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RESEARCH ARTICLE

Lactic acid bacteria waste infusion as a source of attraction and oviposition stimulation of gravid female *Aedes albopictus* mosquitoes

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ABSTRACT

Aedes albopictus poses a public health risk in tropical countries and temperate countries in recent decades due to its capability to transmit various human arboviruses including dengue, yellow fever, and chikungunya. Vector control is the key for preventing transmission of these pathogenic viruses. Improving the effectiveness of currently utilized collection methods, such as ovitraps, is important for best species abundance monitoring, assessment of the threat of arbovirus transmission, and optimizing control activities. Therefore, this study aimed to assess the potential use of lactic acid bacteria (LAB) waste as an infusion-baited ovitrap for *Aedes* collection. The performance of overnight tap water, grass hay infusion and LAB waste infusion were compared for their ability in attracting gravid female *Ae. albopictus*. In this study, the LAB waste infusion was substantially more alluring to *Ae. albopictus* mosquitoes than the two controls grass hay infusion and tap water.

Keywords: Ovitraps; infusion; lactic acid bacteria; vector; *Ae. albopictus*.

INTRODUCTION

Aedes albopictus is the mosquito species that has expanded the most geographically, posing a public health problem in tropical temperate countries due to its capability to spread many human arboviruses, including dengue, chikungunya, and zika (Aranda *et al*., 2006; Medlock *et al*., 2012; Schaffner *et al*., 2013). Thus, it is crucial to survey and monitor the species for preventing new infestations, assessing the danger of arbovirus transmission, and optimizing control actions (Velo *et al*., 2016). Mosquito ovitraps are widely employed in endemic areas to monitor, detect, and manage *Aedes* populations (Chan, 2009; Sivagnaname & Gunasekaran, 2012). They estimate the adult population in certain sites and oblige as an initial cautionary system for potential disease epidemics (Chan, 2009).

Ovitrap is the most commonly used and easiest monitoring tool for *Ae. albopictus* and other species that favour to breed in containers such as *Ae. aegypti*, the primary vector of yellow fever, dengue fever, and zika (Schaffner *et al*., 2013). Ovitrap can be defined as small black plastic container that resembles the favoured breeding environment for mosquitoes, which includes small artificial containers, holes in trees and rocks (Hawley, 1988; Velo *et al*., 2016). According to Obenauer *et al*. (2009), the ovitraps lured with organic infusions are capable to monitor and hence reduce the *Aedes* populations. It has been shown that baiting ovitraps with hay infusion can boost the egg production of *Aedes* (Holck *et al*., 1988; Service, 1993; Allan & Kline, 1995; Sant'ana *et al*., 2006; Burkett-Cadena & Mullen, 2007; Obenauer *et al*., 2009; Ponnusamy *et al*., 2010; Gopalakrishnan *et al*., 2012; Arbaoui & Chua, 2014) and adult mosquito traps (Gama *et al*., 2007;de Santos *et al*., 2012) when compared to ordinary water.

The oviposition reaction is mostly due to the present of semiochemicals in the mixture that act as attractants or repellents (Trexler *et al*., 2003). The leaf infusions produced by fermenting various organic material contains compounds that lure the female mosquitoes to their lay eggs (Trexler *et al*., 2003; Ponnusamy *et al*., 2010; Santos *et al*., 2010; Gopalakrishnan *et al*., 2012). Semiochemicals found in infusions are frequently products of organic matter breakdown by microorganisms (Millar *et al*., 1992; Ponnusamy *et al*., 2008). The purpose of this study was to evaluate the effect of LAB waste infusion on the oviposition responses of *Ae. albopictus* mosquitoes under field condition.

MATERIALS AND METHODS

Lactic acid bacteria waste product

Five different food source isolated strains of lactic acid bacteria were grown in de Man, Rogosa and Sharpe (MRS) broth. The strains used were identified in Yap *et al*. (2021) which are *Lactobacillus plantarum* (strain number L001 to L003) and *Pediococcus pentasaceus* (strain number L004 and L005). The LAB waste was generated from the analysis of gas chromatography-mass spectrometry (GCMS), whereby after the strains were cultured in 100ml of MRS broth, the cell free supernatant was collected and freeze dried. Following that, equal volume of methanol was added and allowed to stand for 48 hours, and the unwanted filtrates from the methanol extract collected on the filter paper (Whatman No. 1) was then collected and used for this study. The LAB waste product was combined and weighed in dry weight (per gram), before resuspending with equal volume of water (per gram per ml) as stock solution (1g/ml).

Table 1. Ovipositional responses of *Ae. albopictus* and number of larvae hatched in ovitraps baited with three different infusions

Ovitrap (Infusion)	n	No. of larvae per infusion (mean ± SE)	Total no. of larvae (%)	% Ovitrap Index (OI)
0.1% LAB waste	49	19.10 ± 27.11	967 (48.62%)	63.27
10% hay infusion	48	13.65 ± 19.55	700 (35.19%)	58.17
Overnight tap water	48	6.39 ± 7.43	322 (16.19%)	54.33

Experimental design

A site in Universiti Malaya, Malaysia, was chosen as the study site. Five random locations were selected for the study. Most of the buildings in the university campus was surrounded by bushes and woodland. The bushes were marked by shrubs of flower pots and grass, while the presence of trees and bushes defined the woodland zones. Each site was approximately 80 metres apart and contained four set of ovitraps. A set of ovitrap consisted an ovitrap with the 10% diluted hay infusion (positive control), an ovitrap with overnight tap water (negative control) and 0.1% diluted LAB waste (experimental). Following the protocol of Polson *et al*. (2002), the ovitraps, (350 ml plastic cup, 91 mm in height and 75mm in diameter) were placed in a set of positive control, negative control and experimental ovitrap at each of the spot. A total of 180 ovitraps were set out between August 2020 and September 2020 in the selected sites for three replicates. All of the ovitraps were collected and their contents were put in plastic containers (16cm x 11cm x 7cm) after being set up for five days. The first instar larvae were fed with liver powder until they reach to third or fourth instar stage. The hatched larvae were identified its species at the third or fourth instar using a compound microscope in accordance with World Health Organization (WHO) guidelines in National Dengue Control Unit Sri Lanka. The abdominal segment of *Ae. albopictus* larvae has straight thorn-like comb scales while of *Ae. aegypti* larvae has pitchfork-shaped comb scales. *Aedes* mosquitoes are differentiated from other mosquito species by their thin, often black body, distinctive pattern of light and dark scales on the abdomen and thorax, as well as alternating light and dark bands on the legs. The *Ae. albopictus* has a white stripes forming median longitudinal line on its thorax while the *Ae. aegypti* has a pair of longitudinal white stripes and a white lyre-shaped marking (Tissera *et al*., 2016). In this experiment, we also identified the pupae to distinguished its sexes. The pupae can be sexed sorted by the differences in the genital lobe shape (at the end of the pupal abdominal segments, below the paddles). The female pupae are often bigger compared to male (Papathanos *et al*., 2009).

Infusion preparation

Traps were prepared with 175 ml hay infusion or tap water and lined with appropriately labelled papers before transportation to the field. According to Polson *et al*. (2002), hay infusion was prepared by soaking 8.4g of dried rice grass (*Oryza sativa*) in 1 litre of overnight tap water in a tightly container. The container remained closed for seven days at room temperature to ferment the grass. The resulting hay infusion was filtered and diluted to 10% in overnight tap water. Whereas for the LAB waste attractant, the infusion was diluted to 0.1% in overnight tap water. The LABs were cultured from food source and the bacteria culture were methanol-extracted for 48h in room temperature. The solution was filtered using filter paper (Whatman, No.1 Filter) and keep for further use. The LAB waste that has been filtered out was used as the mosquito attractant.

Data analyses

The number of larvae obtained from the three different infusion's attractants were counted and statistically evaluated using the non-parametric test, with Kruskal Wallis test. The Shapiro-Wilk test was used to determine the normality of the data and Levene's test was used to determine the homogeneity of the variances. All the data were analyses using the IBM SPSS version 22 program, with statistical significance set at 0.05. According to Sasmita *et al*. (2021), to obtained the ovitrap index (OI), the number of positive ovitraps that comprising of *Aedes* egg or immature mosquitoes were divided by the total number of ovitraps retrieved.

Table 2. Ovipositional responses of *Ae. albopictus* and number of larvae hatched in ovitraps in five different sites

Site	No. of larvae per trap (mean ± SE)	Total no. of larvae per site (%)
A	30.26 ± 6.34	438 (22.02%)
B	9.96 ± 2.82	783 (39.37%)
C	10.993 ± 4.23	378 (19.00%)
D	5.20 ± 1.48	169 (8.50%)
E	7.93 ± 2.00	221 (11.11%)

RESULTS

In general, the number of larvae collected in an individual ovitrap for all three different infusions ranged from zero to 115 larvae of *Aedes* mosquitoes. There is no significant difference between the number of male and female larvae in three different trap infusion observed for all set up sites (Table 3). The number of larvae diverse significantly when infusions of 0.1% LAB waste, 10% hay and overnight tap water were compared using chi square test (H = 9.038; df = 2; P = 0.011). The mean number of larvae was significantly highest in the ovitraps with 0.1% LAB waste infusion (19.10 ± 27.11) compared to both control ovitrap with 10% hay infusion (13.65 ± 19.55) and overnight tap water (6.39 ± 7.43) ($P < 0.05$). Overall, the highest number of larvae recorded in the study was the ovitrap with 0.1% LAB waste infusion, 967 larvae (48.62%) followed by ovitrap with 10% hay infusion, 700 larvae (35.19%) and the least number of larvae recorded was in the ovitrap with overnight tap water, 322 larvae (16.19%) (Table 1). In Table 1, showed that *Ae. albopictus* oviposited its eggs the most in 0.1% LAB waste infusion, which the highest OI is 63.27%, followed by the OI in 10% hay infusion with 58.17% and the lowest OI was 54.33% in overnight tap water.

The Kruskal-Wallis analysis showed that the abundance and distribution of larvae were normally distributed and there is significant difference between the sites. The number of larvae varied significantly per site when compared using chi square test (H = 16.72; df = 4; P = 0.003). The larvae mean rank for site A (30.26 ± 6.3), site B (9.96 ± 2.82), site C (10.93 ± 4.23), site D (5.2 ± 1.48) and site E (7.93 ± 2.00) for all three infusions are shown in Table 2 ($P<0.05$). Site B with the highest level of vegetation and the nearest to the forest regardless of human presence collected the highest number of 783 larvae (39.37%) followed by site A of 438 larvae (22.02%) and site C which surrounded by some limited vegetation area recorded 378 larvae (19.00%). Site D and E with some presence of human and least vegetation collect 169 larvae (8.50%) and 221 larvae (11.11%) respectively (Table 2).

Table 3. Ovipositional responses of *Ae. albopictus* and number of larvae hatched in ovitraps according to the gender in the three different infusions

Ovitrap (Infusion)	Gender	Site A	Mean ± SD	Site B	Mean ± SD	Site C	Mean ± SD	Site D	Mean ± SD	Site E	Mean ± SD	Total	P value	X2
Overnight tap Water	Male	36	12 ± 7.8	30	10.0 ± 10.8	19	6.3 ± 3.1	10	3.3 ± 4.9	23	7.7 ± 6.7	118	X2=5.235, P=0.264	
	Female	49	16.3 ± 10	54	18.0 ± 23.8	47	15.7 ± 16.7	13	4.3 ± 6.7	41	13.7 ± 11.9	204		
0.1% LAB Waste	Male	100	33.3 ± 17.2	180	60.0 ± 70.0	107	35.7 ± 43.1	38	12.7 ± 15.5	36	12.0 ± 12.0	461	X2=8.950, P=0.062	0.011
	Female	100	33.3 ± 14.2	220	73.3 ± 111.6	101	33.7 ± 34.5	34	11.3 ± 11.0	51	17.0 ± 18.7	506		
10% Hay Infusion	Male	74	24.7 ± 16.4	122	40.7 ± 48.8	48	16.0 ± 15.4	35	11.7 ± 8.1	30	10.0 ± 9.5	309	X2=2.977, P=0.562	
	Female	79	26.3 ± 18.4	177	59 ± 78.0	56	18.7 ± 17.0	39	13 ± 3.5	40	13.3 ± 14.0	391		

DISCUSSION

Fay & Eliason (1966) invented oviposition trap that have been standardized by Centres for Disease Control and Prevention (CDC) for use in *Ae. aegypti* monitoring, indicates the manifestation of mature gravid females. This invention was later upgraded by Reiter *et al*. (1991) by using hay infusion than ordinary tap water as the medium and as result female mosquitoes oviposited recurrently in ovitraps with hay infusion. *Ae. albopictus* breeds in both natural and man-made containers, and is utmost common in rural settings. It is found in sylvan settings, but it can quickly colonise suburban and urban areas that have sylvan traits (Hornby *et al*., 1994). In current study, females *Ae. albopictus* was found oviposited in all three types of trap infusions as the study site was surrounded with shrubbery and in woodlands areas. These findings also highlighted the suitable location where *Ae. albopictus* populations should be monitored using ovitraps.

According to Arbaoui & Chua (2014), attractants can be discovered by isolating and identifying bacteria from wide selection or stimulatory substrates, then testing the bacteria in egg laying bioassays. This could result in the advent of new sustainably of attractants, enhancing the efficiency of ovitraps. However, our finding has discovered a new source of attractants which attained from waste of bacteria. In this preliminary study, we have shown that the infusion of LAB waste is able to enhance the production of *Ae. albopictus* eggs compared to the controls. There has been a lot of research on the involvement of microorganisms in *Aedes* mosquitoes' oviposition behaviour and the oviposition-inducing or limiting properties of bacteria. These studies have determined bacteria like *Pseudomonas aeruginosa* (Ikeshoji *et al*., 1975; Hasselschwert & Rockett, 1988), *Aerobacter aerogenes* (Hazard *et al*., 1967; Gopalakrishnan *et al*., 2012), *Bacillus cereus* (Hasselschwert & Rockett, 1988; Pavlovich & Rockett, 2000; Trexler *et al*., 2003) and/or its metabolites to be an efficient egg laying attractant and/or stimulant in mosquitoes. It was discovered that the presence of bacteria in infusion-baited ovitraps increased the attractiveness of ovitraps to gravid by producing volatiles that attracted female mosquitoes more than control water (Obenauer *et al*., 2010). According to a study by Navarro et *al*. (2003), gravid female mosquitoes engage in behaviour that involves responding to visual, olfactory, and chemotactile cues as they look for prospective oviposition sites.

Pheromones correlated with eggs, carboxylic acids, methyl esters, and bacteria in larval water are the chemical stimuli for mosquito oviposition (Obenauer *et al*., 2010). By releasing volatiles created by microbial fermentation, ovitraps augmented with plant infusions draw more gravid females than traps with plain water (Trexler *et al*., 2003). Therefore, our findings support with the previous studies indicating that the product of the bacteria can attract or become stimulant to the gravid female *Ae. albopictus*. The oviposition responses of *Ae. albopictus* mosquitoes differ with respect to type of infusions. The findings indicate that trap infusions may not have the same chemical properties, and that other microorganisms observed in the infusions may yield different composition lures for mosquitos. Our study also demonstrated that overnight tap water did not boost the oviposition response related to the infusion traps. Bacterial action could be crucial in the formation of volatiles, oviposition stimulants, and contact cues, indicating a rich source of sustenance for larvae (Sant'ana *et al*., 2006). As a result, we propose more research and identifying of the compounds generated by these infusions are necessary.

From our finding, we suggested that more gravid female could be attracted to oviposit in 0.1% LAB waste infusion compared to 10% hay infusion. According to Ponnusamy *et al*. (2008), at larger quantities, the bacteria's stimulatory mix can become a limiting factor, discouraging oviposition. The function of attractants is to directly impact behaviour, monitor or diminish mosquito populations while triggering no harm to other animals or humans and leaving no residue in food (Santos *et al*., 2003). Hence, our study involving the LAB which derived from the food source would be considered as safe and harmless to other animals and environment.

CONCLUSION

Ovitraps are convenient surveillance techniques since they are low-cost, non-meddling, and do not require specialised knowledge to use (Polson *et al*., 2002). Utilizing readily available natural resources to control vectors is economical and environmentally sound. The results of this experiment suggest that LAB waste infusions can be used to increase the effectiveness of ovitrap surveillance. The infusions are simple to prepare and suitable to be used for *Ae. albopictus* field surveillance since these LAB can be found abundantly in the environment. This work suggests a potential strategy for controlling arthropod-borne disease vectors: the use of a LAB waste attractant in an ovitrap. The benefits of ovitrap are its low cost of production, ease of maintenance, and versatility.

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Conflict of Interest Statement

The author declares that they have no conflict of interests.

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