

# Characterization of the mechanisms of action and mosquito larva midgut response to a yeast-encapsulated orange oil larvicide

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## Research Article

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# Abstract

Here, we assessed the mechanism of action of a yeast-encapsulated orange essential oil (YEEO) larvicide. YEEO is readily ingested by mosquito larvae leading to midgut epithelial cell responses associated changes in gene expression profiles and severe epithelial damage that were detected shortly after YEEO ingestion. In *Aedes aegypti* 3<sup>rd</sup> instar larvae exposed to YEEO at LC90, greater than 10 fold induction of the effector caspase *CASPS8*, >11 fold induction of the midgut epithelial regenerator *Vein*, and >26 fold induction of the inhibitor of apoptosis gene *IAP1* compared to control larvae by 8 h after YEEO ingestion. For sublethal concentrations (<LC50), we observed significant differential expression of the cytochrome P450 detoxification enzyme families CYP6 and CYP9. Midgut epithelial damage was highlighted by destruction of microvilli, vacuolization of midgut cells, damage to cell junctions and basal lamina as early as 30 min. Curiously, the larval type 2 peritrophic matrix (PM2) structural integrity and porosity appeared to remain unchanged, suggestive that the orange oil released from the digested YEEO capsules seep through the PM2. Of relevance, our results strongly suggest that YEEO effective killing is due to a generalized broad-acting mechanism combining unregulated epithelial damage and apoptosis, with concomitant expression of multiple innate response genes involved in epithelial regeneration and detoxification. YEEO's amenability for use as part of an integrated vector management makes this novel larvicide a practical approach for mosquito larval control in the future.

## Introduction

Mosquito-borne diseases continue to represent a major threat to global health and to the economic development of many countries worldwide. Methods to combat these diseases require strategic planning as effective control of mosquito populations are labor intensive, difficult to sustain, and fraught with issues of vector resistance. Primary approaches to mosquito abatement includes adulticide spraying with synthetic pyrethroids and organophosphates, larval source management with bacterial larvicides such as *Bacillus thuringiensis* (Bti), or a combination of both. Control of *Aedes aegypti*, a major vector of dengue, Chikungunya, and Zika has posed a tremendous challenge for vector-control programs. The endophilic behavior of this species in urban areas has allowed for low mosquito populations to sustain disease outbreaks. Initial successes achieved with insecticide control *Ae. aegypti* have been severely hampered by the emergence of resistance. Recently, methods that apply either transgenic technology via release of insect carrying a dominant lethal (RIDL)<sup>1,2</sup> or mosquitoes carrying the endosymbiont *Wolbachia* to promote cytoplasmic incompatibility of the offspring<sup>3</sup> have been used to reduce mosquito populations. However, these methods of control are still in their infancy, and are still hampered by regulatory barriers, cost, negative public perception, and may require several years of investigation to fully assess their perceived potential.

Larvicides have remained an effective means of larval source management. Bti, for instance, is highly effective in reducing the risk of mosquito-borne diseases as they can be easily administered in urban and rural standing water. Bacterial larvicides are also considered to be more environmentally-friendly than

insecticide spraying, with fewer or no toxic effects on beneficial insects (e.g. bees, butterflies) as well as other non-target species. As such, this class of larvicides are widely accepted by the general public.

Multiple novel approaches for mosquito control are being evaluated, including the use of natural essential oils (EOs)<sup>4-8</sup>. EOs are volatile, aromatic oils that have long been used for many purposes, including medicinal, bactericidal, insecticidal, agricultural, and pharmaceutical applications. Although EOs possess robust mosquito larvicidal traits, they are hydrophobic in the aquatic environment and susceptible to breakdown not only from ultraviolet light but also to temperature and oxidation, highlighting a need to develop an efficient yet effective delivery system to utilize their potency against mosquito larvae.

*Saccharomyces cerevisiae* (Baker's yeast) can serve as a suitable and effective biodegradable microencapsulation container of several compounds and particles including fungicides, drug treatments, and EOs<sup>9,10</sup>. The encapsulation process renders the yeast cells nonviable, but increases water solubility, bioavailability, long-term viability and stability as a lyophilized material, and delivery efficiency as a mosquito larvicide. Further, yeast is readily consumed and digested in the gut by mosquito larvae which possess specialized intestinal enzymes that rapidly break down and digest the yeast  $\beta$ -1,3-glucan cell membranes<sup>11</sup>, which releases the essential oil within the gut, subsequently killing the larvae.

We previously demonstrated that yeast-encapsulated orange oil (YEEO) is an effective larvicide against multiple larval life stages of *Ae. aegypti* and *Culex quinquefasciatus*<sup>12</sup>. Moreover, we also showed that the YEEO LC<sub>50</sub> and LC<sub>90</sub> after 24 h exposure in third larvae instar (L3) *Ae. aegypti* larvae are 12 and 28 ppm, respectively, which is nearly half the required dose necessary when using non-encapsulated orange oil<sup>13</sup>. In this study, we describe the changes in gene expression profiles and midgut epithelial morphology induced by the ingestion of YEEO in *Ae. aegypti* larvae. Innate response gene expression profiles have been extensively investigated in adult mosquitoes<sup>14-17</sup>, however little is known regarding innate responses of mosquito larvae following exposure to larvicides. The killing or toxic effects of larvicides on mosquito larvae presumably lead to changes in gene expression profiles, activation of apoptotic pathways, epithelial regeneration, and the activation of detoxification mechanisms linked with P450 gene expression. Our results point to a mosquito larval killing mechanism that is characterized by a non-specific and unregulated midgut epithelial damage and apoptosis. The combination of the stability of the orange oil provide by the yeast-encapsulated formulation with the irreversible effects on the mosquito larvae without affecting non-target organisms indicate the potential commercial usage of YEEO or other yeast-encapsulated oils as effective mosquito larvae control approaches.

## Materials And Methods

### Larvicide preparation

Lyophilized yeast-encapsulated orange oil (YEEO) was prepared as previously described<sup>12</sup>. Briefly, *Citrus sinensis* essential oil (orange oil, California origin, Sigma- Aldrich, St. Louis, USA) was encapsulated into *S. cerevisiae* (Red Star fresh baker's yeast).

Orange oil, water, and yeast were combined in a baffled flask and agitated for 24 h at 40°C. The resulting mixture was then centrifuged, the supernatant discarded, and the remaining precipitate was subsequently washed three times to remove excess oil and lyophilized prior to storage. The dried larvicide was reconstituted in water between 4.4 – 5% oil in solution prior to bioassay application.

## Larval strains and bioassays

Larvae used in our studies were obtained from mosquito colonies maintained at Uniformed Services University of the Health Sciences (USUHS). *Ae. aegypti* (Liverpool strain – AAE-L and Washington, D.C. strain – AAE-DC strains) and *Anopheles gambiae* (G3 strain - AGA) were tested in our bioassays and the AAE-L strain was used in all other experiments. Larvae were kept in DI water at 28°C and were fed with fish food *ad libitum*. Larvae bioassays were performed according to the standard WHOPES protocol<sup>18</sup>. Briefly, 25 third instar (L3) larvae were placed into cups with 100 ml of DI water. After a 30 min acclimation, different quantities of YEEO larvicide were added to each cup to reach concentrations ranging from 2.5 mg/L to 60 mg/L, and lethality was assessed after 24 hours. In the experiments described here, concentrations of 5, 10, and 30 mg/L of YEEO correspond to LC20, LC50, and LC90, respectively, and were based on our previous results<sup>12</sup> using L3 stage AAE-L. The effects of YEEO on AAE-L larval swimming behavior and lethality can be seen in Supplemental Videos 1-3.

*Gene Expression Analyses.* Individual live whole larvae or guts and carcasses dissected from AAE-L L3 were collected following 1, 4, 8, and 24 h exposure to 10 or 30 mg/L (equivalent to LC50 or LC90, respectively) of YEEO. Samples were placed into 30 µl of RNeasy lysis buffer (Qiagen, Waltham, MA), and frozen at -80°C until RNA extraction. Genes associated with apoptosis (*IAP1* and *IAP2*), autophagy (*ATG1*, *ATG6*, and *ATG8*) and innate immune response genes (*Vein*, *Pirk*, *Serpin-1*, *Serpin-2*, *IMP2*, *CASPS7* and *CASPS8*) were assessed in two independent experiments using RNA samples (in triplicate) obtained from pools of three L3 dissected guts or carcasses for each of the four time points. Here, gut samples were composed of foregut and midgut whereas carcasses included the hindgut, the Malpighian tubules and all remaining larvae-matched body parts such as head, thorax, and abdomen. In contrast, for cytochrome P450 gene expression analysis, one whole L3 individual was used per sample (up to eight samples) for each of the three independent experiments (i.e., three replicates) for both 5 mg/L (LC20) and 10 mg/L (LC50) YEEO concentrations tested after 4 h exposure. Regarding P450, we assessed genes representative of families *CYP6* (*CYP6M11*, *CYP6N12*, and *CYP6Z8*) and *CYP9* (*CYP9J10* and *CYP9M9*). Individual whole larvae were used to determine the systemic insecticide resistance response to YEEO to determine individual gene response variation. In addition, for the P450 analysis specifically, we were not interested in ascertaining spatial expression profiles.

*RNA extraction and cDNA Synthesis.* AAE-L total RNA was isolated using the RNeasy tissue kit (Qiagen, Hilden, Germany) followed by DNase treatment using TURBO DNA-free kit (Invitrogen, Carlsbad, CA). Each RNA sample was quantified on NanoDrop (Thermo Scientific, Waltham, MA) and assessed on 1% agarose-5% formaldehyde in 1x MOPS. RNA samples were stored at -80°C until use. First-strand cDNAs

were obtained using Superscript III reverse transcription kit (Invitrogen) following manufacture protocols with oligo dT12-20 primers and 200 ng of each RNA.

*Quantitative Real-Time PCR.* mRNA levels were quantified with PowerUp SYBR Green Supermix (Applied Biosystems, Foster City, CA) using 10 ng of cDNA with a 50°C for 2 min and 95°C for 2 min hot start initialization, followed by 40 cycles of 60°C for 5 sec and 95°C for 15 sec using a ABI7500 FAST (Applied Biosystems, Foster City, CA). Relative fold changes were assessed using the  $\Delta\Delta C_t$  method<sup>19</sup> and calibrated against the expression of the housekeeping gene *Actin6* (Supplemental Table 1) in L3 larvae control samples fed on blank yeast. Primer sequences for each gene measured are listed in Supplemental Table 1.

*Confocal Microscopy.* Confocal microscopy was used to assess the distribution of YEEO throughout the larva alimentary canal. Whole guts from AAE-L L3 larvae were dissected after exposure to either 30 mg/L YEEO or blank yeast control (30 mg/L) in PBS and fixed for 15-30 min at room temperature with Zamboni's Fixative<sup>20</sup> (4% paraformaldehyde). For experiments focused on the permeability of the type 2 peritrophic matrix (PM2), 20 L3 larvae were exposed to blank yeast control (30 mg/L) or 30 mg/L YEEO in 10 ml DI water at 28°C supplemented with FITC-dextran (Cell Signaling Technology, Danvers, MA) (Molecular weights of 150, 500, or 2,000 kDa) at 0.5 mg/ml for 4 h. Fixed guts were washed 3x in PBS, stained for 5 min with 10 µg/ml of DAPI (Invitrogen, Carlsbad, CA) and washed 3x in PBS before imaging on a ZEISS 710 Two Photon (Carl Zeiss AG, Hoberkochen, Germany) confocal microscope. The permeability of larval PM2 was qualitatively assessed by presence/absence of fluorescein signal in the caeca of dissected guts/larvae<sup>21</sup>. For microscopy, entire alimentary canals were used for viewing clarity.

*TUNEL Analysis.* TUNEL was used to assess apoptosis via DNA fragmentation. AAE-L L3 larvae were exposed to either YEEO (10 mg/L) or yeast blank for 24 h. Guts dissected from insects were fixed for 15-30 min at room temperature with Zamboni's Fixative, washed three times (5 min each) in PBS, twice (2 min each) in PBS containing 0.3% Triton X-100 (PBST), and stained for TUNEL analysis (Roche, Basel, Switzerland) following manufacturer's protocol. Samples were counterstained with 10 µg/mL of DAPI (Invitrogen, Carlsbad, CA), mounted onto slides in Vectashield<sup>→</sup> (Vector Laboratories, Burlingame, CA), and subsequently imaged on a ZEISS 710 Two Photon (Carl Zeiss AG, Hoberkochen, Germany) confocal microscope.

*Transmission Electron Microscopy.* TEM was used to assess cellular damage caused by YEEO ingestion and to verify the structural integrity of YEEO. AAE-L larvae were exposed to either blank yeast control (30 mg/L) or 30 mg/L YEEO. Following 4 h exposure, guts were dissected and fixed overnight at room temperature in freshly prepared 2% formaldehyde and 2% EM grade glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1M cacodylate buffer, pH 7.2. Fixed guts were washed three times in cacodylate buffer (without aldehydes) and incubated in 2% OsO<sub>4</sub> in 0.1M cacodylate buffer for 1 h, After another round of three washes 10 min each in cacodylate buffer, guts were dehydrated in a graduated series of ethanol (10 min each in 30%, 50%, 70%, and 95% ethanol and 2 x 10 min in 100% ethanol), infiltrated in a graduated series of Spurr's epoxy resin (Electron Microscopy Sciences), and then

polymerized at 70°C for 11 h. Polymerized blocks were sectioned in a Leica UC6 ultramicrotome. Thin sections of guts cut transversely and longitudinally were collected on 3 mm copper grids. Grids were post-stained in a Leica EM AC20 grid stainer and then examined on a JEOL JEM-1011 transmission electron microscope (JEOL USA, Peabody, MA). Images were collected on an Advanced Microscopy Techniques (AMT Corp., Woburn, MA) digital camera.

*Statistical Analysis.* Larvae bioassay data to determine LC50 and LC90 YEEO effective concentrations were determined with Nonlinear or Probit regression analyses<sup>22</sup>. Gene expression data of dissected guts and carcasses were subjected to two-way analysis of variance (ANOVA) with repeated measures, with Dunnett's multiple comparisons tests and p-values evaluated at  $\alpha = 0.05, 0.01, \text{ and } 0.001$ . In whole larvae P450 gene expression analyses, data were subjected to one-way ANOVA, and Tukey's Post-Hoc tests with p-values evaluated at  $\alpha = 0.05, 0.01, \text{ and } 0.001$ . Statistics were carried out in SAS 9.4 (Cary, NC) or GraphPad Prism8 (San Diego, CA).

## Results

### Bioassays

In accordance with our prior results<sup>12</sup>, YEEO was effective against larvae of both *Ae. aegypti* strains (AAE-L and AAE-DC), as well as against larvae of *An. gambiae* G3 (AGA). For AAE-DC and AGA, the LC50 was calculated at 11.1 mg/L and 10.3 mg/L, and the LC90 at 17.9 mg/L and 28.1 mg/L, respectively (Figure 1 and Table 1). For AAE-L larvae, the LC50 and LC90 were recalibrated from our original results<sup>12</sup> to 9.4 mg/L and 27.8 mg/L, respectively. These concentrations are consistent with our previous results against AAE-L L3 larvae<sup>12</sup>, and also when assessed using Probit analyses<sup>22</sup> (shown in the Supplemental Figure 1 and the Supplemental Table 1). In addition, the sublethal dose of LC20 was calculated at 5 mg/L.

Table 1

LC50 and LC90 concentrations for L3 larvae of L3 larvae of *Ae. aegypti* – Liverpool (AAE-L), *Ae. aegypti* – Washington, D.C. (AAE-DC), and *An. gambiae* (AGA) after 24 h YEEO exposure. Values were calculated by normalization of dose-response nonlinear regression best fit curves and include upper and lower limits in parentheses (AGA upper limit calculation undetermined). *n*, equals the number of replicates per concentration tested (5 mg/L up to 60 mg/L), with the total combined number of larvae assayed indicated in parenthesis.

	AAE-Liverpool	AAE-DC	AGA
LC50 mg/L	9.4 (7.6-11.1)	11.1 (11.8-21.3)	10.3 (8.7-12.2)
LC90 mg/L	27.7 (21.3-41.6)	17.9 (15.7-20.8)	28.1 (19.8)
n (individuals)	4-6 (1088)	4-8 (1400)	2-6 (1200)

### Gene expression profiles

We investigated the expression of genes associated with cellular processes such as apoptosis and autophagy as well as innate immune response genes in AAE-L L3 larvae after exposure to YE00 in live, motile larvae (no moribund or dead larvae were included for gene expression analyses). Both LC50 and LC90 concentrations led to significant differences in gene expression profiles in apoptosis-related genes, including inhibitors of apoptosis and effector caspases.

In larvae exposed to LC50 concentrations, the inhibitor of apoptosis gene *IAP1* was upregulated more than 7-fold in the carcass 24 h post exposure compared to yeast controls (Figure 2A, p-value = 0.0489). Additionally, at 24 h, the effector caspase *CASPS8* was significantly upregulated 12-fold in the gut (Figure 2B, p-value = 0.0301). The autophagy gene *ATG1* was downregulated 2.5-fold (Figure 2C, p-value = 0.0403) following a 1 h exposure to YE00 at LC50 concentration with expression levels returning to those comparable to the yeast control at 4h and 24 h. Lastly, the midgut epithelial cell regenerator gene *Vein* was upregulated more than 17-fold in the carcass at 24 h post exposure, but did not reach significance (Figure 2D, p-value = 0.059).

Larvae exposed to LC90 concentrations displayed opposing effects for *IAP1* and *IAP2*. For *IAP1*, a 26-fold induction in the carcass at 8 h (Figure 3A, p-value = 0.0001) was observed, whereas for *IAP2* we detected a significant reduction in the carcass at 1 h (Figure 3B, p-value = 0.0389) and gut at 8 h for (Figure 3B, p-value = 0.0298). For the effector caspase *CASPS8*, a 6.7-fold induction in the gut and concomitant 70-fold induction in the carcass were observed at 8 h post-YE00 ingestion (Figure 3C, p-value = 0.0071). In the midgut epithelia regenerator *Vein*, a significant increase by 11-fold was observed in the gut after 4 h (Figure 3D, p-value = 0.0094) and was repressed 7.6-fold in the carcass tissues by 24 h (Figure 3D, p-value = 0.0227).

For other transcripts measured after LC50 or LC90 YE00 exposure, including the IMD negative regulator *Pirk*, *Serpin-1*, *Serpin-2*, and *IMP-2* (genes associated with melanization cascade), the autophagy gene *ATG6*, and the effector caspase *CASPS7*, though a trend suggestive of upregulation was noted, such increases were not statistically significant (LC50 Supplemental Figure 2, and LC90 Supplemental Figure 3). Levels of induction or repression, standard error, and p-values measured for all genes assessed after exposure to LC50 and LC90 concentrations are shown in Supplemental Table 2 and Supplemental Table 3, respectively.

We next assessed if AAE-L L3 larvae exposed to YE00 at sublethal concentrations ( $\leq$ LC50) displayed differential gene expression levels among the CYP6 and CYP9 P450 families. In L3 AAE-L larvae exposed to LC50 concentrations, *CYP6M11* and *CYP6N12* were both significantly upregulated after 4 h exposure at 16.4-fold (Figure 4A right column, p-value=0.0375) and 7.4-fold (Figure 4B right column, p-value = 0.0069), respectively. Interestingly, larvae exposed to a lower, less lethal YE00 concentration of LC20 (5 mg/L) displayed even greater induction of these genes, at 21-fold for *CYP6M11* (Figure 4A left column, p-value = 0.0053) and 9.9-fold in *CYP6N12* (Figure 4B left column, p-value = 0.0002). Notably, *CYP9J10* was significantly repressed compared to controls (Figure 4C, LC20 p-value = 0.0013, LC50 p-value =

0.0021), while *CYP6Z8* and *CYP9M9* displayed no change at either sublethal concentration (Supplemental Figure 4).

### *Cellular and physiological damage in midgut after YEEO exposure.*

Transmission electron microscopy (TEM) was utilized to verify the structural integrity of YEEO. TEM revealed nearly all *S. cerevisiae* cells were loaded with EOs (Figure 5D-F) compared to yeast blank controls (Figure 5A-C). As expected, no cell division was observed in YEEO as the encapsulation process effectively kills the yeast cells. Bud scars which are visible in this population likely occurred before EO loading.

Ingestion of YEEO by AAE-L larvae led to significant morphological and physiological cytotoxic effects, as observed via TEM. Compared to control larvae (Figure 6), there was substantial damage to midgut epithelial cells within 30 mins of exposure (Figure 7), followed by significant damage systemically throughout the midgut epithelia after 4 hours (Figure 8). Microvilli were severely damaged/shortened by 30 min exposure (Figure 7A-C) or destroyed in larvae exposed for 4 h (Figure 8A-B). Further, we noted an increase in cellular vacuolization particularly along the basal lamina (Figure 7D, Figure 8C and 8D), and alterations in the mitochondrial shape and cristae (Figure 7D, Figure 8C and 8D). Interestingly, unlike unexposed larvae which displayed visibly intact yeast control cells within the peritrophic space, no intact YEEO cells were visible in YEEO exposed larvae regardless of the exposure duration. We tested the hypothesis that the breakdown of YEEO in larval guts might be due to pH as no difference in the levels of digestive proteases has been observed (Dr. Fernando Genta, personal communication). As the mosquito larva gut pH ranges from 10 to 7.5 from the anterior to posterior regions<sup>23</sup>, YEEO larvicide was incubated with in HEPES with incremental increases of pH from pH 7 to pH 10 for 30 min, and no changes in the YEEO stability were observed. Lastly, both living and moribund AAE-L larvae were subjected to TUNEL analysis after 24 h exposure of LC50 concentrations. Guts of moribund larvae exhibited increased DNA fragmentation when compared to live larvae. (Supplemental Figure 4). Although the structural integrity of the larval peritrophic matrix 2 (PM2) in AAE-L larvae did not appear to be affected by YEEO exposure up to 4 h, we assessed if its permeability or porosity were altered. Under normal physiological conditions *Ae. aegypti* larva PM2 is not permeable to 2000kDa FITC-labeled dextran particles<sup>21</sup>. Following simultaneous feeding of FITC-labeled dextran at 2000 kDa with YEEO (at LC90 concentration) in 20 AAE-L L3 larvae for 4 h, FITC-related fluorescence was neither detected in the caeca of control larvae as expected (Figure 9A) nor in the larvae fed with YEEO (Figure 9B), suggestive of a lack effect of the YEEO on AAE-L larva PM2 permeability. Simultaneous feeding of FITC-Dextran particles with YEEO was our only option to test permeability as the larvae refused to feed on the particles following YEEO exposure alone.

## **Discussion**

Our work demonstrates the potent effects of a novel, innovative mosquito larvicide on the physiology and innate immune response gene expression profile in L3 *Ae. aegypti* larvae.

Ingestion of the YE00 larvicide, resulted in unregulated cytotoxic events to the larva midgut epithelial cells as well as an upregulation of apoptosis-related genes. Unlike traditional single target insecticides, YE00 displays a multi-modal action affecting multiple cellular pathways. Thus, we predict YE00 activity is independent of specific midgut receptors and killing mechanisms are based upon toxicity to midgut cells. Multi-target larvicides, such as Bti, or multi-modal larvicides like YE00 are thought to be less likely to incur in resistance<sup>8-10</sup>. To date, resistance to EOs has not been reported.

E0s and their derivatives have been investigated *in vitro* against neglected tropical parasites and arboviruses for decades (Reviewed in Luna et al.)<sup>24</sup>. E0s also have been utilized as contact irritants against adult and larval stages of various insect pests, and have been shown to cause testicular apoptosis and morphological damage to the fat body and midgut epithelium of *Spodoptera frugiperda* (lepidopteran) larvae<sup>25,26</sup>. Other natural compounds, such as squamocin and extract from the Amazonian plant *Derris urucu*, have been tested against *Ae. aegypti* larvae on which both cytotoxic and gene expression profiles effects were assessed<sup>27,28</sup>. Encapsulated essential oils offers significant advantage over free E0s. For instance, when sequestered into yeast cells, E0s are effectively protected against photolytic degradation, allowing for long term stability. The lyophilized YE00 can be stored for over a year without any discernible differences between stored vs. freshly generated YE00, as determined by TEM analysis. Moreover, whereas the hydrophobicity of free E0s makes them unlikely candidates for commercial use, the encapsulation approach using *S. cerevisiae* provides an optimal delivery mechanism that facilitates dissemination throughout aquatic environments, and is readily ingested by mosquito larvae. The degradation of the yeast cell wall results in the release of the sequestered E0 within the gut of the insect and allows for targeted delivery of the larvicide.

Though the gene expression profiles only provide indirect evidence, the increase expression of the midgut epithelial cell regenerator *Vein* at 4 h with concomitant upregulation of *CASPS8* at 8 h post YE00 exposure in L3 AAE-L larvae is suggestive of an attempt by the larvae to reconstitute the midgut epithelia damaged by the larvicide, combined with a last-ditch effort to clear dead and dying cells unable to overcome the YE00 non-specific, broad-acting mode of action (MoA). Moreover, the modulation observed in the gene expression levels regarding the inhibitor of apoptosis IAP1 and IAP2 may be associated with their regulatory abilities on initiator or effector caspases<sup>29</sup>. For instance, IAP1 interacts and regulates the expression of both initiator caspases (i.e. Dredd) and effector caspases (CASPS7 and CASPS8)<sup>30,31</sup>. Both CASPS7 and CASPS8 carry out proteolysis and disintegration of proteins during cell death<sup>29-31</sup>. Although the *Ae. aegypti* apoptosis pathway is not entirely understood, studies in *Drosophila melanogaster* have demonstrated that IAP2 not only regulates apoptotic-associated genes, but also negatively regulates the innate immunity (IMD) pathway<sup>32</sup> in a fashion similar to the negative regulator Pirk<sup>33</sup>.

The cytotoxic effects of YE00 are broad, with drastic alteration in cell morphology at both the cellular and subcellular levels in the insect midgut, as demonstrated by the TEM analyses. While YE00 appeared to have produced no specific alterations of the larva PM2, there was substantial damage to midgut epithelia, including loss of microvilli, disordered cellular junctions, and increased cytoplasmic

vacuolization in lamellar bodies. In the cytosol, increased vacuolization and abnormal morphological alterations of the mitochondria and cristae also were observed.

Apoptotic signaling is linked with mitochondrial release of cytochrome *c* and other cofactors to activate effector caspases<sup>34</sup>. The presence of circular-shaped mitochondria observed in our TEM images of midgut cells following YEEO exposure is likely due to the cellular cytotoxicity affecting mitochondrial proteins that coordinate cytochrome *c* release and promote the apoptosis expression cascade suggested by the differential expression of *IAP* and *CASP*, as seen in AAE-L larvae.

With regards to autophagy being mediated by YEEO, no differences were detected for either *ATG1* or *ATG6* when we compared YEEO vs. control yeast ingestion by the mosquito larvae. In addition, no differential expression was detected in *V-ATPase* between YEEO-exposed (LC50) and unexposed L3 larvae. It has been previously reported that the expression of *ATG1*, *ATG6*, and *ATG8* is correlated with the expression of *V-ATPase* following exposure of *Ae. aegypti* larva to the plant-derived fatty-acid compound squamocin<sup>28</sup>. Further, in *Drosophila melanogaster*, autophagic cell death in the midgut is accompanied by markers of apoptosis such as DNA fragmentation<sup>35</sup>. Similarly, in moribund AAE-L larvae exposed to YEEO for 24 h, the typical pattern of DNA degradation present during apoptosis was observed in our TUNEL analysis. Although the relationship between the autophagic and apoptotic pathways is not yet clear, following the ingestion of YEEO by AAE-L larvae, increased levels of cytoplasmic vacuolization and cell death are observed within 30 min (as depicted in our TEM images). However, despite our current lack of evidence, autophagy as an inducible response to the ability of the mosquito larvae to tolerate the non-specific toxic and apoptotic effects associated with YEEO has not yet been discarded.

In our investigations we also noted the disappearance of the YEEO within the gut of the larvae. To exclude the possibility that the breakdown of the yeast cell wall within the larva gut is simply due to pH variations within the mosquito larva gut as previously shown<sup>23,36</sup>, we tested YEEO in increasing concentrations of pH solutions. As no changes in the YEEO stability were detected in a range of pHs from pH7 to pH10, it suggests that other mechanism(s) in the gut are likely responsible for the YEEO breakdown, presumably digestive proteases<sup>11</sup>. It also remains to be determined if such YEEO breakdown is due to the continued action of digestive proteases, if indeed these are involved, or whether it involves a combination of factors that create a domino effect regarding the breakdown of the YEEO cell walls and the release of the orange oil.

Insecticide resistance poses a serious threat to the control of mosquito-borne diseases. Traditional vector management programs either increase insecticide applications to kill resistant populations or must switch to another pesticide to achieve control. These approaches have not generated any significant advantages for mosquito control but have instead contributed to the increase of resistance in mosquito vectors. Continuous exposure to organophosphates and pyrethroids<sup>37-39</sup> have resulted in the overproduction of cytochrome P450 enzymes<sup>37,40,41</sup> that are necessary for detoxification of the various pesticides in *Ae. aegypti*. As is found in nature, it is conceivable that distinct mosquito strains or species possess unique detoxification or innate gene regulatory mechanisms to cope to various pesticide classes,

including organophosphates, pyrethroids, and natural products like YE00. In response to YE00, the differential gene expression between CYP6 family and CYP9 cytochrome P450 family genes suggest that different families of genes may be regulated according specifically to each respective chemical exposure challenge (for example receptor-based pesticides like pyrethroids versus non-receptor based pesticides such as the YE00 larvicide). Additionally, cytochrome P450s are consistent markers of resistance but do not encompass the entire spectrum of underlying resistance mechanisms. Therein lies the possibility that other mechanisms of resistance exist that have yet to be identified against YE00s.

Larviciding approaches such as Bti, which is effective against *Ae. aegypti* larvae, are often still out of reach for many affected communities due to frequent need for reapplications and elevated cost. Thus, gaps exist both in the availability of safe, stable, cost-effective, and efficacious alternatives in mosquito control approaches. YE00 mosquito larvicide is easy to produce and is stable after long-term storage<sup>12</sup>. Moreover, YE00 is affordable as it primarily relies on local resources (essential oils). It is not yet known what effects if any YE00 has on *Ae. aegypti* larval development into adults, including effects on the physiology, fecundity, and fertility of the adult mosquito, especially after sublethal YE00 exposure (i.e. LC20 concentrations). Experiments focused on understanding these effects in larvae in relation to hormesis are currently underway<sup>42</sup>.

In conclusion, YE00 is highly effective against *Ae. aegypti* larvae through mechanisms involving acute midgut cell damage and apoptotic pathways leading to larval death. Because of its multi-target action, resistance and tolerance are unlikely to develop against YE00. Nevertheless, many aspects of how YE00 act remain uncharacterized. Primary components of EOs have been demonstrated to exert their larvicidal effects through at least three different mechanisms: neurotoxicity<sup>43,44</sup>, growth inhibition, and interruption of metabolic pathways<sup>45-47</sup>. Additional work will uncover potential tolerance or resistance mechanisms associated with the non-receptor based larvicides. Identifying its effectiveness against other mosquito species as well as their specific killing mechanisms will provide critical information towards the development of YE00 as a safe, biologically-friendly larvicide for wide spread applications to control mosquito populations.

## Declarations

Ethics and Approval to Participate – All persons include in the study were cleared to participate by their respective institutions.

Consent – All authors have given consent for publication.

Availability of data and material – Data and material used in the studies are available

Competing interests - Authors declare no competing interests

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decision to publish or preparation of the manuscript. PHK is a Henry Jackson Foundation for the Advancement of Military Medicine contractor.

Authors' contributions – PHK was responsible for mosquito rearing, performed the assays, data analyses, and help craft the manuscript; AVY produced and purified the YEEO; AA assisted with the statistical analyzes; IH helped with the study design and helped with crafting the manuscript; MRO coordinated all aspects of the study including design and data analyses and the crafting of the manuscript.

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#### DoD Disclosure

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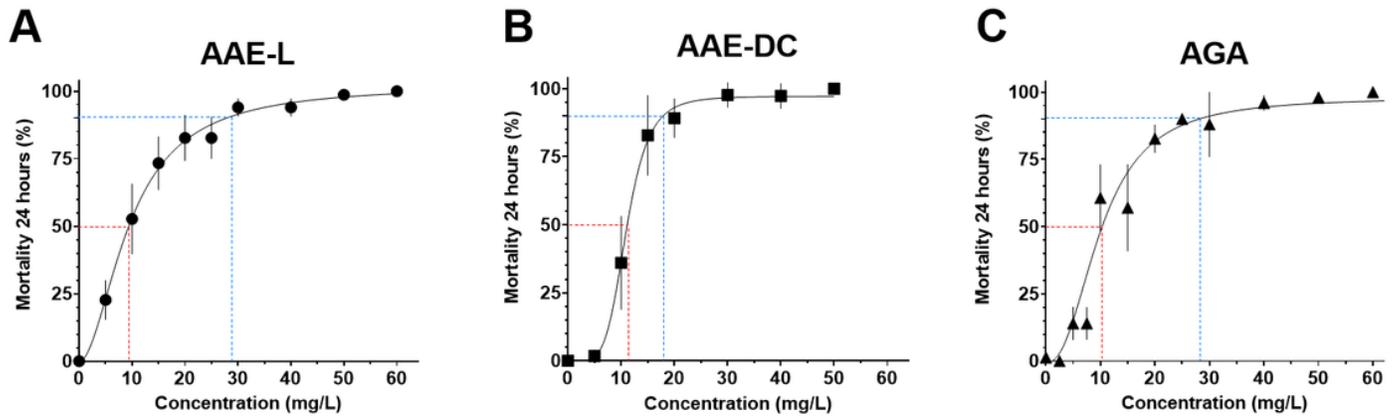
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## Figures



**Figure 1**

**YE00 in larval killing bioassay.** Each larval killing bioassays was performed with 25 L3 larvae placed in a plastic cup containing 100 ml total volume appropriately dissolved YE00 larvicide (X-axis) for 24 h. A= *Ae. aegypti* Liverpool (AAE-L), B= *Ae. aegypti* Washington, D.C. (AAE-DC), and C= *An. gambiae* (AGA). Mortality (Y-axis) was assessed using the logistic procedure in GraphPad Prism 8. Dose-response curves were plotted with nonlinear regression best fit with the means and SEM of each concentration tested. LC<sub>50</sub> (red dotted line) and LC<sub>90</sub> (blue dotted line) are shown. A minimum of two and up to eight replicates (N) were performed for each concentration tested.

**Figure 2**

**mRNA expression profiles in AAE-L after YE00 LC50 (10 mg/L).** Spatial expression of innate response genes in the mosquito larvae. Black bars = dissected gut; and grey bars = carcass. N=4, samples pooled in triplicate, 2-way ANOVA, Dunnett's multiple comparisons post-test, \*p<0.05.

**Figure 3**

**mRNA expression profiles in AAE-L after YE00 LC90 (30 mg/L).** Spatial expression of innate response genes in the mosquito larvae. Black bars = dissected gut; and grey bars = carcass. N=2, samples pooled in triplicate, 2-way ANOVA, Dunnett's multiple comparisons post-test, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

**Figure 4**

**Cytochrome P450 mRNA expression in AAE-L after YE00 LC20 or LC<sub>50</sub> doses.** Expression of cytochrome P450 gene families CYP6 (*CYP6M11* and *CYP6N12*) and CYP9 (*CYP9J10*) were assessed in three

replicates containing 3-8 individual whole larvae exposed to YEEO LC<sub>20</sub> or LC<sub>50</sub> concentrations. 1-Way ANOVA, Tukey's Post-Hoc test. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.



## Figure 5

**Assessment of yeast cells integrity by TEM.** Lyophilized yeast (control) or YEEO were rehydrated in water at 4.4% for 30 min before fixation and subsequent imaging. A-C= yeast control cells; D-F= YEEO. Control yeast shows dividing cells (dc), not present in YEEO. Panels depicting YEEO shows the widespread but uneven loading of the orange oil (eo). cw= Cell wall from control and YEEO are intact and unaffected by essential oil. A, D: 8000x. B, E: 15,000x. C, F: 30,000x.

## Figure 6

**TEM of AAE-L larvae midgut fed yeast cells.** In (A) normal appearance of mosquito larva gut fed on intact control yeast cells (y) sequestered within peritrophic space. The PM2 (arrows) and the extensive microvilli (mv) are visible. The inset shows the microvilli and the PM2 in greater details. (B) The nucleus (n) and integral nuclear membrane are also visible. (C) Distinct cell junctions (arrowhead) between adjacent cells, microvilli (MV), mitochondria (m), and basal lamina (b) are healthy.

## Figure 7

**TEM of AAE-L larvae midgut after 30 min YEEO LC<sub>90</sub> exposure.** Following 30 min after feeding on YEEO, the AAE-L larva midgut shows, in (A) – No detectable microvilli and increased blebbing in the nucleus (n) and nuclear membrane (dashed arrow), and the larval PM2 (pm) still visible (arrows); in (B) - Close-up of apical cell surface with loss of microvilli (#) and blebbing of cell membrane, the PM2 still intact; in (C) A detail of the apical cells (#) with no microvilli, with the PM2 and the peritrophic space (\*) with no visible YEEO cells; and in (D), basal lamina (b) of epithelial cell is shown with slight increase in vacuolization, and still uniformed mitochondria (m).

## Figure 8

**TEM of AAE-L larvae midgut after 4h YEEO LC<sub>90</sub> exposure.** In (A) Complete loss in microvilli (#), increased vacuolization within epithelia cytosol, and no YEEO cells visible within peritrophic space; In (B), a closer view of the apical portion of midgut cells with loss of microvilli and blebbing (#), uncharacteristic cell junction (arrow), yet intact PM2 (pm). In (C) and (D) the basal lamina (b) of epithelial cell displaying

increased vacuolization, blebbing of the nucleus (n) and nuclear membrane (dashed arrow), and abnormal or irregularly shaped mitochondria (m).

## Figure 9

**No effect of YE00 (LC90) PM2 permeability.** Confocal microscopy of dissected L3 AAE-L midguts exposed to either yeast blank controls (A) or YE00 LC<sub>90</sub> concentrations for 4 h (B). No FITC-Dextran (2000kDa) were observed in the caeca of either control or YE00-treated larvae suggestive of no change in PM2 permeability. The auto fluorescence seen in first column due to essential oil leakage and yeast accumulation. Representative image of 20 sampled individuals for each treatment.

## Supplementary Files

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