resistance to pyrethroid/ dichlorodiphenyltrichloroethane (PYR/DDT) insecticides and 2) the substitution of Glycine by Serine at codon 119 (G119S) resulting in resistance to carbamate/organophosphate (CX/OP) insecticides (Djogbénou et al. 2007). These mutations, underlying phenotypic expression of resistance in *Anopheles gambiae* mosquitoes, were known to be associated with the changes of some mosquito physiological processes and could lead to deleterious effects (Rivero et al. 2010). However, to achieve the transition from malaria control to the elimination goal, targeting

some fundamental life-history traits of resistant mosquitoes such as fecundity, will be of a great asset since disruption of this trait could lead to a significant reduction of mosquito density and consequently a decreased malaria parasite transmission in endemic countries.

Malaria elimination and eradication in endemic areas are becoming growing commitments while current vector control interventions are facing repeated failures due to insecticide resistance and exophily (Alonso et al. 2011). However, targeting mosquito vectors has proved to be the most effective among all strategies implemented for malaria control (Bhatt et al. 2015). Therefore, alternative strategies for insecticide-resistant mosquito management are urgently needed. Also, it is important to expand studies towards finding additional tools that could negatively impact the vector life-history traits.

Despite the large-scale implementation of preventive strategies against malaria vectors through LLINs and IRS, the 2020 world malaria report has shown that endemic countries are still dealing with high levels of malaria burden (World Health Organization 2020). Innovative alternatives need to be explored to develop new tools and design efficient strategies to strengthen the efforts of endemic countries towards malaria elimination. An innovative alternative to controlling malaria-transmitting vectors is by applying ivermectin to animals (Fritz et al. 2009, Poché et al. 2015). Indeed, ivermectin was demonstrated to be lethal to *Anopheles* mosquitoes when ingested in sufficient doses upon blood-feeding on treated animals (Chaccour et al. 2013). Recently, the development of ivermectin as a complementary malaria vector control tool has been launched (The Ivermectin Roadmappers et al. 2020). Also, trimethoprim and thiostrepton antibiotics have been able to hamper the *Plasmodium* development inside *An. stephensi* mosquitoes (Delves et al. 2012). However, antibiotics such as penicillin and streptomycin have been reported to increase the vectorial capacity of *An. gambiae* mosquitoes (Gendrin et al. 2015). Amoxicillin and erythromycin antibiotics were found to be toxic to *Tilapia nilotica* fish and to *Culex pepans* mosquito larvae at concentrations up to 150 µg/liter (El-Nahhal and El-Dahdouh 2015). Antibiotics usage could therefore have a potential influence on the life-history traits of mosquito vectors.

The oxytetracycline antibiotics are inexpensive products and are largely used to prevent and treat bacterial infections in animals (Ratasuk et al. 2012). Following veterinary administration in animals, residues of oxytetracycline could be excreted in urines or feces and might be found in different environmental compartments. For instance, antibiotics have been detected in wastewater, groundwater, and surface water in the environment (Lindsey et al. 2001, Watkinson et al. 2009). Thus, during the life cycle of *Anopheles* mosquitoes, they could be exposed to antibiotic oxytetracycline in a given environment either by blood-feeding on animals (at the adult stage) or in the breeding sites (during the larval developmental period). It was reported that the presence of higher concentrations of oxytetracycline and ciprofloxacin antibiotics found in wastewater may harm larval development in *Culex quinquefasciatus* (Pennington et al. 2016). In addition, researchers reported that, the effects of genetic resistance on some mosquito life-history traits such as parasite infection and longevity have been influenced by environmental conditions (Bourguet et al. 2004, Lambrechts et al. 2006). Therefore, investigating the impact of antibiotic oxytetracycline on some life-history traits in *Anopheles gambiae* carrying resistance genes could be helpful for malaria vector management. In the present study, we hypothesized that, oxytetracycline could have negative effects on the fecundity in insecticide-resistant *Anopheles* mosquitoes. We investigated the impact of the commercial antibiotic oxytetracycline on egg production in *An. gambiae* females carrying the knock-down resistance and acetylcholinesterase insensitivity alleles.

Materials and Methods

Mosquito Strains and Rearing

To assess the impact of antibiotic oxytetracycline on the fecundity of malaria vectors, four laboratory strains of *Anopheles gambiae* sensu stricto were used. Among these, one is an insecticide susceptible reference strain (Kisumu) collected from Kenya in 1953 and has been kept in a colony ever since (Shute 1956). The three others are insecticide-resistant strains: pyrethroids/dichlorodiphenyltrichloroethane (PYR/DDT) resistant strain called KisKdr which is homozygous [kdr^{RR}] for the L1014F mutation (Alout et al. 2013); the AcerKis strain which is homozygous [$ace\text{-}1^{RR}$] for the *ace-1* (G119S) mutation that confers cross-resistance to carbamate/organophosphate (CX/OP) insecticides (Djogbénou et al. 2007) and AcerKdrKis strain which is homozygous [kdr^{RR}, $ace\text{-}1^{RR}$] for both *kdr* (L1014F) and *ace-1* (G119S) mutations which result in resistance to both pyrethroid/dichlorodiphenyltrichloroethane (PYR/DDT) and carbamate/organophosphate (CX/OP) insecticides (Assogba et al. 2014). All these laboratory mosquito strains share the same genetic background as that of the *An. gambiae* susceptible reference, Kisumu [kdr^{SS}, $ace\text{-}1^{SS}$]. Mosquitoes were reared in the laboratory at the Tropical Infectious Diseases Research Centre, University of Abomey-Calavi (Benin), under insectary conditions (insecticide-free environment, 27 ± 2°C ambient temperature, 70 ± 8% RH and 12:12 (L:D) h photoperiod). At an aquatic developmental stage, larvae were fed ad libitum with TetraMin Baby fish food. After emergence, adult mosquitoes were fed ad libitum on a 10% honey solution.

Mosquito Feeding Assays

Given the intensive use of antibiotic oxytetracycline for the treatment and prevention of bacterial infections in several animals such as rabbits, cows, cattle (Coetzee et al. 2005, Holman et al. 2019), we performed mosquito blood-feeding experiments using rabbits (for the purpose to prevent bacterial infections in these rabbits according to the guidelines for rabbit breeding in Benin (Food and Agriculture Organization of the United Nations (FAO) 2018)). Commercial oxytetracycline with the trade name Terramycin 10% (Kuipersweg 9 WOEDEN-HOLLAND) was injected into laboratory rabbits *Oryctolagus cuniculus* (Lagomorpha: Leporidae) through intravenous administration at a concentration of $39{\cdot}10^{-5}$ M according to manufacturer's instructions. Briefly, the rabbits were weighed and oxytetracycline injected according to 1 ml/10 kg body weight. After 24 h, adult mosquito females aged 3–4 d were allowed to feed on the treated rabbit. Others batches of mosquitoes were fed on untreated rabbits serving as controls. Three biological replicates were performed.

Assessment of Mosquito Fecundity

Twenty-four hours following the blood-feeding, all the fully gravid mosquito females were individually transferred into dry plastic cups with access to 10% honey solution until mosquito ovary dissection on the third day following blood-feedings. Ovarian follicles were dissected from individual mosquito ovaries into physiological saline solution (0.9% NaCl) under a stereomicroscope (Leica Microsystems EZ4HD). The eggs were counted from each gravid mosquito female and the number of individuals that developed eggs was recorded.

Statistical Analysis

Data were analyzed using R statistical software version 3.4.4 (R Core Team 2015) and GraphPad Prism 8.0.2 software (San Diego, CA). The normality of data was assessed using the Shapiro–Wilk test (Shapiro and Wilk 1965). The mosquito fecundity of each strain was assessed as the number of eggs counted over the number of females dissected. Descriptive statistics were used to calculate the percentages of mosquitoes developing eggs (number of ovaries positive for eggs/number of mosquitoes dissected) and the mean numbers of eggs developed in each mosquito strain. The unpaired Student's *t*-test was performed to compare the mean numbers of eggs developed between the treatment groups. The impact of genotype on fecundity in oxytetracycline-exposed mosquitoes was assessed using the negative binomial model (NBM) defined as follow: log (Fe) = Genotype + ε where Fe is the number of eggs per female, Genotype is the four-level factor corresponding to the different genotypes tested ([kdr^{SS}, *ace-*1^{SS}]; [kdr^{RR}]; [*ace-*1^{RR}]; [kdr^{RR}, *ace-*1^{RR}]), ε is the error parameter which follows a negative binomial distribution. All the analyses were set at a confidence interval of 95%.

Results

In total, 83 Kisumu females, 97 KisKdr females, 83 AcerKis females, and 94 AcerKdrKis females exposed to oxytetracycline were allowed to develop eggs. For the controls, 87 Kisumu females, 82 KisKdr females, 86 AcerKis females, and 89 AcerKdrKis females fed on the blood without oxytetracycline, were allowed to develop eggs. In all cases, no mortality was recorded after blood-feedings.

We first investigated whether the presence of oxytetracycline affected fecundity within each mosquito strain by comparing both the percentages of mosquitoes that developed eggs and the mean numbers of eggs developed between the exposed females and their controls. For Kisumu females, no significant difference was observed in percentages of mosquitoes developing eggs between control and treated females (Controls: 77.01%; Exposed: 72.29%, $\chi^2 = 0.283$, df = 1, $P = 0.59$) as well as in mean numbers of eggs they developed (Controls: 58. 01 ± 26.44 eggs; Exposed: 57. 42 ± 19.28 eggs, $t = 0.14$, df = 125, $P = 0.66$). The percentage of oxytetracycline-exposed KisKdr females that developed eggs was significantly lower (12.37%, $\chi^2 = 22.35$, df = 1, $P = 2.27 \times 10^{-6}$) than that recorded in their controls (45.12%) as illustrated in Fig. 1A. The mean number of eggs developed was significantly reduced in the KisKdr females that fed on oxytetracycline-treated blood compared to that of the unexposed individuals (Controls: 55. 68 ± 20.88 eggs; Exposed: 32. 83 ± 12.79 eggs, $t = 3.57$, df = 47, $P = 0.08 \times 10^{-2}$) (Fig. 1B). No significant difference was observed in the percentage of AcerKis females that developed eggs between the exposed females (53.01%, $\chi^2 = 0.48$, df = 1, $P = 0.49$) and their controls (46.51%) (Fig. 1A). By contrast, the mean number of eggs was significantly higher in AcerKis mosquitoes that fed on oxytetracycline-treated blood (45. 34 ± 23.71 eggs) compared to controls (32. 53 ± 14.59 eggs) ($t = 2.95$, df = 82, $P = 0.04 \times 10^{-1}$) as shown in Fig. 1B. In AcerKdrKis females, no significant difference was observed in the percentage of mosquitoes that developed eggs (Controls: 64.04%; Exposed: 58.51%, $\chi^2 = 0.38$, df = 1, $P = 0.54$) as well as in the mean numbers of eggs they developed (Controls: 61. 09 ± 38.14 eggs; Exposed: 72. 44 ± 41.01 eggs, $t = 1.52$, df = 110, $P = 0.13$) (Fig. 1A and B).

We then analyzed the impact of interaction between oxytetracycline and the resistance genotypes on mosquito fecundity. First, when comparing fecundity between unexposed resistant and susceptible strains (Table 1a), the percentage of mosquitoes that developed eggs was significantly lower in both KisKdr females (45.12%, $\chi^2 = 16.82$, df = 1, $P = 4.12 \times 10^{-5}$) and AcerKis

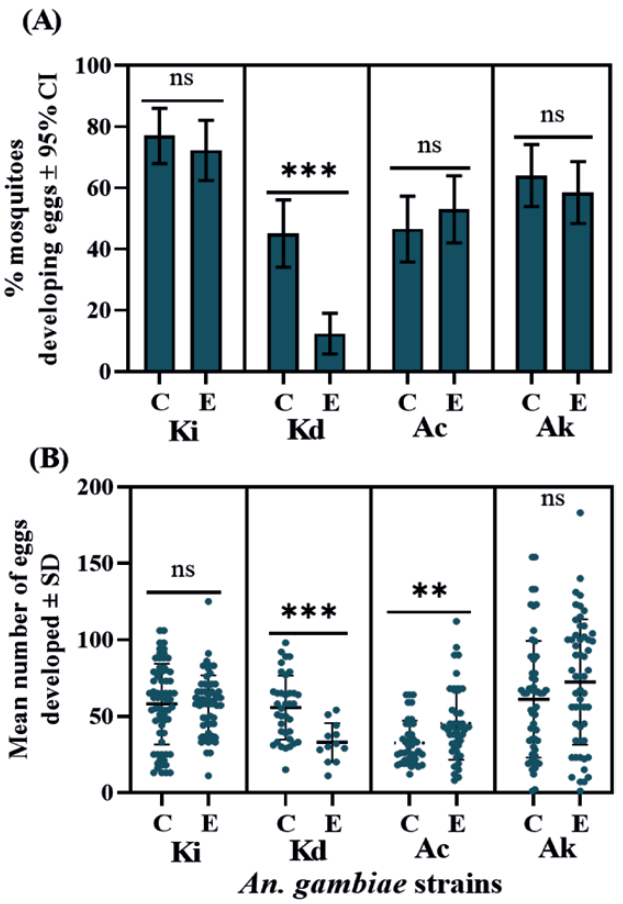

Fig. 1. Fecundity in oxytetracycline-exposed and unexposed *An. gambiae* strains. The histograms in panel (A) and scatter dot plots in panel (B) show, respectively, proportions of mosquitoes developing eggs and number of eggs developed among three biological replicates. C, E, Ki, Kd, Ac and Ak mean, respectively, Control, Exposed, Kisumu, KisKdr, AcerKis, and AcerKdrKis. CI, confidence interval; SD, standard deviation. ns, **, and *** indicate, respectively, no significance; $P < 0.01$ and $P < 0.01 \times 10^{-1}$.

females (46.51%, $\chi^2 = 15.78$, df = 1, $P = 7.11 \times 10^{-5}$) compared to that of Kisumu individuals (77.01%). For the mean number of eggs developed, the KisKdr and AcerKis females displayed, respectively, no significant difference (55. 68 ± 20.88 eggs, $t = 0.46$, df = 102, $P = 0.64$) and a significant reduction (32. 53 ± 11.59 eggs, $t = 5.60$, df = 105, $P = 0.01 \times 10^{-2}$) when compared to that of the Kisumu females (58. 01 ± 26.44 eggs). In AcerKdrKis mosquitoes, no significant difference was observed in both percentage of females developing eggs (64.04%, $\chi^2 = 2.96$, df = 1, $P = 0.08$) and mean number of eggs developed (61. 09 ± 38.14 eggs, $t = 0.53$, df = 122, $P = 0.60$) compared to those of the Kisumu females (77.01%, and 58. 01 ± 26.44 eggs, respectively). Second, when comparing fecundity between oxytetracycline-exposed resistant and susceptible strains (Table 1b), the percentage of mosquitoes that developed eggs was significantly reduced in both KisKdr females (12.37%, $\chi^2 = 64.44$, df = 1, $P = 9.98 \times 10^{-16}$) and AcerKis females (53.01%, $\chi^2 = 6.22$, df = 1, $P = 0.01$) compared to that of the Kisumu ones (72.29%). The mean number of eggs developed was significantly reduced in KisKdr females (32.83 ± 12.79 eggs, $t = 4.22$, df = 70, $P = 0.01 \times 10^{-2}$) as well as in AcerKis females (45.34 ± 23.71 eggs, $t = 2.86$, df = 101, $P = 0.05 \times 10^{-1}$) compared to that of Kisumu females (57.42 ± 19.28 eggs). The AcerKdrKis mosquitoes displayed no significant difference in percentage of females developing eggs (58.51%, $\chi^2 = 3.09$, df = 1, $P = 0.08$)

Table 1. Fecundity in unexposed (a) and exposed (b) resistant *An. gambiae* (KisKdr, AcerKis, and AcerKdrKis) compared to that of unexposed and exposed Kisumu females, respectively

An. gambiae strains	% Mosquitoes developing eggs	95% CI	Chi-squared test (χ^2)	*P*-value	Mean number of eggs ± SD	*P*-value
(a)						
Kisumu	77.01	67.99–86.03			58. 01 ± 26.44	
KisKdr	45.12	34.12–56.12	16.82	**4.12×10^{-5}**	55. 68 ± 20.88	0.64
AcerKis	46.51	35.76–57.27	15.78	**7.11×10^{-5}**	32. 53 ± 14.59	**0.01×10^{-2}**
AcerKdrKis	64.04	53.88–74.21	2.96	0.08	61. 09 ± 38.14	0.60
(b)						
Kisumu	72.29	62.46–82.12			57. 42 ± 19.28	
KisKdr	12.37	5.70–19.04	64.44	**9.98×10^{-16}**	32. 83 ± 12.79	**0.01×10^{-2}**
AcerKis	53.01	42.05–63.98	6.22	**0.01**	45. 34 ± 23.71	**0.05×10^{-1}**
AcerKdrKis	58.51	48.36–68.66	3.09	0.08	72. 44 ± 41.01	**0.01**

Bold P-values for the significant differences. SD, standard deviation; CI, confidence interval.

but a significantly higher mean number of eggs (72.44 ± 41.01 eggs, $t = 2.55$, df = 113, $P = 0.01$) when compared to those of the Kisumu females (72.29% and 57. 42 ± 19.28 eggs, respectively).

Overall, the fecundity of KisKdr females exposed to oxytetracycline decreased by 53% (GLM.NB: $F = 50.31$, $\Delta df = 1$, $P = 5.53 \times 10^{-5}$) and 56% (GLM.NB: $F = 181.47$, $\Delta df = 3$, $P = 9.29 \times 10^{-4}$) when compared to that of their controls and exposed wild-type Kisumu females, respectively. The fecundity of AcerKis females fed on oxytetracycline-treated blood, increased by 33% (GLM.NB: $F = 86.32$, $\Delta df = 1$, $P = 1.64 \times 10^{-3}$) compared to that of their controls but decreased by 24% (GLM.NB: $F = 181.47$, $\Delta df = 3$, $P = 0.02$) when compared to that of the exposed Kisumu females. In the exposed AcerKdrKis females, the fecundity was increased by 23% (GLM.NB: $F = 181.47$, $\Delta df = 3$, $P = 0.02$) compared to that of the exposed wild-type Kisumu females.

Discussion

The development of decreased susceptibility to the existing insecticides in malaria vectors has occurred as a major challenge to the control strategies implemented across endemic regions (World Health Organization 2017). Targeting parameters like mosquito life-history characteristics in association with insecticide resistance mechanisms could help to improve the strategies for breaking the chain of malaria transmission. To assist in these efforts, the present work investigated the effect of an antibiotic (oxytetracycline) on the fecundity of *An. gambiae* sensu stricto bearing two target-site resistance mechanisms.

Our findings showed a significantly reduced fecundity in the oxytetracycline-treated blood exposed KisKdr females compared to that of the controls. It seemed that the presence of oxytetracycline has induced a fitness cost on the fecundity of mosquitoes carrying the kdr^R (L1014F) resistance genotype. This likely interaction between oxytetracycline and *kdr* genotype would probably have involved the endosymbionts that grow especially in pyrethroid-resistant *Anopheles* mosquitoes (Dada et al. 2019). Some vertically transmissible bacteria were suggested to be involved in fecundity in *Anopheles*, *Culex* and *Aedes* mosquito species (Minard et al. 2013, Coon et al. 2014). Antibiotic-mediated bacterial microbiota inhibition has already been reported in mosquito species. For instance, antibiotics including oxytetracycline were demonstrated to inhibit specific bacterial microbiota in *Culex quinquefasciatus* (Pennington et al. 2016). Although the present study was not performed to assess the direct effect of oxytetracycline on bacterial communities in *An. gambiae* mosquitoes carrying *kdr* resistance genotype, our findings would indicate an implication of this antibiotic in reducing mosquito fecundity. However, a study comparing the microbial community in both oxytetracycline-treated and untreated resistant mosquito strains will help to explore our assumption above. An alternative assumption might be that the antibiotic oxytetracycline inhibits the activity of some enzymes such as superoxide dismutase and catalase which are often involved in the detoxification of the reactive oxygen species in pyrethroid-resistant mosquitoes as observed in *Anopheles arabiensis* (Müller et al. 2008). Specifically, it has been demonstrated that inhibition of catalase activity led to a significant fecundity reduction in *An. gambiae* females (DeJong et al. 2007).

Altogether, the antibiotic oxytetracycline could contribute to decreasing the density of the field populations of *An. gambiae* mosquitoes carrying the kdr^R (L1014F) genotype. Moreover, it was reported that the knock-down resistance genotype (kdr^R (L1014F)) and resistance to dieldrin (Rdl^R) reduce the mating competitiveness in *An. gambiae* males (Platt et al. 2015). It will therefore be interesting to investigate if the antibiotic oxytetracycline can induce a fitness cost on the competitive mating ability of male *An. gambiae* mosquitoes carrying the *kdr* genotype for the purpose of integrated management of these malaria vectors. Furthermore, it has previously been demonstrated that the presence of the kdr^R (L1014F) genotype increases the susceptibility to *Plasmodium* infection in *An. gambiae* mosquitoes (Ndiath et al. 2014). Accordingly, the antibiotic oxytetracycline could be possibly, an alternative tool to reduce the transmission of malaria parasites (by reducing the density of their vectors) in endemic countries of Africa where the frequency of *kdr* allele is almost fixed. It will be interesting to ascertain these assumptions through experimental field studies.

As we hypothesized, the antibiotic oxytetracycline induced a fitness cost on fecundity in *An. gambiae* mosquitoes carrying the homozygote knock-down (kdr^R (L1014F)) genotype. These lay the groundwork for further studies and could help to design a novel strategy for integrated management of resistant *An. gambiae*.

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Author Contributions

Conception and design of the work: A.A.M., E.G.S., and L.S.D. Acquisition of data: A.A.M., E.G.S., E.A., E.B.S., and L.D. Analysis and interpretation of data: A.A.M. and O.Y.D. Drafting and substantial revision of the manuscript: A.A.M., O.Y.D., A.B., and L.S.D. All authors read and approved the final version of manuscript.

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