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ORIGINAL ARTICLE

The effect of coexistence between larvae of *Anopheles gambiae* and *Culex quinquefasciatus* on larvicidal efficacy of *Bacillus thuringiensis* var. *israelensis*

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ABSTRACT

Background: The efficacy of *Bacillus thuringiensis* var. *israelensis* (*Bti*) is affected by several factors including the species of the mosquito. Mosquito larvae of different species are found to coexist in larval breeding habitats. This study evaluated whether the coexistence between *Anopheles gambiae* and *Culex quinquefasciatus* affect the larvicidal activity of *Bti*.

Methods: Two parallel larval bioassay experiments were conducted to test *A. gambiae* sensu stricto (s.s) and *C. quinquefasciatus* larvae susceptibility to *Bti*. They were followed by three parallel bioassays in which *A. gambiae* s.s and *C. quinquefasciatus* larvae were mixed in different proportions such that the earlier species contributed three quarters, half and a quarter of the larvae in each testing cup respectively. In each bioassay, six *Bti* concentrations were tested in four replicates and repeated on three different days. Larvae mortality was scored 24 hours after application of *Bti* and subjected to Probit analysis.

Results: *C. quinquefasciatus* was significantly more susceptible to *Bti* than *A. gambiae* s.s at both lethal concentration values (LC_{50} and LC_{95}). In coexisting scenario, LC_{50} of *Bti* was significantly lower when the proportion of *C. quinquefasciatus* exceeded 50%. No significant variation in susceptibility to *Bti* was observed at LC_{95} in any proportion of coexistence between the two species.

Conclusion: The findings show that larvae of *C. quinquefasciatus* were significantly more susceptible to *Bti* than those of *A. gambiae* s.s. Moreover, when larvae of the two species coexisted, there was a general trend of increase in sensitivity to *Bti* with higher proportion of *C. quinquefasciatus*. Although this increase in sensitivity of coexisting larvae to *Bti* is worth noting, our findings suggest that it will not impact larval control where *A. gambiae* s.s and *C. quinquefasciatus* coexist.

Keywords: *Anopheles gambiae* sensu stricto, *Culex quinquefasciatus, Bacillus thuringiensis* var. *israelensis,* Larval bioassays.

INTRODUCTION

Insecticide-based malaria vector control interventions have contributed significantly to the reduction of global malaria transmission and consecutively renewed interest in global malaria elimination.[1] However, new novel tools are urgently needed not only to complement the core malaria vector control interventions (insecticide treated nets and indoor residual spraying) but also with the potential to tackle threats posed by insecticide resistance and behavioral adoptions by malaria vectors.[2] Application of bacterial larvicide products based on *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) has been found to be effective and with potential to control both indoor and outdoor biting malaria vectors and possibly delay the evolution of insecticide resistance.[2] Furthermore, the potential role of larviciding for malaria vector control increases as malaria continues in a declining trend, creating a more appropriate condition for the interventions as summarized elsewhere.[3]

Bacterial larvicide *Bacillus thuringiensis* var. *israelensis* (*Bti*) (Bacillales: Bacillaceae) is gram-positive, spore-forming aerobic bacteria isolated from a multitude of aquatic larval habitats.[4] It has been used extensively as a larvicide for mosquito (Diptera: Culicidae) and black fly (Diptera: Simuliidae) control globally.[4,5] The larvicidal activity of *Bti* is based on delta-endotoxins produced by this bacterium at the time of sporulation.[5] When ingested by susceptible mosquito larvae, these toxins bind to the surface membranes of the epithelial cells of the larval midgut disrupting osmotic balance and resulting in the death of the larvae.[5] Based on review of bacterial larvicides, 13 *Bti* based products have been evaluated and found to be effective for malaria vector control in sub-Saharan Africa (SSA).[3] However, only the *Bti* strain AM65-52 (Vectobac® granules (GR) and VectoBac® water dispersible granules (WG)) has been prequalified by the World Health Organization (WHO) to be used for malaria vector control.[6] In addition to its proven effectiveness in mosquito control, *Bti* is generally safe to other non-target organisms coexisting with mosquito larvae in aquatic habitats.[7] The reported efficacy and safety of *Bti* when used for malaria vector control make it ideal for inclusion in Integrated Vector Management (IVM) operations to supplement other vector control strategies.[3] A study conducted in western Kenya demonstrated the potential role of integrating larviciding into adult malaria mosquito vector control interventions in reducing malaria transmission.[8]

Efficacy of *Bti* in mosquito control has been reported to vary greatly, mainly due to factors related to target mosquitoes (species of mosquito, their respective feeding strategies, age and density of larvae), larval habitat conditions (temperature, solar radiation, depth of water, turbidity, organic contents and presence of vegetation) and larvicide properties (application rates, toxin contents, type of carrier, how effective the material reach the target, settling rate, means of application and frequency of treatment).[4,5] In this regard, control efficacy and persistence of *Bti* based products have been shown to vary greatly in different ecological settings in SSA. [3] Understanding factors that cause variation of the effectiveness of *Bti* is important, particularly when planning larviciding interventions in different geographical settings. Like any other larviciding intervention, the *Bti* application must be guided by adequate knowledge of the prevailing mosquito vectors species, including their ecology, and the properties of the larvicide used.[5]

Studies have reported contradictory results on the level of susceptibility of larvae of *Anopheles gambiae* complex (Diptera: Culicidae) and *Culex* species (Diptera: Culicidae) to *Bti*. In controlled conditions, studies have shown that these two-mosquito species are equally susceptible to *Bti*.[9–11] On the other hand, in laboratory settings, *Culex quinquefasciatus* (Diptera: Culicidae) larvae were reported to be up to 4 times more susceptible to *Bti* than *A. gambiae* complex.[12] A field study conducted on the Kenyan coast showed that *A. gambiae* complex were more susceptible to *Bti* than *C. quinquefasciatus*.[13] From an ecological perspectives, it has been documented that the larvae of *A. gambiae* complex spend much more time feeding on the water surface whereas *C. quinquefasciatus* larvae feed under the surface of the water.[14] The nature and properties of a particular larvicide (including its settling rate) is likely to affect the larvicidal exposure rate between surface and bottom feeding larvae.[15]

Immature stages (larvae and pupae) of *A. gambiae* complex and *C. quinquefasciatus* have been found to coexist freely in the natural larval habitats.[16,17] In some instances, the coexistence of *A. gambiae* complex and *C. quinquefasciatus* has been detected in all potential anopheline larval habitats surveyed.[16] This coexistence has shown to harm the fitness of resulting adult mosquitoes due to competition for resources.[18] The coexistence may lead to competition for available larvicide toxins in a treated larval habitat which may subsequently impact on the effectiveness of *Bti*. It has been shown that *C. quinquefasciatus* has a relatively

higher particulate filtration rate than *A. gambiae*.[15] In the coexistence scenario, it is assumed that *C. quinquefasciatus* may filter more *Bti* toxins due to its inherent effective feeding behaviour and will succumb more to the lethal effect of the larvicide than *A. gambiae* complex. The current study was designed to evaluate whether co-existence between *A. gambiae* sensu stricto (s.s) and *C. quinquefasciatus* in the same larval habitat could influence the larvicidal activity of *Bti*.

MATERIALS AND METHODS

Study area and test mosquitoes

The study was conducted at the insecticide testing facility of the National Institute for Medical Research, Amani Medical Research Centre in Muheza, Tanga, Tanzania. Larvae of *A. gambiae* s.s (Kisumu strain) and *C. quinquefasciatus* (Tropical Pesticide Research Institute stain), both of which were fully susceptible to insecticides were used for the laboratory bioassays. The tested colony of *A. gambiae* s.s has been maintained for 30 generations at the insectary of the Amani Medical Research Centre whereas *C. quinquefasciatus* (at their 40th generation) were obtained from the Tropical Pesticide Research Institute, Arusha, Tanzania. *A. gambiae* s.s (Kisumu strain) is a reference strain, considered susceptible to insecticides and has been used extensively in bioassay experiments across Africa.[19]

Adult *A. gambiae* s.s and *C. quinquefasciatus* were maintained at an average room temperature of 26.5 °C (24–29°C) and an average relative humidity of 77.5% (65–90%) whereas larvae were kept at an average room temperature of 32 °C (29–35 °C). Before larval bioassays, first and second stage (early instar) larvae of *A. gambiae* s.s were fed on instant yeast (Istanbul, Turkey) whereas third and fourth stage (late instars) were fed Aquafin® fish food (Quanzhou, China). Larvae of *C. quinquefasciatus* were fed Whiskas® cat food (Mars Africa, South Africa). The feeding strategy was such that, 0.25–0.5 g of instant yeast was reconstituted in 4 mL of water and then 1 mL solution was added to 1500 mL chlorine free tap water, which was sufficient to feed around 500 respective first and second stage larvae of *A. gambiae* s.s for one day. For third and fourth stage larvae of this species, 0.1g Aquafin® was added to 1500 mL chlorine free tap water to feed around 500 larvae per day. For *C. quinquefasciatus*, 1g of Whiskas® cat food was added in 1500 mL of chlorine free tap water to feed approximately 500 larvae per day.

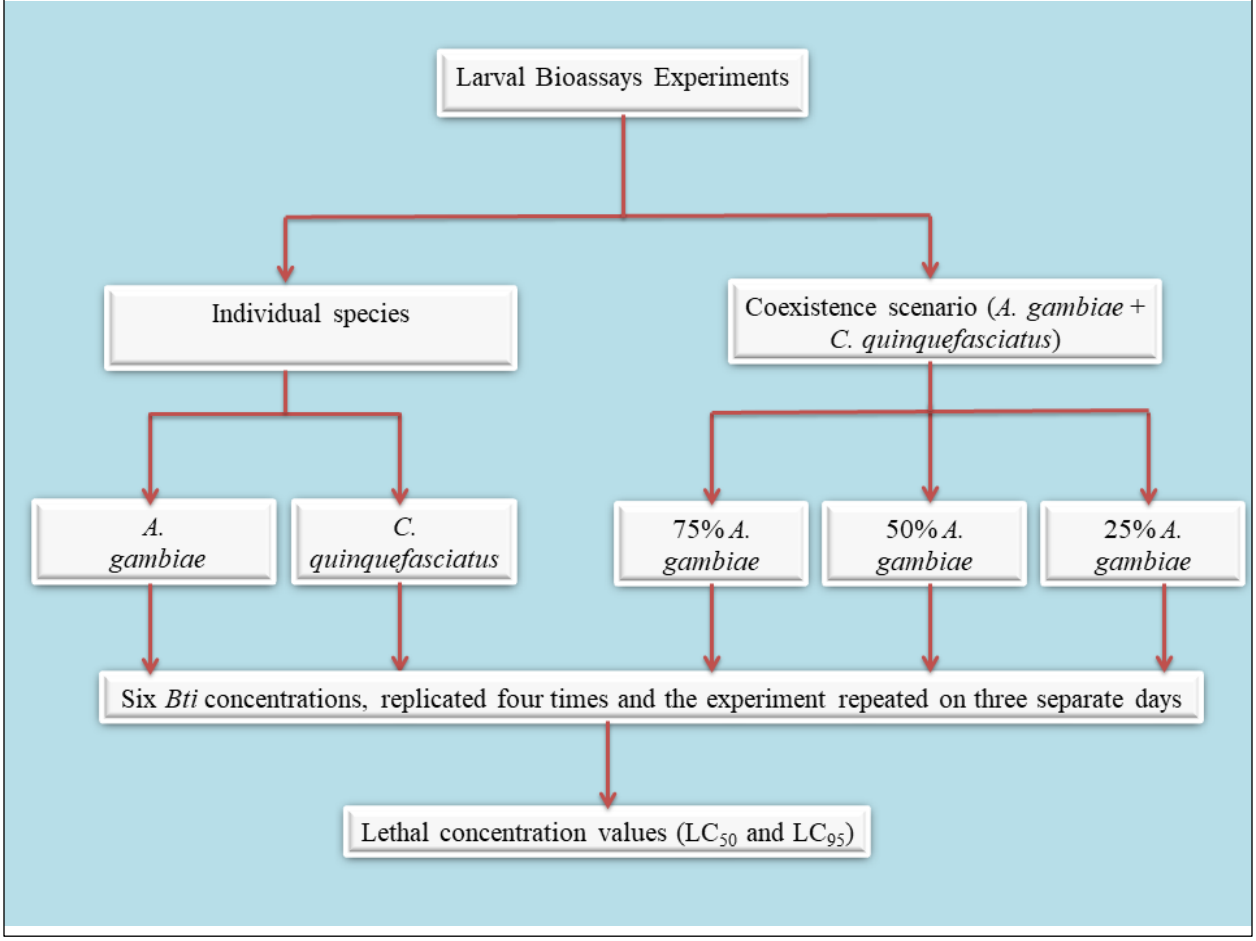
Preparation of Test Larvicides

Larval bioassays experiments were conducted with *Bti* strain Becker Microbial Products (BMP) 144 (potency 7000 ITU [International Toxic Units]/mg) form Becker Microbial Products, Inc, (11146 NW 69th Place, Parkland, FL 33076, USA). Testing solution of *Bti* larvicide was prepared and serially diluted as per recommended practice.[20] In brief, a stock solution was made by dissolving 200 mg of *Bti* powder in 20 mL distilled water. The resultant 10 mg/mL stock solution was kept frozen in 2 mL aliquots until use. On the day of the experiment, one aliquot of the stock solution of *Bti* was thawed and serially diluted in distilled water as recommended. Ten-fold dilution series was prepared by first transferring 2 mL of stock solution to 18 mL of chlorine-free tap water to make 1.0 mg/mL concentration, and then by subsequently repeating this procedure by transferring 2 mL of the latest solution to 18 mL of chlorine-free tap water to make 0.1, 0.01 and 0.001 mg/mL concentrations of *Bti*. The last three concentrations (0.1, 0.01 and 0.001 mg/mL) were used in the subsequent larvicide bioassays.

Study design

Larval bioassay experiments with *A. gambiae* s.s and *C. quinquefasciatus* were run from August to October 2019. At the beginning, two parallel larval bioassay experiments were conducted to separately test the susceptibility of *A. gambiae* s.s and *C. quinquefasciatus* larvae to *Bti*. They were then followed by three parallel larval bioassay experiments in which *A. gambiae* s.s and *C. quinquefasciatus* larvae were mixed (coexistence scenario) such that *A. gambiae* s.s contributed three quarters (15 out of 20), half (10 out of 20) and a quarter (5 out of 20) of the larvae in each testing cup (Figure 1).

FIGURE 1: Larval bioassay experiments



Larval bioassay experiments

At the start of each experiment, 20 third stage larvae were transferred from the larval rearing pans to the labelled disposable paper cups with 100 mL of chlorine-free tap water by use of disposable Pasteur pipettes. Using a pipette with disposable tips, and starting with the lowest concentration, appropriate volumes established in range finding bioassays (0.5 to 0.158 mL) of each of the three last dilutions of *Bti* were then added to the experimental cups (with mosquito larvae in 100 mL of chlorine-free tap water). In each larval bioassay, six concentrations of *Bti* (including a negative control) were tested in four replicates and repeated on three different days. In negative control test cups, 0.5 mL of chlorine free tap water was used. The test cups were held at an average ambient temperature of 29.0 °C and photoperiods of 12 hours light followed by 12 hours of darkness. Larval mortality was recorded at 24 hours after treatment with *Bti*. Only experiments with control larval mortality below 5% were included for further analysis.

Data analysis

Data on larval mortality were entered in Microsoft (MS) Excel (Microsoft Corporation, 2007) spread sheets and subsequently analysed to establish lethal concentrations (LC) of *Bti* that caused 50 and 95% mortality of test larvae (LC_{50} and LC_{95}), lethal concentration ratios (LCR) including their 95% confidence limits by using Probit/Logit analysis software Polo Plus (2002-2003 LeOra Software, Petaluma CA, USA)[21]. Polo Plus has been shown to be robust enough for analysis of mortality-concentration regression and its output compares fairly well with other analysis softwares.[22] The variation in LCs and

LCRs among the tested mosquitoes were compared by examining their 95% confidence limits, a common way to compare lethal concentrations or other point estimates. If the confidence limits overlap, then the LCs or LCRs do not differ significantly.[21] Experiments were considered valid if control mortality did not exceed 5%.

RESULTS

Overall, for the five larval bioassay experiment conducted, the lethal concentration of *Bti* that caused 50% and 95% mortality of the tested larvae (LC_{50} and LC_{95}) ranged from 0.021 mg/L to 0.065 mg/L and 0.105 mg/L to 0.423 mg/L, respectively (Table 1). *A. gambiae* s.s and *C. quinquefasciatus* displayed a different level of sensitivity to *Bti*, with the latter species being significantly more susceptible at both LC_{50} and LC_{95} (Table 1). In the coexisting scenario, LC_{50} of *Bti* was found to be significantly lower when the proportion of *C. quinquefasciatus* exceeded 50%. At LC_{95}, there was no significant variation in susceptibility between the tested larvae at any level of coexistence, although a small trend of increased sensitivity to *Bti* was observed with increasing proportion of *C. quinquefasciatus*. (Table 1). These findings were confirmed by examining lethal concentration ratios of *Bti* calculated by comparing LC values of the tested larvae with that of *A. gambiae* s.s (Table 2). At LC_{50}, the lethal concentration ratio (LCR) of coexisting larvae was significantly different when the proportion of *C. quinquefasciatus* exceeded 50% and no variation in susceptibility was observed in LCR at LC_{95} (Table 2).

TABLE 1: Laboratory bioassay results of *Bacillus thuringiensis* var. *israelensis* against larvae of *Anopheles gambiae* s.s and *Culex quinquefasciatus* in different proportions of coexistence

Mosquito species (proportion in %)	No. tested‡	LC_{50}† (95% CI)	LC_{95}† (95% CI)	Slope ±SE	χ^2 (df)	Heterogeneity
A. gambiae s.s (100)	1440	0.065 (0.056–0.075)	0.359 (0.283–0.484)	2.211±0.113	101.34 (58)	1.747
A. gambiae s.s (75) + *C. quinquefasciatus* (25)	1440	0.059 (0.053–0.067)	0.423 (0.345–0.540)	1.925±0.097	53.923 (58)	0.930
A. gambiae s.s (50) + *C. quinquefasciatus* (50)	1440	0.039 (0.035–0.045)	0.387 (0.303–0.519)	1.659±0.083	63.867 (58)	1.100
A. gambiae s.s (25) + *C. quinquefasciatus* (75)	1440	0.038 (0.033–0.042)	0.300 (0.241–0.391)	1.821±0.090	62.916 (58)	1.085
C. quinquefasciatus (100)	1440	0.021 (0.019–0.023)	0.105 (0.088–0.130)	2.358±0.135	52.042 (58)	0.897

Note: ‡1200 subjects and 240 controls in all tests (control mortality did not exceed 1.3% in any experiment); †mg/litre at 24 hours

Abbreviations: CI, confidence interval, SE, standard error; df, degrees of freedom, s.s, sensu stricto

TABLE 2: Lethal dose ratios for *Bacillus thuringiensis* var. *israelensis* against larvae of *Anopheles gambiae* s.s and *Culex quinquefasciatus* in different proportions of coexistence

Mosquito species (proportion in %)	Bti-Lethal Concentration Ratios† at	
	LC_{50} (95% CI)	LC_{95} (95% CI)
A. gambiae s.s. (75) + *C. quinquefasciatus* (25)	1.093 (0.927–1.289) a	0.848 (0.626–1.147) a
A. gambiae s.s. (50) + *C. quinquefasciatus* (50)	1.639 (1.391–1.933) b	0.928 (0.672–1.281) a
A. gambiae s.s. (25) + *C. quinquefasciatus* (75)	1.723 (1.466–2.026) b	1.195 (0.881–1.621) a
C. quinquefasciatus (100)	3.101 (2.674-3.597) c	3.436 (2.593–4.554) b

†Compared to *An. gambiae* sensu stricto (s.s)

Values in the columns followed by the same letter are not statistically significant (overlapping confidence interval)

DISCUSSION

Bti has been used extensively for the control of mosquitoes and black flies.[4,5] However, the activity of *Bti* based products is affected by a multitude of factors related to the target mosquitoes, their ecology, and inherent properties of the larvicide formulations.[5] Of relevancy to the current study, the control efficacy of *Bti* is known to vary with the species of the mosquito, mainly due to variation in larval feeding strategies.[23,24] In larvae ecology, the coexistence of different species of mosquito larvae in aquatic habitats, particularly anopheline and culicine species is not uncommon. The current study was designed to establish whether this coexistence could affect the larvicidal activity of *Bti* under laboratory settings. Understanding factors that affect the activity of larvicide has both epidemiological and economic advantages in the control of mosquito-borne diseases using *Bti* based products.

The findings have shown that larvae of *A. gambiae* s.s and *C. quinquefasciatus* tested were readily susceptible to *Bti* at relatively low application rates, which corroborates well with the results of other studies as summarized elsewhere[4]. A comparison between the two species revealed that *C. quinquefasciatus* were up to three times more susceptible to *Bti* than *A. gambiae* s.s. The high sensitivity of *C. quinquefasciatus* observed in the current study has also been reported in other studies,[12,23,24] and has been linked to the inherent high particulate filtration rate of this mosquito species.[25] In addition to particulate filtration rate, the larvicide settling rate has been identified to impact the activity of bacterial larvicides.[5] In this regard, the rapid settling rate of *Bti* toxins has been shown to lower larvicidal activity of surface feeding *Anopheles* mosquitoes.[25,26] Although the settling rate of *Bti* was not measured in this study, the test solutions were found to settle to the bottom of storage tubes and required gentle shaking before dispensing to the testing cups. Vigorous to gentle shaking or stirring of tubes containing re-suspended bacterial larvicide preparations before application to the target larvae has been emphasized in many testing protocols.[20] Although higher particulate filtration rate is known to increase the sensitivity of *C. quinquefasciatus* to *Bti*, it also appears likely that this species is considerably more exposed due to its bottom-feeding habit when tested with products with relatively high settling rate. The findings suggest that, if other factors that affect larval susceptibility to *Bti* remained constant, *C. quinquefasciatus* may respond better to *Bti* intervention than *A. gambiae* s.s.

In larval bioassay experiments in which larvae of *A. gambiae* and *C. quinquefasciatus* were mixed to represent various proportions of coexistence, an increased in sensitivity to *Bti* was recorded when compared to the LC values of *A. gambiae* s.s. At LC_{50}, the increase in larval sensitivity to *Bti* was significantly higher when the proportion of *C. quinquefasciatus* in test cups exceeded 50%. Likewise, relatively lower *Bti* concentrations were required to cause 95% mortality (LC_{95}) of test larvae with increased proportion of *C. quinquefasciatus* but this did not reach statistical significance in any of the three coexistence experiments. Our findings suggest that the increase in sensitivity of coexisting larvae to *Bti* was possibly due to relatively high susceptibility of *C. quinquefasciatus* with an overall effect of increasing sensitivity of coexisting larvae. This assumption is supported by previous findings showing rapid onset of toxic manifestation and reduced feeding in *C. quinquefasciatus* after an initial period of feeding on *Bti* toxins ranging from 15-20 minutes.[26] Provided that LC_{95} represents the minimum effective dose by which field application rates are based,[20] lack of significant variation in susceptibility of coexisting larvae at LC_{95} has important practical implications relevant to larviciding. In this regard, it can be safely generalized that larval control interventions using *Bti* can be scaled-up with little consideration to the level of coexistence between *A. gambiae* complex and *C. quinquefasciatus*.

In practical field applications, when anophelines and culicines coexist, treating the two species as a single unit in terms of susceptibility to *Bti* is appropriate and it has been previously documented.[9–11] This approach reduces logistical challenges pertaining to resources, time and efforts that would have been required in the larvicide intervention and monitoring if coexistence had an influence on activity of *Bti*. Thus, this assumption allows for rapid scale-up of larviciding interventions in different ecological settings where association between anopheline and culicine species is common. Although the efficacy of *Bti* is affected by a multitude of factors, when these factors are considered, *Bti* is effective and can be successfully incorporated in integrated vector management programs.

CONCLUSIONS

The findings of the current study have shown that larvae of *C. quinquefasciatus* were more susceptible to *Bti* than those of *A. gambiae* s.s and when the two species coexisted, there was a general increase in sensitivity to *Bti, which* increases proportionally with *C. quinquefasciatus*. Although this increase in

sensitivity of coexisting larvae to *Bti* is worth noting, our findings suggest that it will not impact larval control where *A. gambiae* and *C. quinquefasciatus* coexist.

Authors' contributions

YAD, EJK, WNK, GY, AKG and FWM conceived and designed the study. YAD conducted laboratory experiments and performed data analysis. YAD drafted the manuscript with contributions from EJK, WNK, GY, AKG and FWM. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethical statement

Not applicable.

Availability of data and materials

All relevant data supporting the conclusions of this article are included in the article.

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