INVESTIGATION OF FLY-BACTERIA'S ASSOCIATION ON DECOMPOSING TISSUES AND ANTIMICROBIAL EVALUATION OF FLY LARVAE NATIVE EXCRETIONS/SECRETIONS

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ABSTRACT

The interaction between flies and microbes on decomposing tissue is mediated by a vast community of bacteria. Currently, the knowledge of this relationship is limited by technological barriers. Besides, the antimicrobial activity of native excretions or secretions (NES) of fly larvae is important to be evaluated. The objective of this preliminary study is to understand the fly-bacteria association on decomposing tissue through bacteria identification along with the antimicrobial activity of NES. Two fish baits (*Rastrelliger brachysoma*) weighing 120 grams each were installed at two locations in Universiti Teknologi MARA, Puncak Alam Campus, Selangor, Malaysia from 17th April 2021 to 24th April 2021. Specimens of adult flies, larvae, pupae, and swabs of the bait surface were taken daily. The bacteria colonies were isolated from four different locations: blowflies' external surface, blowflies' internal microbiome, larvae external surface, and bait surface. A total of 23 flies were identified represented by four species from three families. *Chrysomya megacephala* (Family: Calliphoridae) was the most abundant species followed by *Sarcophaga* sp. (Family: Sarcophagidae), *Ophyra spinigera* (Family: Muscidae), and *Atherigona orientalis* (Family: Muscidae). However, *Sarcophaga* sp. larvae were the dominant larva species. Culture-based bacterial investigation showed that both Gram-positive and Gram-negative bacteria were present on the external surface and the internal microbiome of *Ch. megacephala.* Meanwhile, temporal changes of the Gram-negative bacteria group from non-lactose fermenter to lactose fermenter were observed on larvae external surface samples as well as samples from the decomposing bait. The investigation of NES collected failed to show any antimicrobial activity. A larger sample size is recommended for the future antimicrobial properties of NES studies. In conclusion, the data obtained from this preliminary

study can be used as a basic direction for future studies. This study can be extended by using metagenomic methods to understand more about the bacteria-fly association.

Keywords: Forensic entomology, blowfly, bacterial community, fly-bacteria interaction, antimicrobial, native excretions/secretions (NES)

ABSTRAK

Sebilangan besar bakteria merupakan pengantara interaksi di antara lalat dan mikrob pada tisu pengurai. Pada masa ini, pengetahuan mengenai hubungan ini adalah terhad dan dibatasi oleh kesesuaian teknologi. Selain itu, aktiviti antimikrob dari hasil perkumuhan atau rembesan asli (NES) larva lalat juga penting untuk dikaji. Objektif kajian awal ini adalah untuk memahami hubungan bakteria-lalat pada tisu yang sedang mengalami proses penguraian melalui proses pengecaman bakteria serta aktiviti antimikrob NES. Dua umpan ikan (*Rastrelliger brachysoma*) seberat 120 gram masing-masing diletakkan di dua lokasi di Universiti Teknologi Mara (UiTM), Kampus Puncak Alam, Selangor, Malaysia dari 17 April 2021 sehingga 24 April 2021. Spesimen lalat dewasa, larva, pupa dan calitan permukaan umpan diambil setiap hari. Koloni bakteria diasingkan dari empat sumber yang berbeza: permukaan luaran lalat, mikrobiom dalaman lalat, permukaan luaran larva, dan permukaan umpan. Sejumlah 23 ekor lalat telah dikenal pasti mewakili empat spesies daripada tiga famili lalat. *Chrysomya megacephala* (Famili: Calliphoridae) adalah spesies yang paling banyak diikuti oleh *Sarcophaga* sp. (Famili: Sarcophagidae), *Ophyra spinigera* (Famili: Muscidae), dan *Atherigona orientalis* (Famili: Muscidae). Walau bagaimanapun, larva *Sarcophaga* sp. adalah yang paling dominan. Kajian terhadap kultur bacteria menunjukkan kedua-dua kumpulan bakteria Gram-positif dan Gram-negatif terdapat di permukaan luaran dan mikrobiom usus *Ch. megacephala*. Sementara itu, perubahan koloni bakteria iaitu pertukaran bakteria Gram-negatif kumpulan penapai bukan laktosa kepada kumpulan penapai laktosa diperhatikan pada sampel permukaan luar larva serta sampel daripada umpan. Hasil daripada kajian NES yang dijalankan gagal menunjukkan terdapatnya aktiviti antimikrob. Saiz sampel yang lebih besar dicadangkan untuk kajian antimikrob NES pada masa hadapan. Kesimpulannya, data yang diperoleh daripada kajian awal ini dapat digunakan sebagai petunjuk asas untuk kajian masa hadapan. Kajian ini dapat dilanjutkan dengan menggunakan kaedah metagenomik untuk memahami dengan lebih lanjut mengenai hubungan bakteria-lalat.

Kata kunci: Entomologi forensik, langau, komuniti bakteria, interaksi lalat-bakteria, antimikrob, perkumuhan/rembesan asli

INTRODUCTION

Flies are among the earliest group of necrophagous insects to arrive and colonise a decomposing body (Amendt et al. 2011). Blowflies (Diptera: Calliphoridae) are usually the earliest coloniser of a decomposing body and followed by flesh flies (Diptera: Sarcophagidae) and house flies (Diptera: Muscidae) (Verheggen et al. 2017). The presence of these fly families dominates the decomposing corpse during the early stages of decomposition. In Malaysia, *Chrysomya megacephala* and *Chrysomya rufifacies* are the two common forensically significant blowfly species (Kavitha et al. 2013; Syamsa et al. 2017). The colonisation of blowflies on a decomposing tissue begins with the oviposition by gravid female blowflies. The eggs will then hatch on the decomposing body, and the larvae will undergo three stages of development (Anderson 2000). Before reaching adulthood, the larvae become pupae and hatch as adult blowflies.

The attraction of necrophagous insects such as flies to decomposing tissue is mediated by volatile organic compounds (Verheggen et al. 2017). Bacteria are known to be responsible to produce volatile organic compounds by breaking down amino acids present on a corpse (Liu et al. 2016). For blowflies, the species of bacteria present and the volatile odour compounds released lead to different responses towards a decomposing tissue (Chaudhury et al. 2010; Chaudhury et al. 2016). However, a mixed bacteria community is more attractive to blowflies than a single species of bacteria. The effects of volatile odour compounds on blowflies are primarily based on sex and ovarian status (Tomberlin et al. 2017). However, volatile odour compounds may also signal the presence of beneficial bacteria for the development of larvae (Liu et al. 2016).

The presence of necrophagous flies at bacteria-rich environments such as decomposing tissue during various stages of life lead to the exposure to various bacteria (Junqueira et al. 2017). The continuous exposure to pathogens and deleterious bacteria leads to the development of the immune system that is capable to produce and secrete antimicrobials as a measure of self-preservation (Thompson et al. 2013; Yakovlev et al. 2019). The Native Excretions/Secretions (NES) of fly larvae are rich in natural compounds with activity against Gram-negative and Gram-positive bacteria (Dallavecchia et al. 2021).

The current understanding of bacteria-fly association is largely limited by technological barriers (Thompson et al. 2013; Tomberlin et al. 2017). However, a deeper understanding of this association can be made through the identification of bacteria and understanding the effect of NES on the bacterial community. Moreover, the fly-bacteria associations for Malaysian native species have not yet been reported. Thus, this initial study was aimed to understand bacteria-fly association through bacteria identification and antimicrobial activity of NES of local fly larvae.

MATERIALS AND METHODS

Study Sites

Sample collection was conducted at two different sites at Universiti Teknologi MARA, Puncak Alam Campus, Bandar Puncak Alam, Selangor, Malaysia (3.2012°N, 101.4480°E). One trap was set up at each sampling location. The first location was located at the Faculty of Pharmacy named as location A. Meanwhile, the second location was located near the student residential area named as location B.

Fly Collection

The collection was conducted for eight days from 17th April 2021 to 24th April 2021. The samples were collected daily between 10 am to 11 am. The light intensity of the study site was measured using a lux meter during sample collection. A modified commercial hanging trap was used for fly collection (Azwandi et al. 2020). The trap was installed with fish bait of *Rastrelliger brachysoma* weighed 120 grams each. When flies were caught, the trap was replaced but the bait was reused. The filled trap was then transferred into a zip lock bag. Then, the trapped flies were killed by freezing at -20°C for 30 minutes (Sukontason et al. 2007). Later, the collected flies were transferred into sterile vials according to the species.

Bait Swab Collection

Sterile cotton tips were used to collect bacteria samples from the surface of the bait. The cotton tips were kept in 2 ml vials filled with 1 ml of buffered peptone water.

Larvae Collection
Larvae present on the bait were transferred into 2 ml sterile vials filled with 1 ml of buffered peptone water. For the first instar larvae, a sterile cotton swab was used to pick up the larva from the bait. The tip of the cotton swab was then left in the vial along with the larva. The second and third instar larvae were transferred using sterilised forceps. Forceps sterilised using 70% alcohol solution were used to transfer the larva. Third instar larvae for NES collection were transferred into sterile 50 ml vials with caps using sterilised forceps.

Identification of Fly Adults, Larvae, and Pupae
Adult flies were pinned and identified based on the morphology aided by a stereomicroscope (Leica, Zoom 2000). The selected larva was dissected using a scalpel. Observation of the posterior spiracle was done under a stereomicroscope. The structure of the posterior spiracle was used to determine larva species based on established dichotomous keys (Ishijima 1967). Meanwhile, pupae present on bait were transferred using forceps into sterile 50 ml vials with caps. Then, the caps were replaced with parafilm to allow the presence of sufficient air for any hatched flies. The vial was kept in an upright position in a room temperature area. Emerged adults were identified by established dichotomous keys (Kurahashi et al. 1997).

Isolation of Bacteria from Adult Fly Samples
Isolation of bacteria from *Ch. megacephala* was done on the external surface and the internal microbiome. The prepared samples were used for streaking on MacConkey Agar and Columbia Agar with 5% Sheep Blood. The isolation of bacteria from the external surface of *Ch. megacephala* was done by soaking the flies in buffered peptone water. 1 ml of buffered peptone water was used for each *Ch. megacephala* in a vial. The content was swirled to ensure adequate distribution of bacteria to the buffered peptone water. A small volume of buffered peptone water was aseptically transferred for streaking.

The isolation of internal bacteria from samples started with external sterilization. The method used was modified from Deguenon et al. (2019). The flies were swirled in the 70% alcohol solution to ensure adequate sterilization. The alcohol solution was drained, and the flies were rinsed three times using buffered peptone water. The isolation of the internal bacteria method was based on Zhang et al. (2017). Following the third wash, the buffered peptone water was drained, and new buffered peptone water was added to the container. Then, the flies were crushed and then shaken to ensure the distribution of internal bacteria in buffered peptone water. Later, a small volume of the buffered peptone water was aseptically transferred for streaking. The agar plates were incubated at 37°C overnight.

Isolation of Bacteria from Bait Swab and Larvae
The isolation of bacteria from bait swabs and larvae was conducted by aseptically transferring a small volume of buffered peptone water from the vials containing the samples for streaking. Streaking was done on MacConkey Agar and Columbia Agar with 5% Sheep Blood. The agar plates were incubated at 37°C overnight.

Microscopic Identification of Bacteria
Microscopic identification was done on selected bacteria colonies. The identification of bacteria started with the preparation of bacterial smears. A drop of sterile water was transferred onto a microscope slide using a micropipette. Then, a small amount of the isolated colony was picked aseptically by using an inoculating loop. Later, the bacterial colony was mixed with sterile water and spread thinly on the microscope slide. Next, the slide is allowed to air dry. Lastly, the air-dried smear was heat fixed by passing the slide through the flame of a gas burner three times.

The bacterial smears were then stained using Hucker's modification of the Gram stain method (Smith & Hussey 2005). Firstly, the prepared smear is flooded with crystal violet staining reagent for one minute. Then, the glass slide is washed using an indirect and gentle stream of tap water for 2 seconds. Following that, Gram's iodine is used to flood the slide for 1 minute. A second wash using tap water was done on the slide for 2 seconds. Next, a 95% ethanol solution was used to flood the smear for 15 seconds. Later, safranin was used to flood the slide for 30 seconds. Lastly, the slide was washed with gentle and indirect stream of water and blotted dry by using absorbent paper. Observation on the morphology of bacteria was done by using a light microscope.

Collection of Pooled Larvae Native Excretions/Secretions

The method used for the collection of NES was modified from Dallavecchia et al. (2021). The identified third instar larvae of the same species from both sampling sites were pooled together and used for the collection. The pooled larvae were transferred to 4°C water to slow down their movements to ease mass measurement on a semi-analytical scale. Subsequently, excess water was drained, and the larvae were dried on Petri dishes lined with filter paper. Later, they were placed into a conical centrifuge tube containing a volume of sterile purification water equivalent to 100 µL per gram of larvae and incubated at 37°C for 1 hour. After the incubation, the supernatant was collected and transferred to a microcentrifuge tube. The supernatant was centrifuged at 12000 g for 10 minutes at 4°C to separate insoluble particulates. The remaining supernatant was filtered through a 0.45 µm membrane filter. To study the sterility of NES, a 10 µL aliquot of the NES sample was spread on the surface of the Mueller-Hinton agar plate. Then, the agar plates were incubated at 37°C overnight. After the incubation period, the plate was analysed for the growth of bacterial colonies.

Disk Diffusion Assay

Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli and *Staphylococcus aureus* broth culture suspensions were used for disk diffusion assay. The bacterial culture suspensions were matched against the turbidity of 0.5 McFarland standard. The turbidity was adjusted using sterile saline. Using a micropipette, 100 µL of the bacterial culture was withdrawn. The culture was aseptically transferred to Mueller-Hinton agar and a sterile disposable hockey stick was used to spread the culture on the surface of the agar. Then, two blank discs and one antibiotic disc were placed evenly on the surface of the Mueller-Hinton agar plate by using sterile forceps and 15 µL of NES sample was added to one of the blank discs. Contrarily, for the negative control, purified water was used. An antibiotic disk was used for positive control. The plates were inverted and incubated at 37°C overnight and the diameters of bacterial growth inhibition were measured the following day.

RESULTS

Flies' Collection

A total of 23 flies were captured from both traps during the seven days of sampling. Four species of flies from three different families were identified in the sample collection (Table 1). *Ch. megacephala* (Calliphoridae), *Sarcophaga* sp. (Sarcophagidae), *Atherigona orientalis* (Muscidae), and *Ophyra spinigera* (Muscidae) were identified. During the sampling period, it was found that the trap at location A contained fewer flies compared to trap B. Besides that, only one species was caught at location A while four species were at location B. Only two individuals of *Ch. megacephala* were caught at location A. Trap at location A was set at the Faculty of Pharmacy and had a mean light intensity of 91.87 lux.

Table 1. Number of adult flies collected in the study

Species	Flies collected in location A	Flies collected at location B	Total number of flies collected
Chrysomya megacephala	2	16	18
Sarcophaga sp.	0	2	2
Atherigona orientalis	0	2	2
Ophyra spinigera	0	1	1
Total	**2**	**21**	**23**

At location A, only one individual of *Ch. megacephala* was caught on Day 3 and Day 4 respectively (Table 2). At this location, eggs and larvae were present before *Ch. megacephala* was caught. Eggs were discovered on the bait container cover on Day 1 of decomposition and larvae were present on bait from Day 2 until the end of sample collection (Table 3). However, no pupa was obtained during the sampling period. Although only a single species of fly was obtained at location A, morphological identification confirmed that the larvae belonged to the Sarcophagidae family.

Table 2. Adult fly attendance during the study period in trap at location A

Species	Day of decomposition							
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Chrysomya megacephala				+	+			
Sarcophaga sp.								
Atherigona orientalis								
Ophyra spinigera								

"+" indicates presence in the trap while blank indicates absence in the trap

Table 3. Stage of fly collected in Trap A

Fly stage	Day of decomposition							
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Egg		+						
Larvae			+	+	+	+	+	+
Pupae								
Adult				+	+			

"+" indicates presence in the trap while blank indicates absence in the trap

A total of 21 flies were caught at location B which comprised four different species that belonged to three different families. Location B was located near a student residential area with a recorded average light intensity of 3,290 lux during sampling time. *Ch. megacephala* was the most dominant species at location B with 16 individuals were captured. The highest number caught were on Day 1 with six flies, followed by four flies on Day 3 (Table 4). Meanwhile, two flies were collected on Day 2 and Day 5 respectively.

Besides *Ch. megacephala*, three other species were present at location B (Table 4). *Sarcophaga* sp. were collected on Day 1 and Day 5 of decomposition. A single fly from this

family was collected on those days. Other than that, *A. orientalis* and *O. spinigera* were also found. A single *A. orientalis* was caught on Day 1 while two *O. spinigera* were caught on Day 5. For location B, larvae were consistently present since Day 1 (Table 5). The presence of eggs was recorded only on Day 1 while adult flies were present more frequent during the sample collection period. Morphological identification confirmed that larvae present at location B also belong to *Sarcoph*aga sp. On the other hand, pupa was present at location B at the end of the sample collection period. A total of seven pupae were collected from Day 6 and Day 7. From the total of seven pupae collected, three pupae were hatched into *Sarcophaga* sp. The remaining failed to survive.

Table 4. Adult fly attendance during the study period at location B

Species	**Day of decomposition**							
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Chrysomya megacephala		+	+	+		+		
Sarcophaga sp.		+				+		
Atherigona orientalis		+						
Ophyra spinigera						+		

"+" indicates presence in the trap while blank indicates absence in the trap

Table 5. Stage of fly at location B

Fly stage	**Day of decomposition**							
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Egg		+						
Larvae		+	+	+	+	+	+	+
Pupae							+	+
Adult			+	+		+		

"+" indicates presence in the trap while blank indicates absence in the trap

Bacteria Isolation from *Chrysomya megacephala* Fly

Adult *Ch. megacephala* was chosen for bacterial isolation because it was the most collected species. Bacteria isolation was carried out to isolate bacteria present on the external surface of *Ch. megacephala.* It was found out that Gram-negative bacteria were present on the external surface of *Ch. megacephala*. Two different types of Gram-negative bacteria colonies were observed that representing lactose fermenting bacteria and non-lactose fermenting bacteria (Figure 1A). The lactose fermenting colonies were found to be Gram-negative bacilli (Figure 1B) while the non-lactose fermenting colonies were found to be Gram-negative cocci (Figure 1C). Only a single type of bacterial colony was found on Columbia Agar with 5% Sheep Blood (Figure 1D). The colonies were white and circular. The bacteria in the colonies performed alpha-haemolysis and were found to be Gram-negative cocci (Figure 1E).

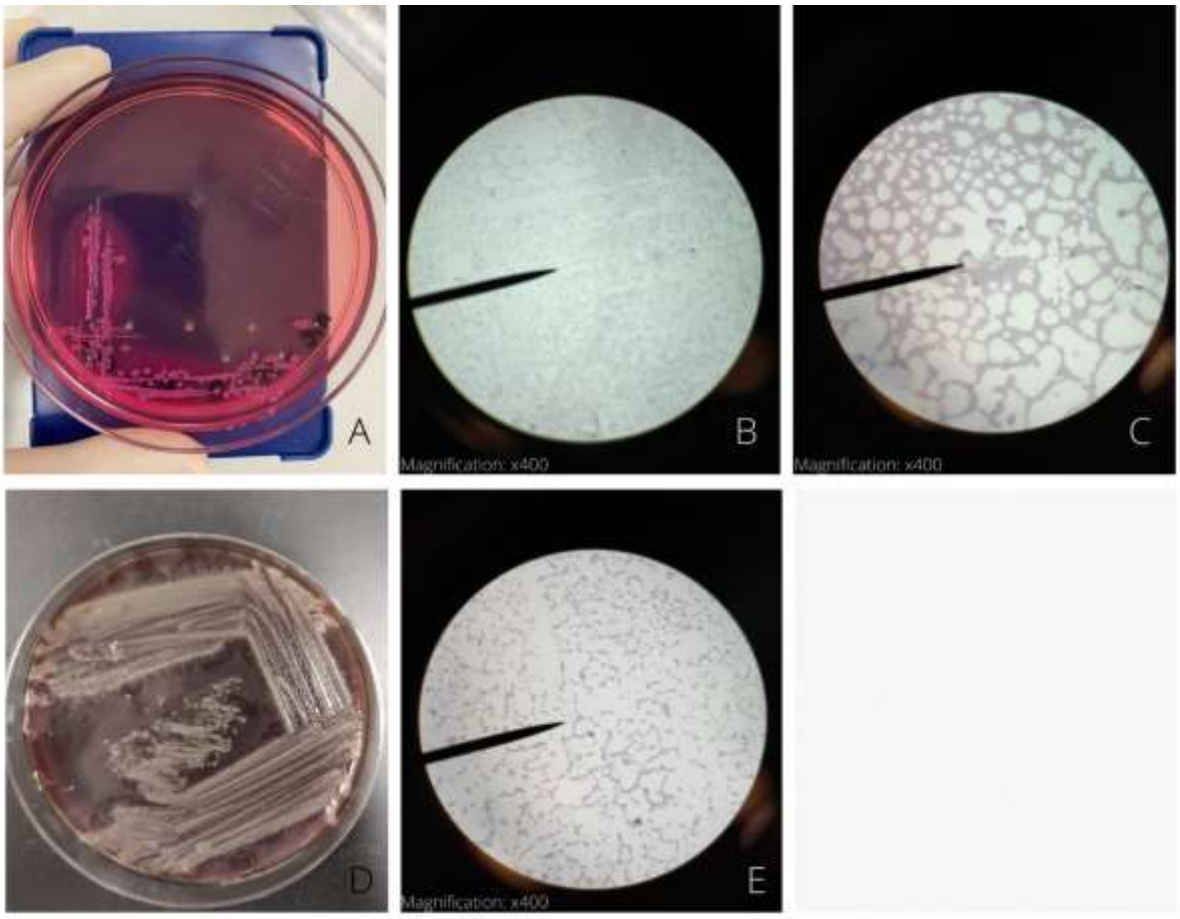

Figure 1. Bacteria isolated from the external surface of adult *Chrysomya megacephala* (A: External surface bacteria on MacConkey Agar; B: Lactose fermenter Gram-negative bacilli isolated on MacConkey Agar; C: Non-lactose fermenter Gram-negative cocci isolated on MacConkey Agar; D: External surface bacteria on Columbia Agar with 5% Sheep Blood; E: Gram-negative cocci isolated on Columbia Agar with 5% Sheep Blood.)

The isolation of bacteria from the internal microbiome of *Ch. megacephala* on MacConkey agar shows the presence of both lactose fermenting and non-lactose fermenting bacteria (Figure 2A). Both types of colonies belong to Gram-negative bacilli (Figure 2B & 2C). Two types of alpha-haemolytic colonies grew on Columbia Agar with 5% Sheep Blood (Figure 2D). The white and circular colonies belong to Gram-negative bacilli (Figure 2E) while the translucent and flat colonies are Gram-positive bacilli (Figure 2F).

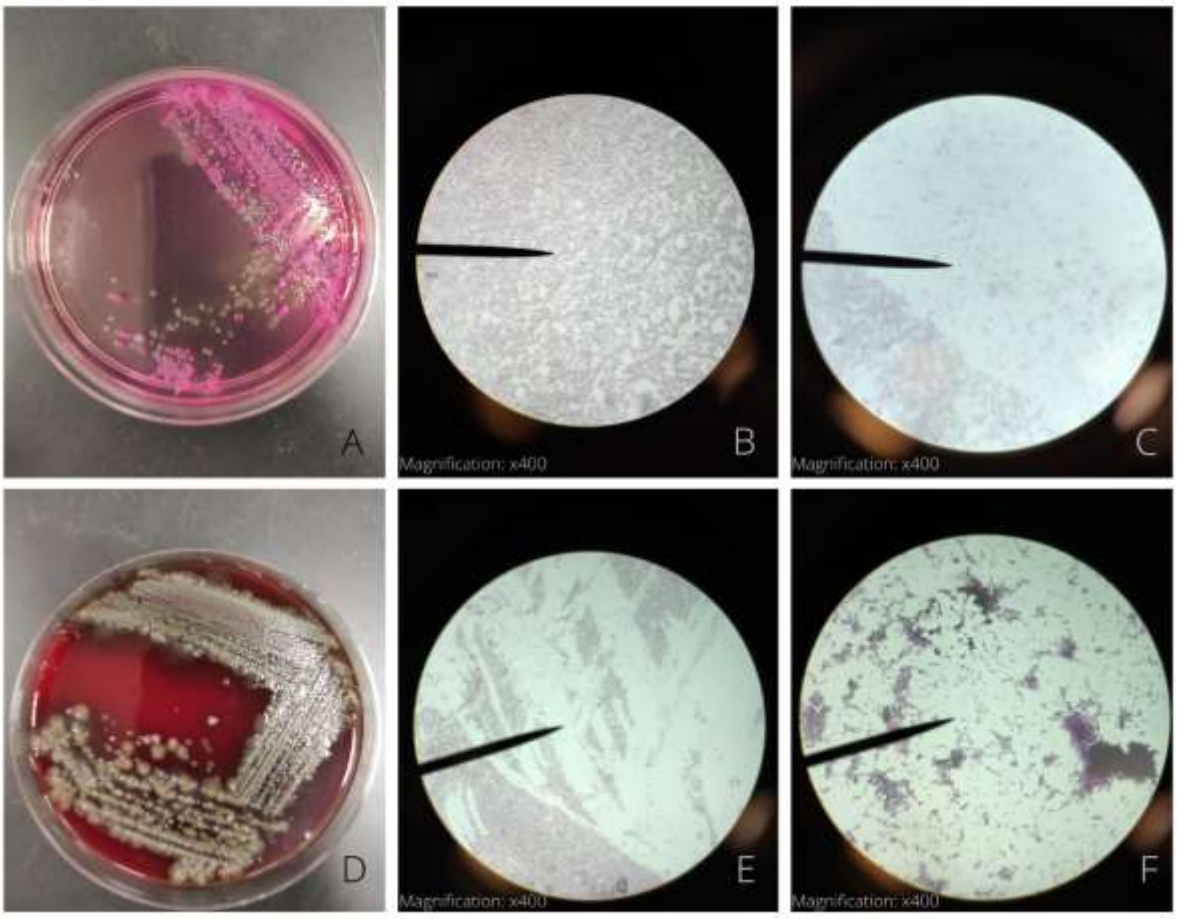

Figure 2. Bacteria isolated from the internal microbiome of adult *Chrysomya megacephala* (A: Internal microbiome on MacConkey Agar; B: Lactose fermenter Gram-negative bacilli isolated on MacConkey Agar; C: Non-lactose fermenter Gram-negative bacilli isolated on MacConkey Agar; D: Internal microbiome on Columbia Agar with 5% Sheep Blood; E: Gram-negative bacilli isolated on Columbia Agar with 5% Sheep Blood; F: Gram-positive bacilli isolated on Columbia Agar with 5% Sheep Blood.)

Bacteria Isolation from Bait

The presence of bacteria isolated from the bait can be visually observed based on the colony growth on both types of agars as the bait ages. For bacteria isolated from the surface of fresh bait, Gram-negative lactose fermenter colonies are dominant on the MacConkey Agar (Figure 3A). Colonies of non-lactose fermenting bacteria were not visually observable on the agar. Then, from the 3-days old bait, both lactose fermenter and non-lactose fermenter Gram-negative bacteria colonies grew on MacConkey Agar (Figure 3B). The dominance of lactose fermenters can be visually seen drop compared to fresh bait. Lastly, it was visually observable that non-lactose fermenting bacteria were more abundant on 7-days old bait compared to fresh and 3-days old bait (Figure 3C). Non-lactose fermenting Gram-negative bacteria were the dominant colonies on the seven days old bait. Microscopic investigation of the colonies found that both types of colonies belong to Gram-negative bacilli (Figure 3D & 3E).

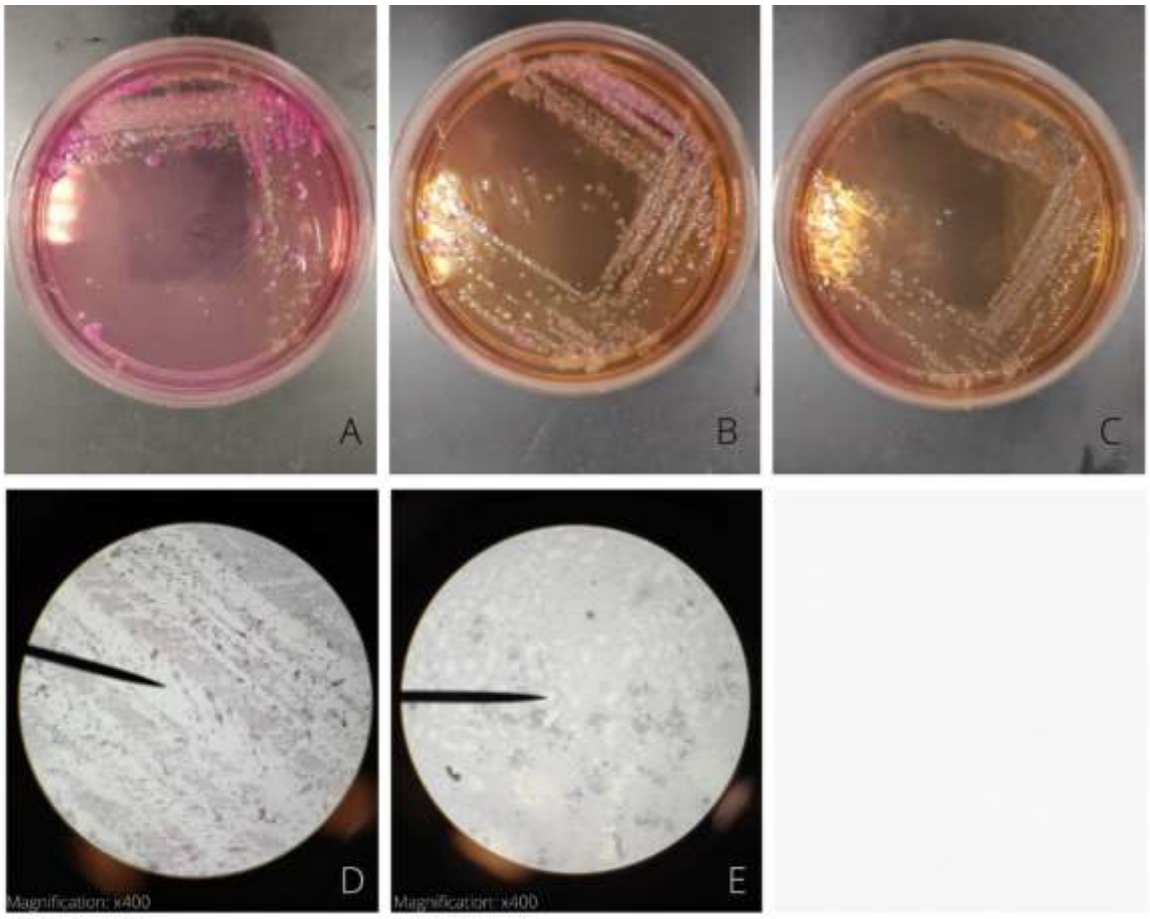

Figure 3. Bacteria isolated from the surface of bait on MacConkey Agar (A: Fresh bait on MacConkey Agar; B: 3 days old bait on MacConkey Agar; C: 7 days old bait on MacConkey Agar; D: Lactose fermenting Gram-negative bacilli isolated on MacConkey Agar; E: Non-lactose fermenting Gram-negative bacilli isolated on MacConkey Agar.)

Besides, bacteria isolated on Columbia Agar with 5% Sheep Blood also has shown differences in colonies growth according to the age of the bait. For fresh bait, it can be visually seen that circular white colonies are dominant for this sample (Figure 4A). Then, yellowish colonies can be seen among the white colonies. A different observation can be made from the 3-day old bait. The colonies of bacteria are pinkish in colour and circular (Figure 4B). Lastly, the colonies present from the 7-days old bait are white colonies that are flat and punctiform (Figure 4C). All colonies grown on the agar can perform alpha haemolysis. The different colonies that grew on the agar belong to Gram-positive bacilli (Figure 4D-4H).

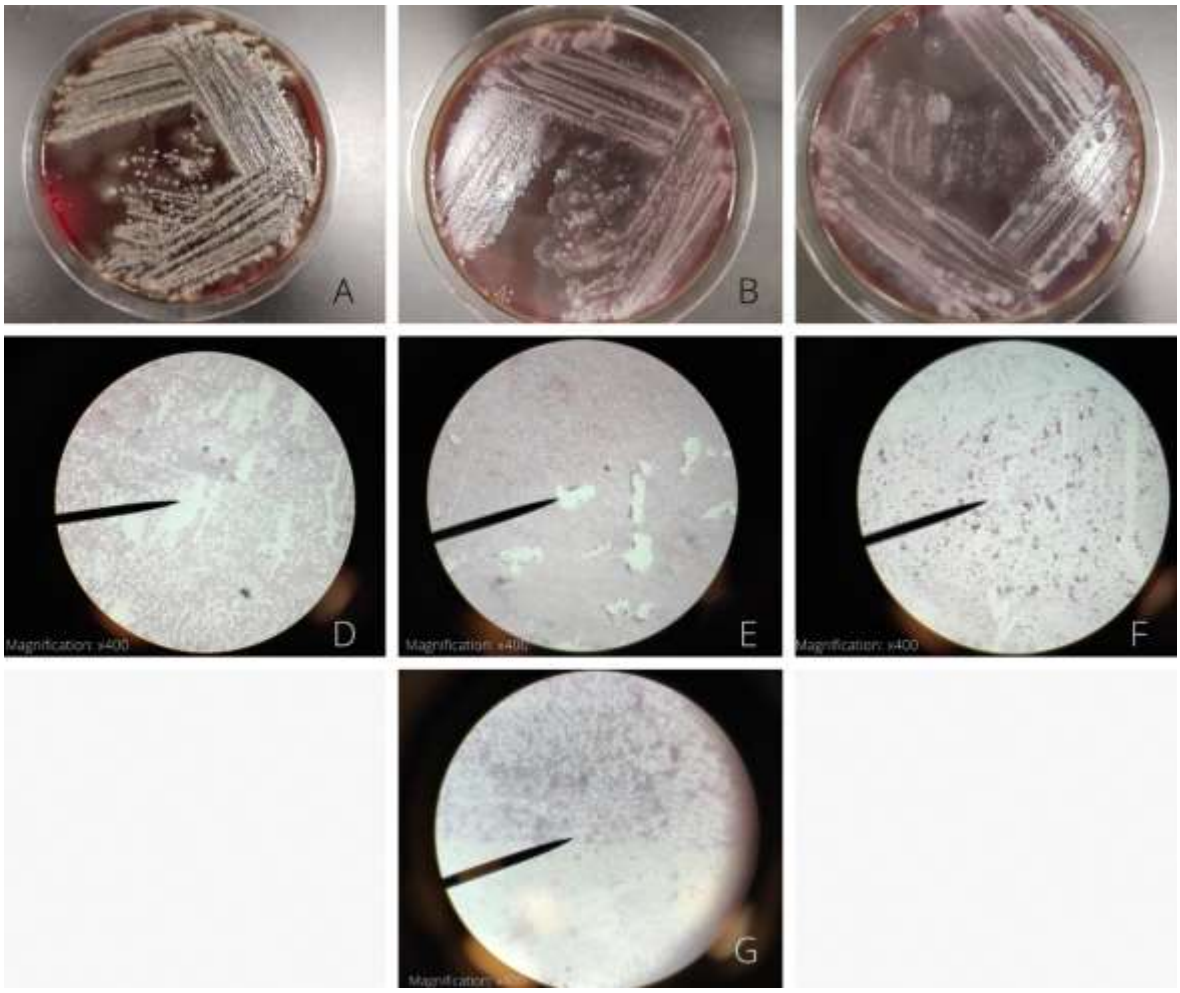

Figure 4. Bacteria isolated from the surface of bait on Columbia Agar with 5% Sheep Blood (A: Fresh bait on Columbia Agar with 5% Sheep Blood; B: 3 days old bait on Columbia Agar with 5% Sheep Blood; C: 7 days old bait on Columbia Agar with 5% Sheep Blood; D: Gram-positive bacilli from white colonies of fresh bait isolated on Columbia Agar with 5% Sheep Blood; E: Gram-positive bacilli from yellowish colonies of fresh bait isolated on Columbia Agar 5% Sheep Blood; F: Gram-positive bacilli from 3 days old bait on Columbia Agar with 5% Sheep Blood; G: Gram-positive bacilli from 7 days old bait on Columbia Agar with 5% Sheep Blood.)

Bacteria Isolation from Flesh Fly Larvae

In the study, only the larvae of *Sarcophaga sp*. were identified on the fish bait. Thus, the larvae of this species were used for the bacteria isolation. The bacteria isolated from the external surface of the flesh fly larvae shown microbial succession according to the larval development stage. Isolation of bacteria from the external surface of first instar larvae on MacConkey Agar indicated the presence of lactose fermenting Gram-negative bacteria (Figure 5A). This was consistent with the results from fresh bait. This lactose fermenting bacteria belongs to Gram-negative bacilli and was dominant on the surface of the first instar larvae (Figure 5B).

In contrast, isolation of bacteria from the external surface of second instar larvae sampled on the third day of decomposition showed the presence of non-lactose fermenting bacteria (Figure 5C). This finding is similar to the results from the third day's old bait. However,

isolation of bacteria from third instar larvae sampled from seventh days old bait showed only non-lactose fermenting bacteria were present. (Figure 5D). This finding is also similar to the observation made on bacteria isolated from the seventh day's old bait. Both colonies from second and third instar larvae belong to Gram-negative bacilli (Figure 5E).

Lastly, isolation of bacteria from the external surface of third instar larvae on Columbia Agar with 5% Sheep Blood showed the presence of bacteria with alpha-haemolytic ability. The white colonies present on the agar were different from the bacteria colonies present on seven days old bait (Figure 5F). The colonies present on the agar belong to Gram-negative bacilli (Figure 5G).

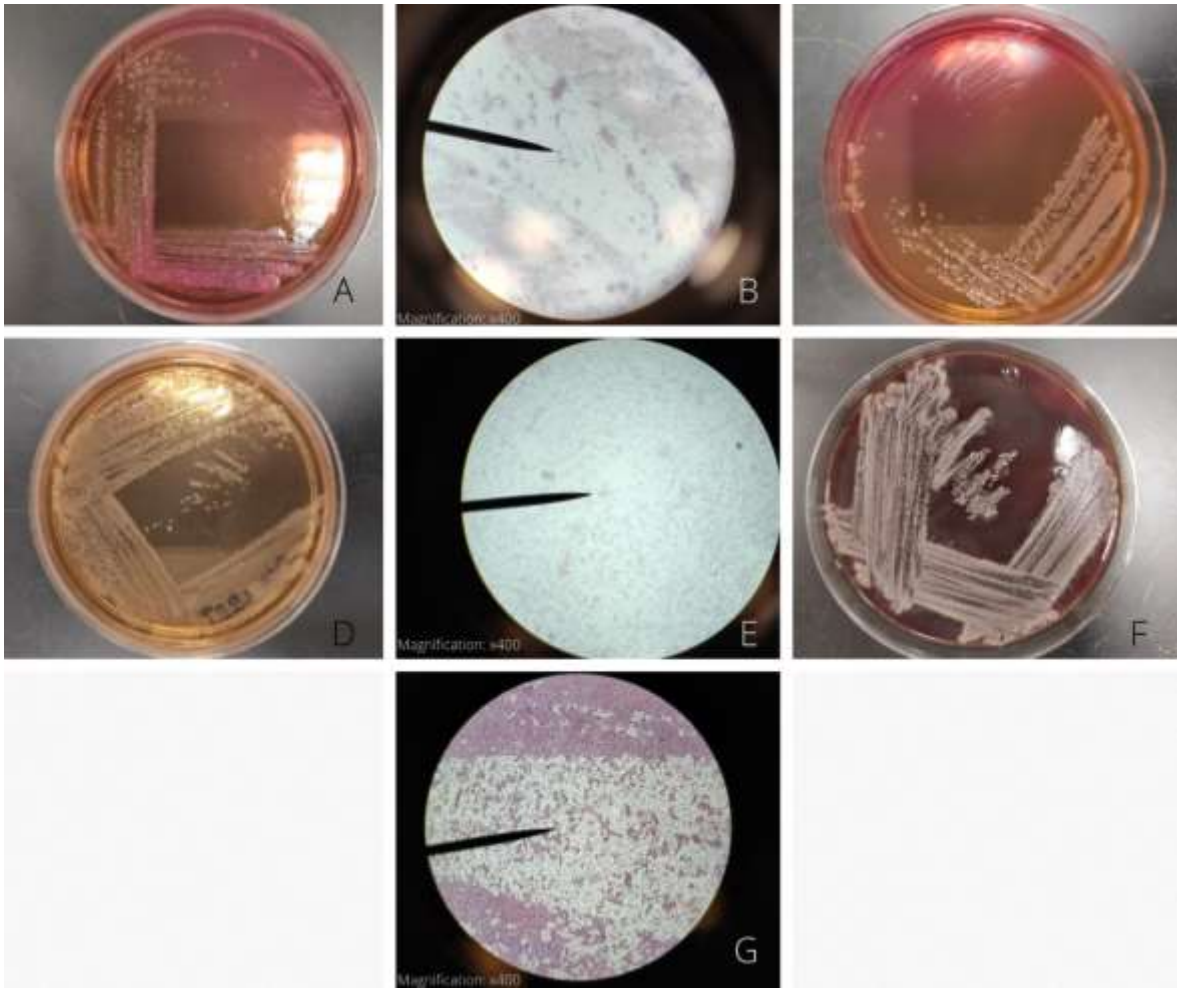

Figure 5. Bacteria isolated from the external surface of larvae (A: First instar larvae on MacConkey Agar; B: Lactose fermenting Gram-negative bacteria from first instar larvae on MacConkey Agar; C: Second instar larvae on MacConkey Agar; D: Third instar larvae on MacConkey Agar; E: Non-lactose fermenting Gram-negative bacteria from larvae on MacConkey Agar; F: Third instar larvae on Columbia Agar with 5% Sheep Blood. G: Gram-negative bacteria isolated from third instar larvae on Columbia Agar with 5% Sheep Blood.)

Disk Diffusion Assay of Pooled Larva Native Excretions/Secretions

The 15 µL of NES extracted from 8.44 grams of pooled larva from both study sites failed to cause any inhibition on all four bacteria of interest (Table 6). The NES obtained from a small number of pooled larvae for the disk diffusion assay was ineffective antibiotics as the bacteria concentration surrounding the disk experienced no changes. Besides that, the negative control also recorded no inhibition of bacterial growth. This shows that purified water used for the extraction of NES had no antimicrobial properties.

Positive control shows that most bacteria of interest are susceptible to the antimicrobial effect of the designated antibiotics apart from *Pseudomonas aeruginosa* where the antimicrobial activity of ceftriaxone is intermediate.

Table 6. Evaluation of disk diffusion assay for the antimicrobial activity of native excretions/secretions (NES) of collected pooled fly larvae

Bacteria	**Zone of Inhibition (mm)**		
	NES Sample	**Positive Control**	**Negative Control***
Bacillus subtilis	-	19 (gentamicin)	-
Staphylococcus aureus	-	19 (ampicillin)	-
Escherichia coli	-	22 (ceftriaxone)	-
Pseudomonas aeruginosa	-	15 (ceftriaxone)	-

*Purified water was used as a negative control

DISCUSSION

In this study, a total of four species of flies from three different families were collected. Blowflies (Diptera: Calliphoridae), flesh flies (Diptera: Sarcophagidae), and house flies (Diptera: Muscidae) are the families of flies known to have forensic importance due to their association with a decomposing body (Joseph et al. 2011). In this study, *Ch. megacephala* was the only species of blowfly caught. Several local studies performed on different types of carcasses recorded the arrival of *Ch. megacephala* during the early stages of decomposition (Adrus & Rahim 2018; Azwandi & Ahmad 2009; Azwandi et al. 2013; Silahuddin et al. 2015). Besides that, *Ch. megacephala* was discovered to be a prevalent species found from human remains in Malaysia (Kavitha et al. 2013; Kumara et al. 2012; Syamsa et al. 2017).

The colonisation of flesh flies on decomposing tissue usually succeeded in the colonisation of blowflies (Verheggen et al. 2017). In this study, two unidentified fly species from the family Sarcophagidae were collected. Based on records, there are several species of forensically important flesh flies found in Malaysia such as *Boettcherisca highlandica, Sarcophaga peregrina*, and *Sarcophaga ruficornis* (Kumara et al. 2012; Silahuddin et al. 2015). In our study, *Sarcophaga* sp. was caught in the fish bait during the first and fifth day of sampling at location B. Flesh fly is known to be active in all stages of decomposition (Silahuddin et al. 2015). However, the type of carcass may influence Sarcophagidae activity on the carcass (Adrus & Rahim 2018).

In this study, two species of muscids from different genera were found. The species present during the early stage of decomposition is *A. orientalis.* This fly or commonly known as the pepper fruit fly is a known pest for several types of crops (Suh & Kwon2016; Suputa et

al. 2010). However, *A. orientalis* activity is not limited to crops as this species was found on various types of carcasses at various stages of decomposition (Azwandi et al. 2013). The second species of muscids found on the bait was *O. spinigera.* This species was found at location B during the fifth day of sampling. Generally, *O. spinigera* did not arrive on decomposing carcasses until the bloating stage of decomposition (Wang et al. 2021). Immature stages of this fly can be found during advanced decay carcasses and dry remains (Wang et al. 2016). *Ophyra spinigera* has been found in various types of carcasses from different environments in Malaysia (Adrus & Rahim 2018; Azwandi et al. 2013; Kumara et al. 2012).

In our study, the larvae of *Sarcophaga* sp. were the most dominant in both locations. Unlike blowflies, flesh flies reproduce through larviposition (Singh & Bharti 2008). This means, when deposited on decomposing tissue, blowflies start as eggs while flesh flies start as first instar larvae. This reproduction strategy is an advantage to *Sarcophaga* sp. which can give an earlier head start to *Sarcophaga* sp. larva to grow and enable it to dominate their food source. Besides that, rapid development and the size of the flesh fly larvae which is relatively larger than blowflies could be the second factors. For instance, a flesh fly species, *Sarcophaga caerulescens* was reported to have a rapid development and larger size (Cherix et al. 2012). These characteristics are making them favourable to be collected in forensic cases.

The trap set in the student residential area was found to have a higher number of flies caught compared to the trap set at the Faculty of Pharmacy buildings. The two traps were set at places with different environments and availability of food. The trap set up at the student residential area was close to several dumpsters and the cafeteria which provides food sources and breeding sites for the flies. This location is also adjacent to a forested area which may lead to a variety of species being captured. Variability of species composition according to different habitats was reported in previous studies (Klong-Klaew et al. 2018; Silahuddin et al. 2015). Besides that, light intensity is one of the environmental abiotic factors that can affect the activity of flies. At a low light intensity, *Ch. megacephala*, was reported to prefer to walk towards the carrion compared to flying (Smith et al. 2016). As our trap was hanged, with a low light intensity that less than 100 lux at Faculty of Pharmacy buildings, a lower number of blowflies were caught.

Identification of bacteria on the external surfaces and internal microbiome confirmed the presence of both Gram-positive and Gram-negative bacteria. From the external surface of the blowfly, Gram-negative cocci and Gram-negative bacilli were isolated. However, for the internal microbiome, Gram-negative bacilli and Gram-positive bacilli were isolated. A study by Sukontason et. al (2007) reported that up to eight bacterial species were isolated from a single fly by using the conventional method. The caught *Ch. megacephala* from the same study yielded a total of 39 bacterium species, with coagulase-negative staphylococci and non-fermentative Gram-bacilli being the most prevalent. (Sukontason et al. 2007). A different study by Chaiwong et al. (2014) found that coagulase-negative staphylococci and *Streptococcus* group D non-enterococci were the most common bacteria isolated from *Ch. megacephala*. Human pathogens such as *Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus* and *E. coli* O157:H7 (EHEC), *Staphylococcus aureus*, and *Shigella* sp. were also present (Chaiwong et al. 2014).

In the study, different species of flies were caught at different stages of tissue decomposition. The dominant species, *Ch. megacephala* were caught at different days of bait decomposition while *Sarcophaga* sp. was caught on the first and fifth sampling day. Besides, *A. orientalis* was only caught on the first day of sampling while *O. spinigera* was caught on the

fifth day. It was proven that decomposition stages play an important role in the timing of fly colonisation (Azwandi & Ahmad 2009). The volatile odour compounds produced by bacteria from breaking down essential amino are responsible for the attraction of necrophagous insects to decomposing tissue (Verheggen et al. 2017). These compounds are capable to attract gravid female blowflies and stimulating oviposition (Chaudhury et al. 2010). Volatiles from different bacteria species has been found to have a different effect on the attraction of gravid blowflies and stimulating oviposition. A study on the attraction of eight species of Enterobacteriaceae towards gravid blowfly, *Cochliomyia hominivorax* found that *Klebsiella oxytoca, Proteus mirabilis, Proteus vulgaris, Providencia rettgeri,* and *Providencia stuartii* are more attractive than *Enterobacter cloacae, Enterobacter sakazakii,* and *Serratia liquefaciens* (Chaudhury et al. 2010). A different study on the attraction of *K. oxytoca, P. mirabilis, P. vulgaris, P. rettgeri,* and *P. stuartii* attraction towards gravid *Cochliomyia macellaria* found substrates with *P. mirabilis* is the most attractive (Chaudhury et al. 2016). The substrates with *K. oxytoca* are the least attractive. According to the same study, *C. macellaria* and *C. hominivorax* use similar chemical cues for attraction and oviposition. However, both studies recorded volatiles from mixed species of bacteria to have a greater effect on attraction and oviposition.

Our seven days sampling for adult flies using fish bait shows the dissimilarity of flies' arrival. In this study, most adult flies were trapped during the early stages of bait decomposition. Subsequently, the number of adult flies caught per sampling day reduced gradually towards the end of the sampling period. Duration of bait incubation can affect the attraction of gravid flies to attend it for oviposition. Chaudbury et al. (2010) reported volatiles from substrates incubated for 48 hours and 72 hours showed a higher effect on attraction and stimulation of oviposition of female gravid blowflies when compared to substrates incubated for 24 hours and 96 hours. However, it was reported that substrates were incubated for 72 hours to have a better attraction towards gravid female blowflies than substrates incubated for 24 hours, 48 hours, and 96 hours (Chaudhury et al. 2016). High responses towards volatile odour compounds were at sex and ovarian status (Tomberlin et al. 2017). However, volatile odour compounds can be used as signalling for bacteria-blowfly symbiosis (Liu et al. 2016). *P. mirabilis* is responsible for the production of phenylacetic acid. This compound only attracts gravid female *Lucilia sericata* as the presence of this compound is an indication of beneficial bacteria for larvae development. The unresponsiveness of male blowflies towards phenylacetic acid could be due to the absence of a possible mate (Tomberlin et al. 2017). The presence of volatile odour compounds can affect the presence of necrophagous flies on decomposing bait.

The observation from this study recorded changes in the microbial community on the bait. Fresh bait has a higher composition of lactose fermenter Gram-negative bacilli. However, as the bait ages, the presence of non-lactose fermenter Gram-negative bacilli is more prominent on the surface of the bait. Investigation on Columbia agar with 5% Sheep Blood concluded a succession of Gram-positive bacilli. The haemolytic bacteria colonies from a different age of bait shared the same microscopic features but the morphology of the colony shows the stark difference between the colonies present. A study on human cadavers shows that microbial succession occurs in decomposing bodies (Vass 2001). The study deduce bacteria community from early decomposition is succeeded by anaerobic bacteria and the microbiome can be affected by bacteria from the environment and necrophagous insects. Additionally, studies by Pechal et al. (2013) and Pechal et al. (2014) found that significant changes occur in the bacteria community on decomposing bodies throughout the decomposition process. Furthermore, it has been discovered that necrophagous flies have a significant effect on the bacterial community formation during the decomposition process (Pechal et al. 2013).

Flies were shown to be dependent on bacteria and their metabolic products for growth and development during the immature stages (Zurek et al. 2000). Larvae rely on bacteria to break down corpses into nutrients. The presence of bacteria in diet has been found to have a positive impact on larval growth and a mixed bacterial diet is favourable for larval development. However, the effect of bacteria on growth may vary depending on the species of blowfly (Crooks et al. 2016) and species of bacteria on the substrate (Ahmad et al. 2006; Crooks et al. 2016). Other than positive effects on larval growth, some species of bacteria may present a detrimental effect on larval growth (Ahmad et al. 2006). In the study, we found that bacteria isolated from the external surface of larvae have similar morphology to the bacteria isolated from the surface of fish bait. The succession and similarity of bacteria on the decomposing bait and external surface of the larvae may be influenced by the larval Native Excretions/Secretions (NES).

Even though our study failed to detect any antimicrobial activities against bacteria of interest, there are numerous pieces of evidence of larvae NES ability to suppress bacteria activity. Several antimicrobial peptides such as lucifensin, lucimycin, attacins, cecropins, diptericins, proline-rich peptides, and sarcotoxins have been identified in inoculated *Lucilia sericata* larvae (Pöppel et al. 2015). Chymotrypsin found in *L. sericata* larvae has been found able to disrupt the biofilm of both *Staphylococcus aureus* and *Staphylococcus epidermidis* (Harris et al. 2013). Furthermore, recent molecular and biochemical studies on third-instar larvae of *Ch. megacephala* revealed that trypsin and chymotrypsin in their NES products have antibacterial activity against a range of bacteria (Mohamed 2015). A study conducted on *Calliphora vicina* larvae shows that their NES has high antimicrobial activity against Gram-negative bacteria such as *Klebsiella pneumoniae* and *E. coli*, moderate action against *Candida albicans*, and short-lived action against Gram-positive bacteria such as *Staphylococcus aureus* (Dallavecchia et al. 2021). Larva of Sarcophagidae is also capable to produce antimicrobial NES as a study deduced *Sarcophaga peregrina*, synthesizes multiple antimicrobial peptides with different microbial specificity in which it is primarily active against Gram-negative bacteria but shows moderate activity towards Gram-positive bacteria (Natori 2010).

CONCLUSION

The study outcomes possess some limitations. Firstly, no bacterial taxa were discovered to the highest taxonomic rank in the study. This can be achieved by metagenomic analysis through the 16S ribosomal RNA sequencing method. This method was previously performed on the microbiome of *Ch. megacephala* and 316 bacteria taxa were identified by Junqueira and team in 2017. Secondly, the small sampling effort conducted in this preliminary study resulted in a small sample collection. The current sample size is insufficient to conclude the fly-bacteria association with decomposing tissue. Hence, a larger sampling effort is recommended for subsequent studies. Thirdly, the mass of larvae used in the study is less than 10% of larvae mass from the adapted method. A low concentration of antimicrobial peptides in NES may be insufficient to detect any antimicrobial activity. Therefore, an increased sample is suggested for future research.

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