CHARACTERIZATION OF ENTERIC BACTERIA FROM HOUSE FLIES IN HEALTH CAMPUS, USM

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by

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CERTIFICATE

This is to certify that the thesis entitle “Characterization of Enteric Bacteria from House Flies in Health Campus, USM” is fide record of research work done by Ms Sitti Nanda binti Zainal during the period from February 2018 to December 2018 under my supervision.

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other masters at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the thesis for teaching, research and promotional purposes.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	degree Celcius
BPW	Buffered Peptone Water
CFU/ml	Colony-forming units per milliliter
FBD	Food Borne Diseases
FDA	Food and Drug Administration
g	gram
MAC	MacConkey agar
MOH	Ministry of Health
MRVP	Methyl red-Vogues Proskauer
NA	Nutrient agar
pH	Potential of hydrogen
PPSK	Pusat Pengajian Sains Kesihatan (School of Health Sciences)
SSA	Salmonella-Shigella agar
SIM	Sulphide-Indole-Motility
TCBS	Thiosulphate-citrate-bile salt-sucrose agar
TNTC	too numerous to count
TSI	Triple Sugar Iron
USM	Universiti Sains Malaysia
WHO	World Health Organization

PENCIRIAN BAKTERIA ENTERIK DARIPADA LALAT RUMAH DI KAMPUS KESIHATAN, USM

ABSTRAK

Musca domestica, atau dikenali sebagai lalat rumah merupakan serangga sinantrofik yang memainkan peranan penting sebagai vektor mekanikal dalam penyebaran penyakit. Lalat rumah boleh menyebabkan pencemaran makanan berlaku apabila mereka mendarat pada makanan, air dan perkakasan. Lalat rumah telah dikaitkan dengan pelbagai patogen, termasuklah yang boleh menyebabkan penyakit bawaan makanan seperti kolera, salmonelosis dan shigelosis. Maka, kajian ini dijalankan untuk memencil dan mengenalpasti bakteria enterik daripada lalat rumah yang dikumpul di Kampus Kesihatan USM. Pemencilan dan pengenalpastian bakteria enterik dilakukan melalui kaedah konvensional. Terdapat 16 pencilan yang berjaya dikenalpasti daripada enam lokasi terpilih iaitu tiga kafe; Harmoni, Murni dan PPSK, kolam kumbahan, tempat pembuangan sampah dan rumah haiwan. Bakteria daripada genus *Escherichia, Enterobacter, Klebsiella, Proteus* dan *Serratia* telah dikenalpasti. Bakteria *E. coli* dan *Proteus* spp. menunjukkan bilangan pencilan paling banyak iaitu masing-masing dengan n=5, diikuti oleh *Enterobacter* spp., n=3, *Klebsiella* spp., n=2 dan *Serratia* sp., n=1. Kajian ini menunjukkan bahawa lalat rumah boleh mengangkut bakteria enterik bersifat patogen dan menyebarkannya ke habitat manusia serta terdapat kemungkinan untuk menyebabkan berlakunya penyakit bawaan makanan.

CHARACTERIZATION OF ENTERIC BACTERIA FROM HOUSE FLIES IN HEALTH CAMPUS, USM

ABSTRACT

Musca domestica, or known as house flies are synantrophic insect that play a role in being a mechanical vector in transmitting diseases. They are able to cause food contamination easily by landing on food, water or utensils. House flies have been associated with various pathogens, especially those that can cause food borne diseases such as cholerae, salmonellosis and shigellosis. Hence, this study was conducted to isolate and identify enteric bacteria from house flies collected from various locations in Health Campus, USM. Isolation and identification of bacteria was performed using conventional culture and identification methods. Total of 16 isolates as identified from six locations in the vicinity of Health Campus, USM which were three of the cafes; Harmoni, Murni and PPSK, sewage pond, garbage dumping site and animal house. Bacteria from genera *Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus* and *Serratia* were identified. Both *E. coli* and *Proteus* spp. exhibited the highest number of isolates, n=5, respectively, followed by *Enterobacter* spp., n=3, *Klebsiella* spp., n=2 and *Serratia* sp., n=1. This study has confirmed the ability of house flies in carrying pathogenic enteric bacteria and may spread them into human habitations which have the possibility to cause food borne illnesses.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Food-borne disease (FBD) is one of the public health concerns around the world. Most cases of FBD with high prevalence occur in under-developed countries due to health and economical loss (Parvez et al., 2016). The contributing factors to FBD are contamination of food with microorganisms, especially pathogenic microbes and intoxication with biological or chemical components ingested with the food (Parvez et al., 2016). Meanwhile, food contamination can occur at any stages, which includes food processing, preparation and delivery. The sources of contamination may come from environmental factor such as air and water which can deliver microbes. However, most of the time food contamination is caused by poor hygiene behavior of food handlers and lack of sanitation of the surroundings.

Since pests such as house flies are usually considered as an indicator of unhygienic and poor sanitation environment, its presence also contributed to food contamination. This is because *Musca domestica*, or also known as house fly is a synantrophic insect and can be easily found in human surroundings, thus, it can easily contaminate food, water or utensils. In addition, the behavioral characteristics of the insect ensure its contact with microorganisms from food,

garbage, excreta and other sources of filth on their legs, mouthparts and other body parts, and then transferred to food when they landed on it (Nazni et al. 2005).

House flies are an important mechanical vector for numbers of pathogenic microorganisms. This insect has been recognized by the US Food and Drug Administration (FDA) as a major agent in spreading diseases such as cholera, shigellosis, and salmonellosis (Nazari et al., 2017). Most bacteria associated with house flies include food-borne pathogenic bacteria such as *Escherichia coli*, *Vibrio cholerae, Salmonella* spp., *Shigella* spp., and others (Barreiro et al. 2013). The house fly also acts as a carrier for *Campylobacter jejuni*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus fecalis* (Bahrndoff et al., 2017).

1.2 RATIONALE OF STUDY

House flies as the mechanical vector in bacterial transmission and causing FBD is often overlooked by people even though previous studies has shown there are few possibilities of how the microbes can be transmitted from the insect, such as Kelly et al. (1994) showed that fecal-oral route of transmission is feasible since viable bacteria was isolated from feces. Another study by Nazni et al. (2005) in Malaysia has shown that the feces and vomitus carried by the house flies contain bacteria such as *Bacillus* sp. and *Staphylococcus* sp. It also reported that the external body parts of house flies can come into contact with viable microorganisms and carries them around.

Additionally, the development of antibiotic resistance among clinical bacterial isolates and commensal bacteria of people and animals, as well as bacteria in other habitats, raises a concern that house flies can potentially be a competent vector not only for specific pathogens such as enteric bacteria, but also for nonpathogenic bacteria carrying antibiotic resistance genes (Barreiro et al., 2013). Plus, there are only few studies on the identification of microorganism from the common flies has been conducted in Malaysia (Nazni et al., 2005; Tan et al., 1997). This study was conducted in Kelantan since it is endemic to cholera, shigellosis and salmonellosis, besides having one of the highest number of cases of FBD in the country (Ang et al., 2010; Baddam et al., 2012). The level of hygiene in various sectors of the society and in institutional of higher studies are not satisfactory at all. Hence, the bacteriological characterization of the house flies in Health Campus, USM is performed.

1.3 OBJECTIVES OF STUDY

1.3.1 General objective

To isolate and identify bacteria from house flies sampled from various locations in Health Campus, Universiti Sains Malaysia, USM.

1.3.2 Specific objectives

1. To determine the bacterial load carried by house flies.
2. To isolate enteric bacteria from house flies using conventional culture methods.
3. To identify the isolated enteric bacteria using conventional identification methods.

CHAPTER 2

LITERATURE REVIEW

2.1 HOUSE FLY

Musca domestica, or also known as the common house fly, belongs to the order Diptera and family Muscidae (Nazari et al., 2017). There are about 70 species of flies in the genus *Musca* but *M. domestica* is the most common species worldwide (Service, 2012). It is categorized as synantrophic flies which are flies adapted to live in close association with human habitations and are capable of transmitting microorganisms either mechanically or biologically through this close interaction (Chaiwong et al., 2012). The fly species in the family Muscidae such as house fly has evolved to live and adapt in close association with human habitations changes and development. The ability of house flies to act as the mechanical vector in transmitting microorganisms into human habitat is contributed by of their morphology and biology, behavioral characteristics and habitat.

2.1.1 Morphology and biology

According to the Medical Entomology for Students (2012), house flies are non-metallic flies with the length of about 6–9 mm long and color variation from light to dark grey with some darker markings. They have four black longitudinal stripes, or specifically four longitudinal black vittae on the dorsal surface of the thorax, also known as the mesonotum. Each fly has antenna with prominent hair known as arista. This insect has a proboscis, or the mouthpart which are adapted for feeding. There are three pairs of legs

on a *M. domestica*, which the end of them are equipped with paired claws and a pair of pulvilli, a fleshy pad-like structures supplied with grandular hairs. These external morphologies of house flies become the factor that allow them to become the mechanical vector in transmitting microorganism.

The life cycle of a house fly consist of four distinct stages; egg, larva, pupa and adult (Figure 2.1). Temperature plays a big role in the development of the eggs into adult house flies, in which it usually takes from six to 42 days; the cooler the condition, the longer the development takes place (World Health Organization, WHO 1991). A female *M. domestica* lays around 75 to 100 eggs on any breeding sites, with the length of 1 to 1.2 mm banana-shaped appearance and creamy-white-in-color. The eggs can hatch in between 10 to 16 hours under optimum condition and it takes longer time if it is in cooler weather. However, the eggs are unable to withstand desiccation and may die if being exposed to extreme temperatures, such as below 15°C and above 40°C (Service, 2012). After the eggs hatch, it is followed by the larvae stage, also known as maggots. The development of maggots passes through three instars and usually can be completed from three to five days. It may take longer period of time depending on the temperature and availability of food. Pupation stage occurs when the larvae migrates to a sufficiently dry place that offers protection and hide into it, for example, into soil. The puparium, a capsule-like case is formed and the transformation from larva to adult house fly takes place usually from two to ten days and under cold environment it may last up to 14 days. The body of adult house fly that emerges from the puparium dries and harden before it can fly. It takes a few days before the adult house flies are capable to mate and reproduce, thus the life cycle starts again. An adult female house fly can lay eggs for a

few times in a lifetime but as mentioned by WHO (1991), under natural conditions they can rarely lay eggs more than five times.

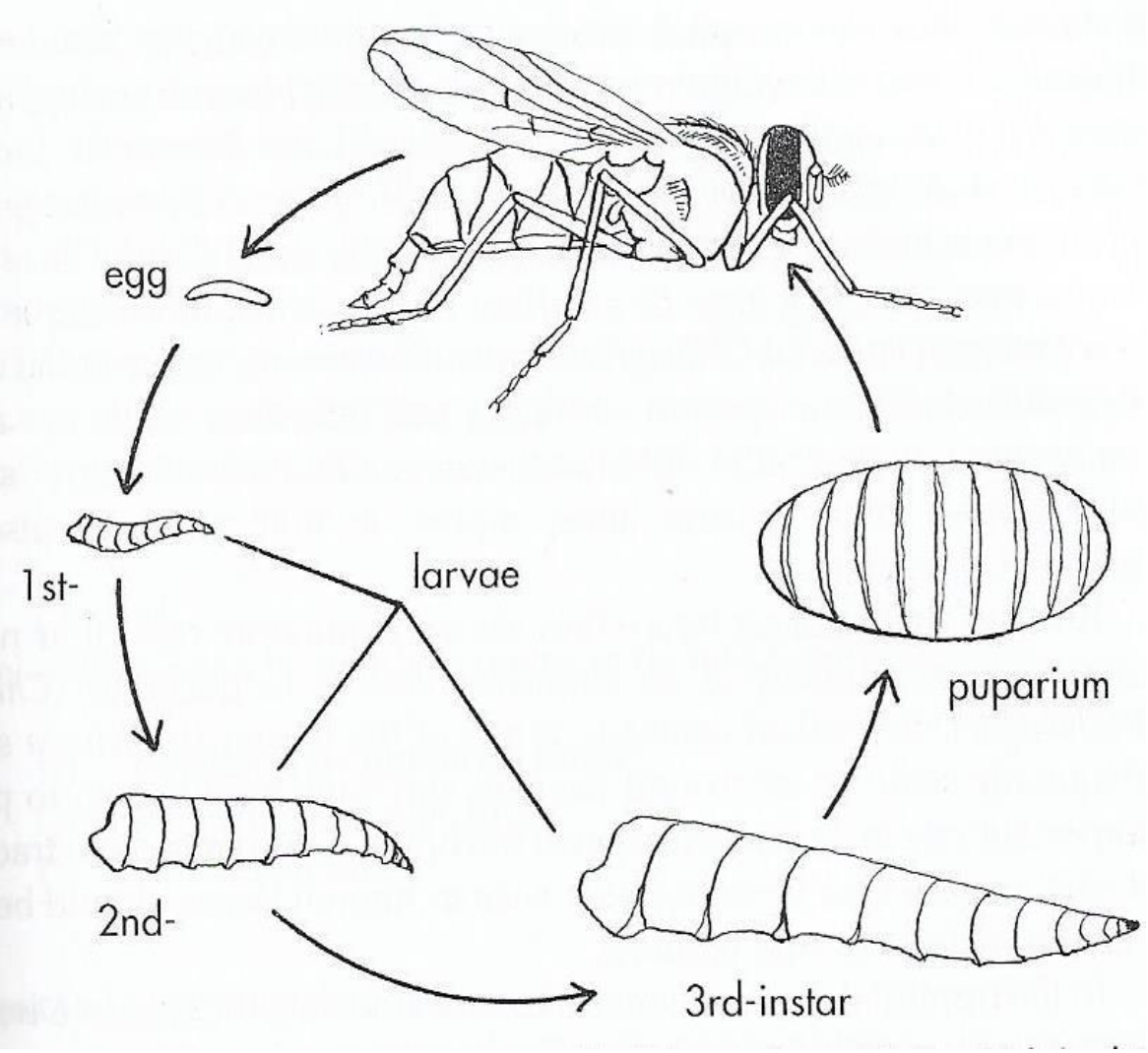

Figure 2.1 Life cycle of house fly, *Musca domestica* (Source: Service, 2007)

2.1.2 Behavioral characteristics and habitat

House flies feed on all kinds of substances including food, garbage and excreta from humans and animals – almost any organic material. It has been reported that their feeding sites includes horse manure, cow manure, human excreta, fermenting vegetables and fruits, garbage and kitchen wastes and commonly exposed human foods (Ahmadu et al., 2016). The proboscis is the most important part for feeding as it is specially adapted for sucking up fluid and semifluid foods (Service, 2007). The structure of proboscis consists of a pair of oval-shaped labella, with very fine channels named pseudotracheae. The fluids are sucked up through those channels. When a house fly feeds, the proboscis will be extended downwards towards the food source and when it is not in use, the proboscis is partially withdrawn into the head capsule.

The flies have different approaches of feeding which are affected by the physical state of the food (Service, 2007). When the flies feed on fluids such as milk, the labella are placed directly in contact with the food and it will be sucked up through small openings in the pseudotracheae. If the feedings are sourced from semisolid state such as vomit or animal dung, the labella are completely everted and the food is sucked up directly into the food channel. As for foods in solid state, the flies have to wet it with their saliva or perform regurgitation in the crop before ingestion occurs. The acts of regurgitation and moistening the food are very conducive in the spreading of variety of pathogens. Since house flies have the same feeding sites and breeding sites, another factor that helps with the widespread of pathogens is when the flies landed on these sites, the microorganisms are able to attach to the pulvilli on the flies' legs. The sticky hairs enable the house flies to adhere onto any surfaces, including smooth surface such

as glass and plastics. Not to mention, the exterior surfaces of the house flies are exposed to the surroundings, making them having the potential to carry the pathogens. A study in 2007 by Yap et al. has shown that the wings of the house flies have the capability to carry *V. cholerae* in droplets. However, they have concluded that the wings did not play a significant role in mechanical transmission of non-adhering liquid medium because of the low transfer rate of the bacteria to the wings and poor retention of bacteria on the wings during normal house fly activities. Still, the exposure of the exterior body parts of the flies towards sources of microorganisms in the surroundings helps in making them a very important mechanical vector to introduce pathogens into human habitat.

In addition, the house flies distribution is greatly influenced by several factors (WHO, 1991). This comprises by their reactions to light, temperature, humidity, surface color and texture. During the day, they are mainly gathered near their feeding and breeding sites, where mating and resting also take place. While during the night, they are normally inactive so most of the time they are at their resting sites like ceilings. House flies favor the temperature from 20°C to 25ºC, which is also the temperature of when they reach the highest density of distribution in the environment. As mentioned by WHO (1991), the preferred temperature for resting is between 35°C to 40°C. If the temperature gets lower than 10°C or higher than 40°C, the flies distribution can be undetectable. All of these details about the house flies may give an insight towards their role as carriers of diseases and why they become a public health importance.

2.1.3 Public health importance

House flies can be an important nuisance towards people, especially when a large number of them are involved. Plus, their presence is considered as a sign of unhygienic conditions (WHO, 1991). According to Nayduch and Burrus (2016), house flies serve as bridges between clean and unclean environments, moving freely between contaminated materials such as waste to domestic and peridomestic environments, food and water sources. Their synantrophic nature strengthened their potential as mechanical vector in transmitting diseases to humans. Other than the two ways they are able to transmit the microorganisms; through their exterior body surface and feeding method, there is another one possible route of transmission, which is by defecation. According to Sasaki et al. (2000), house flies defecate while feeding or resting, leaving specks and organisms passing through their digestive system. This is a simple mechanical transfer of microbes by a house fly that act as the vector, whose behavior places the contaminants from filth sources onto new food or host source they visit (Holt et al., 2007)

Few studies in the past had shown the microorganisms associated with house flies and the diseases they cause. According to Sukontason et al. (2000), the house flies are able to carry microbes that can cause eye infection, such as trachoma, caused by *Chlamydia trachomatis*. It was also considered as a potential carrier of bird flu virus, which can cause harm towards humans' health and livestocks industry (Nazari et al., 2017). House flies can also transmit polio virus, which causes poliomyelitis and leads to paralysis (WHO, 2017). In 1997, Grubel et al. had stated that house flies probably can act as vectors in the transmission of *Helicobacter pylori* if they carry the bacterium and contaminate human food. The house flies were also reported to act as carrier for

Campylobacter jejuni, Staphylococcus aureus, Pseudomonas aeruginosa and *Enterococcus fecalis* (Bahrndoff et al., 2017). Most importantly, this insect has long been associated with foodborne pathogens such as *E. coli, V. cholerae, Salmonella* spp., *Shigella* spp. and others (Barreiro et al., 2013) that may cause foodborne diseases such as diarrhea, cholera, typhoid, shigellosis and other food poisoning conditions, respectively. According to the study conducted by Chavasse in 1999, the use of effective measures for controlling the population of house flies would reduce the prevalence of gastrointestinal symptoms like diarrhea.

2.2 FOOD-BORNE DISEASES

Foodborne diseases (FBD) are responsible for a large burden of illnesses (morbidity) and death (mortality) in both resource-rich and resource-poor countries (Kirk et al., 2015). Globally, an estimated 2 million people died from diarrheal diseases in 2005; approximately 70% of diarrheal diseases are foodborne. Diarrheal diseases alone, a considerable proportion of which is foodborne, kills 1.8 million children every year worldwide (WHO, 2007). There are more than 200 diseases that can be transmitted to people from ingesting contaminated food biologically (with microorganisms) or the ones contaminated with chemicals. Food contamination can occur at any stages of food production and the source of contamination can arise from water, soil and air pollution, or through lack of good food-handling practices such as unwashed hands before handling food or usage of dirty utensils, plus low level of sanitation in the surroundings.

According to WHO (2011), the population in developing countries is more prone to suffer from foodborne illnesses because of multiple reasons, including lack of access

to clean water for food preparation; inappropriate transportation and storage of foods; and lack of awareness regarding safe and hygienic food practices. According to Kirk et al. (2015) again, FBD usually presented with gastrointestinal symptoms such as stomach cramps, diarrhea and vomiting. These are the most common symptoms for any FBD cases. However, some FBD can have symptoms that affect other parts of the body or causes serious sequelae. For example, certain infection with other *E. coli* strains can lead to kidney failure (Nordstrom et al., 2013). Generally, symptoms of FBD are self-limiting or mild. However, severe cases can occur towards the high risk groups. These include infants, young children, the elderly and immunocompromised people (Fleury et al., 2008). Even though FBD are prevalent (Hoffman et al., 2005), but the magnitude of illness and associated deaths are not accurately reflected by the data available in both developed and developing countries (Jahan, 2012). It can be considered as an unknown burden, especially towards the specific causative agents of FBD cases that occurred. This is mainly because the person involved does not seek medical attention whenever they came up with FBD, leading to lack of laboratory-tested results that may be used to fill the gaps of FBD prevalence data (WHO 2011).

Recognizing that contaminated food is an important cause of human disease, estimates of disease burden of the various FBD has been sought to enable advocacy for improved food safety and to assist governments to prioritize efforts for enhancing food safety (Kirk et al., 2015). Many different diseases, including those due to bacteria, viruses, parasites, chemicals, and prions, may be transmitted to humans by contaminated food (Scallan et al., 2011). Thus, some of these factors that cause food contamination

can be related to house flies as the mechanical vector in transmitting pathogens, causing cross-contamination between the filth to the food sources.

2.2.1 Food-borne diseases in Malaysia

In Malaysia, the reported food and waterborne diseases in 2009, such as cholera, dysentery, typhoid and Hepatitis A were low, ranging from 0.14 to 1.07 cases per 100,000 people in a population (MOH, 2009). In contrast, food poisoning cases is on the rise as evident by the incidence rate of 62.47 cases per 100,000 population in 2008 and 36.17 in 2009 according to the Ministry of Health (MOH 2009, 2010). In 2007, MOH has stated that there are five food and waterborne diseases (FWBD) that must be reported and notified under the Prevention and Control of Infectious Diseases Act 1988 (Act 342). These are cholera, typhoid/paratyphoid fevers, viral hepatitis A, food poisoning and dysentery. FBD outbreaks are believed to be lead by locations of foods consumed in institutions and other food services which were demonstrated by Olsen et al. (2000). Most of the implicated food settings occurred in schools' and academic institutions' food preparation premises and inappropriate food handling practices, meals prepared too early and kept at ambient temperature until served and unhygienic practices were the causes of food poisoning cases (Soon et al., 2011).

In Malaysia, the main contributing factor was identified as insanitary food handling procedures and lack of cleanliness in food preparation establishments which accounted for more than 50% of the poisoning episodes (MOH 2007). It is hereby believed that there are association between the presence of house flies as the indicator of unsanitary environment, them being the mechanical vector of food borne pathogens and

transmission of the microbes into human habitations. Hence, this study is conducted to have preliminary observation of the variation of microorganisms carried by the house flies in the vicinity of USM's Health Campus, an academic institution in the state of Kelantan. In addition, Kelantan always has higher incidence of typhoid compared to other states in Malaysia (Baddam et al., 2012). There was also a cholera outbreak in this state from November to December 2009 which *V. cholerae* O1 was isolated (Ang et al., 2010). Thus, this study was performed to see whether these bacteria can be isolated from the house flies collected, which may in fact strengthened the hypothesis of having this insect associated with being the mechanical vector in transmitting food borne pathogen.

2.3 ENTERIC BACTERIA

Enterobacteriaceae is a family of bacteria that consist of around 53 genera with hundreds of species. They are a group of facultatively anaerobic Gram negative rod bacteria and they are distributed worldwide, which can also be found in soil and plants. Also, their natural habitat is in the intestinal tract of human and animals (Kayser, 2005). However, some of the bacteria in this family are facultative pathogenic. The nomenclature of the Enterobacteriaceae is complicated and has been based on biochemical and antigenic characteristics. The application of new technologies such as DNA hybridization has resulted in numerous changes in classification of the Enterobacteriaceae (Hong et al., 2007). Many new genera and species have been discovered, some unusual and rare, and many species have also been reclassified to other genera. Characteristics of Enterobacteriaceae also included having peritrichous flagella except bacteria from genus *Tatumella*, *Shigella* and *Klebsiella* which are non-motile. Usually bacteria from this family are divided by their ability of fermenting lactose.

There are a few of genera in Enterobacteriaceae that are considered medically important according to the UK Standards for Microbiology Investigations (2015). First, is from genus *Citrobacter*. Ten out of eleven species bacteria from this genus has been isolated from clinical material (Euzeby, 2013). They may be found in the faeces of humans and animals as part of the normal flora. Second is bacteria from genus *Enterobacter*. They have the general characteristics of *Klebsiella* species but can be differentiated because they are motile and ornithine positive. *Enterobacter* species are widely distributed in nature. They are found in the soil, water, dairy products, and in the intestines of animals as well as humans. Only 10 out of 26 species in this genus has been isolated from clinical materials (Euzeby, 2013). Third, bacteria from genus *Klebsiella* which there are four species related to humans and they include *K. pneumoniae* subspecies *pneumoniae, ozaenae*, and *rhinoscleromatis*; *K. oxytoca*; *K. granulomatis* and *K. variicola*. Bacteria from this genus are known to cause bacteremia and hepatic infections. They also have been isolated from a number of unusual infections, including endocarditis, peritonitis, acute cholecystitis, crepitant myonecrosis, pyomyositis, necrotising fasciitis, psoas muscle abscess, fascial space infections of the head and neck, and septic arthritis (Janda & Abbott, 2006).

Then, there is a genus well-known in this family, which is *Proteus* spp. *Proteus* includes pathogens responsible for many human urinary tract infections (Guentzel, 1996). *P. mirabilis* is often found as a free-living organism in soil and water. It can cause wound and urinary tract infections. Once attached to the urinary tract, *P. mirabilis* can affect and infect kidney more commonly than *E. coli*. The other species well-known under this genus is *P. vulgaris*. It occurs naturally in the intestines of humans and a wide

variety of animals, and in manure, soil, and polluted waters. However, this organism is isolated less often in the laboratory and usually only targets immunosuppressed individuals. Next are the most commonly known bacteria from this family, which are *Escherichia, Salmonella* and *Shigella*. The genus *Shigella* consists of four species; *Shigella dysenteriae, Shigella flexneri, Shigella boydii,* and *Shigella sonnei* (Euzeby, 2013). They can cause shigellosis, and dysentery which can be classified into different level of symptoms severity; fever, stomach cramp and diarrhea with blood (Kaper et al., 2004). Mostly, bacteria from Enterobacteriaceae can cause diarrheal symptoms in human, only with different level of severity according to the risk groups affected.

Together with the family of Vibrionaceae, the genus of *Vibrio* is focused in this study. This bacterium is a gram negative with comma shape and motile with a single polar flagellum, giving the 'darting' motion. 10 species of bacteria from this genus have cause gastrointestinal and extra-intestinal diseases in man; most importantly cholera, caused by *V. cholerae*.

CHAPTER 3

METHODOLOGY

3.1 MATERIALS

Lists of chemicals and reagents, consumables as well as laboratory apparatus used in this study are shown in Appendix A, B and C respectively. Preparation methods for media are shown in Appendix D and E while chemical and reagents used in this study has been prepared by the laboratory staff and also purchased ready-to-use packages or kits.

3.2 STUDY DESIGN

This is a cross-sectional study in which the house flies, *Musca domestica* from several locations within the vicinity of Health Campus, USM were collected. The study was done between the months of May to October 2018. Bacteria were isolated from the house flies using conventional culture method for heterotrophic plate counts as well as enteric bacteria isolation and identification. Figure 3.1 shows the flowchart of the study.

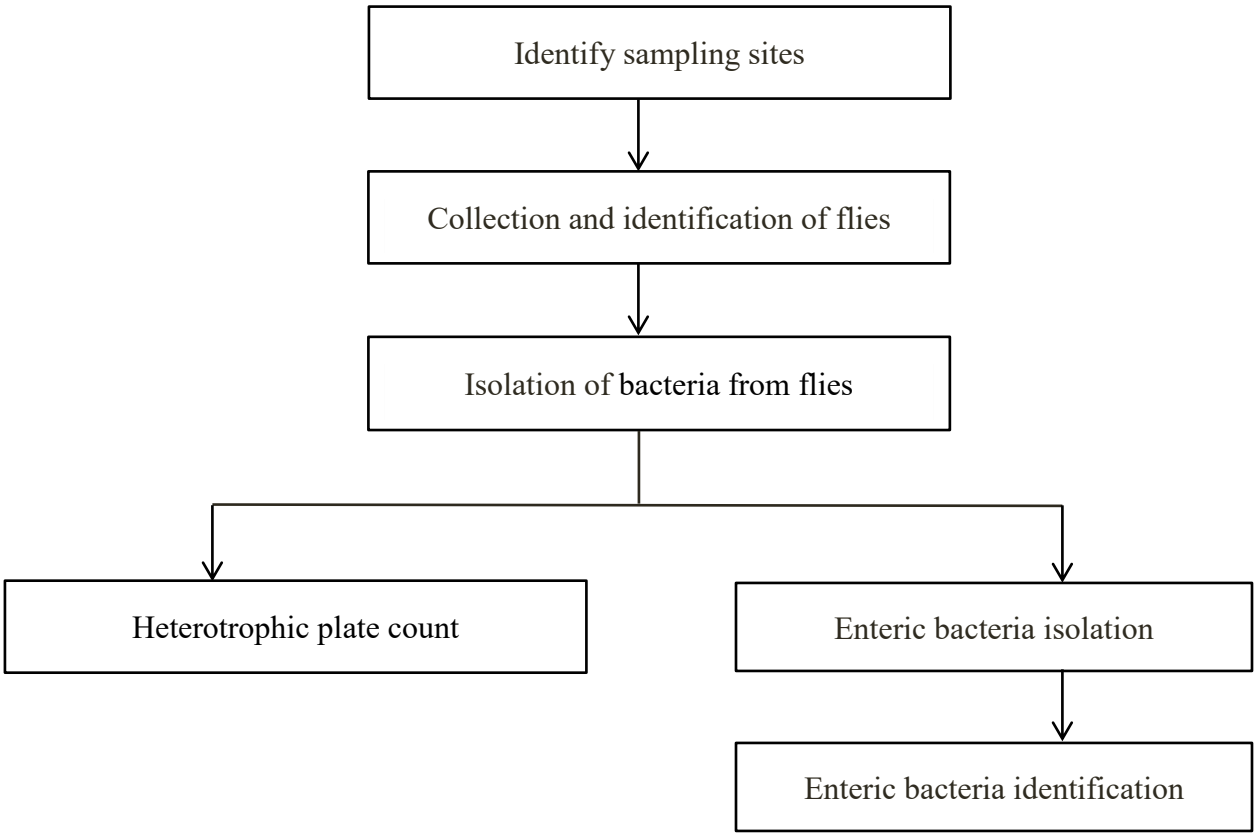

Figure 3.1 Flowchart of study

3.3 STUDY LOCATION

This study was conducted in Health Campus, USM. Sampling sites where house flies, *M. domestica* are commonly found were chosen for sampling. These include cafeteria in the School of Health Sciences, Harmoni cafeteria and canteen in Desasiswa Murni, garbage collection site, animal house and sewage pond.

3.4 COLLECTION AND IDENTIFICATION OF HOUSE FLIES

Flies were collected randomly using sterile insect net from cafeterias, canteen, garbage collection site, animal house and sewage pond. The flies were immediately transferred

from the net into the individual sterile containers from each collection site. The flies were then stored at 4°C overnight to immobilize them. Following immobilization, the flies were aseptically handled and identified according to Nazni *et al*. (2011). Flies other than *M. domestica* were not included for bacterial isolation in this study.

3.5 ISOLATION OF BACTERIA FROM *M. domestica*

About 1 g of *M. domestica* were added to 9 ml sterile buffered peptone water (BPW) and allowed to stand for 15 minutes before vigorously agitated thrice at interval of one minute to dislodge all bacteria from the flies. The flies' body parts were removed from the BPW and the solution was used in the determination of heterotrophic plate counts and isolation and identification of enteric bacteria.

3.6 DETERMINATION OF HETEROTROPHIC BACTERIAL COUNTS

Serial dilutions were performed in which 1 ml of BPW solution from Section 3.5 was diluted into a series of test tubes containing 9 ml of sterile BPW up to 1 x 10^{-6}. Aliquots of 0.1 ml from each tube were inoculated onto the surface of nutrient agar (NA) using spread plate technique for bacterial enumeration. The plates were incubated at 37°C for 24 hours. Discrete colonies that grew were counted to obtain the colony forming units (CFU/ml), and the total number of bacterial load from the flies can be estimated (Davari et al., 2010). After 24 hours incubation period, the plates are viewed with the bottom side up and the number of colony on the surface and embedded within the agar were counted. Ideally, only plates with the range of 30 to 300 colonies are used. Hence, any plates with number of colony of more than 300 is considered as "too numerous too

count" (TNTC). To calculate the concentration of viable cells in the sample, the following equation by Lammert (2007) was used:

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{number of colonies on plate}}{\text{dilution of sample} \times \text{plated volume}}$$

For example, suppose that 30 colonies were counted on the 10^{-5} plate, and when these numbers were used in the equation above it will be,

$$\frac{\text{CFU}}{\text{ml}} = \frac{30}{10^{-5} \times 0.1} = 30 \times 10^{6} = 3.0 \times 10^{7}$$

Thus, the viable cells load from the sample is estimated to be 3.0×10^{7} CFU/ml. Since the plate count method has at least 10% margin of error, only two significant numbers appear in the final value.

3.7 IDENTIFICATION OF BACTERIA

MacConkey agar (MAC) and Salmonella-Shigella agar (SSA) were primarily used for the detection and isolation of enteric bacteria. Thiosulphate citrate-bile salt sucrose agar (TCBS) was used to detect and isolate *Vibrio* spp. Dilutions from previous BPW solutions were plated onto each agar medium and the plates were incubated at 37°C for 24 hours. The bacterial colonies that emerged from the agar plates were differentiated based on the colony morphology. The presumed different colonies were sub-cultured onto new NA plates to obtain pure colonies. Then, the pure colonies were stored on nutrient agar slant.

The pure isolates were identified at least up to genus level by conventional identification method; microscopy and biochemical tests. First, Gram staining was

performed to determine bacterial morphology microscopically. Then, biochemical tests were performed which involve oxidase, catalase, triple-sugar iron (TSI), sulphide-indole-motility (SIM), indole, methyl-red and Voges-Proskauer (MRVP), citrate utilization and urease test. For bacteria that were suspected as *Vibrio* spp., hanging-drop method was performed to observe 'darting' movement, which is a distinct characteristic of *V. cholerae*. Identification of bacteria was performed by referring to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

3.7.1 Selective and/or differential media

As mention before, MAC, SSA and TCBS were used to primarily isolate bacteria as these three types of agar are considered as selective and/or differential media. For MAC (Oxoid UK), it was used to detect and differentiate among gram-negative enteric bacilli based on their ability to grow on the medium and to ferment lactose. Lactose was added in this media, making it differential to which bacteria that are able to ferment lactose and produce acids will have pink to red colonies because of color change in neutral red, a pH indicator in the medium. For non-lactose fermenting bacteria, they will produce a colorless colony. Gram positive bacteria are unable to grow on MAC since their growth are inhibited by the presence of crystal violet and bile salts, making MAC as a selective media. Few examples of lactose-fermenter (LF) Enterobacteriaceae are from genus *Escherichia, Klebsiella,* and *Citrobacter*, while for non-lactose fermenter (NLF) they came from genus *Salmonella, Shigella* and *Proteus*. For SSA (Oxoid UK), it was used to primarily detect the presence of *Salmonella* and *Shigella* from the house fly external wash, based on the formation of colorless colony with black center from sulphide production that may be the indicator of *Salmonella typhimurium* presence and colorless

colony without black center that is indicative of *Shigella sonnei* presence. SSA contains some selective inhibitory components such as bile salts, thiosulphate and citrate that are able to inhibit the growth of gram positive and coliform organisms. As for TCBS, it was used to primarily detect the presence of *Vibrio* spp., specifically *V. cholerae* and *V. parahaemolyticus*, based on their morphology on the agar plate, which are flat, circular, yellow colonies and blue to green centered colonies, respectively (Hardy Diagnostics USA). TCBS also able to inhibit the growth of gram positive bacteria and coliforms.

3.7.2 Biochemical tests

Before biochemical tests were performed, all the isolates were subcultured onto NA to obtain pure colonies and identified microscopically using Gram stain method in order to confirm the morphology of the bacteria which are gram negative rod for enteric bacteria and gram negative with "comma" shape or slightly curved rod for *Vibrio* spp. Then, the biochemical tests were performed based on the techniques in microbiology (Lammert, 2007) which include the followings:

3.7.2.1 Oxidase test

This test was performed to determine whether the bacteria have cytochrome oxidase, a participant in electron transport chain during respiration. When electrons are added to the oxidase reagent by cytochrome oxidase, a positive outcome will turns the reagent color from dark blue to purple within few seconds. All Enterobacteriaceae are oxidase-negative microorganism except for *Plesiomonas shigelloides*, which caused the reagent to turn colorless. While for *Vibrio* spp., it will give positive result.

3.7.2.2 Triple Sugar Iron

This test was performed to differentiate among the Enterobacteriaceae as to their ability to ferment glucose, lactose and sucrose, also their ability to produce H_2S. This media contain 1% of lactose and sucrose and 0.1% glucose. If any of these sugars are fermented by the bacteria, it will produce organic acids as waste products, causing drop in pH level. The acidic condition will turn the phenol red color that act as pH indicator into yellow, while alkaline end product will produce red color of medium. TSI are prepared in slant. The interpretations of this test are; entire tube (slant and butt) are yellow, glucose and lactose and/or sucrose are fermented, if red slant and yellow butt, only glucose are fermented, if entire tube is red then no sugar are fermented, indicating bacteria presence are not from Enterobacteriaceae. Bubbles produced in agar indicates byproduction of gas from fermentation and finally black precipitate formation indicates that H_2S was produced.

3.7.2.3 Sulphide-Indole-Motility

Through this test, three conditions of bacteria biochemical and morphology can be observed which are their ability to produce H_2S, their ability to split the amino acid tryptophan into indole and pyruvic acid since some bacteria able to use tryptophan as the energy source and producing indole as their byproduct, and their motility. *E.coli* is positive in indole and form a 'red-ring' when Kovac's reagent are added into the tube. While black precipitate is produced if the bacteria is positive in H_2S production, a byproduct from the ability of bacteria to reduce thiosulfate, such as *Proteus* spp. The motility of the bacteria can be observed through their growth along the stabbing line, in

which growth along the line means the bacteria is non-motile and if they grow spreaded from the stabbing line they are motile.

3.7.2.4 Methyl Red

This test was performed in order to determine the ability of bacteria to ferment glucose via mixed-acid fermentation. Methyl red is added to the medium as it act as the pH indicator. Whenever the pH of the medium is 4.5 and below, methyl red reagent will remain red in color, giving a positive result of acid formation from glucose mixed-acid fermentation.

3.7.2.5 Citrate utilization

This test was performed to determine whether a bacterium is able to utilize citrate as the sole source of carbon and energy. Bacteria that carry citrate permease have the ability to convert citrate into pyruvate and carbon dioxide. Bromothymol blue is used as pH indicator in this medium, which it changes color to blue, denoting a positive result from alkaline pH changes. For example, *E. coli* does not have the ability to utilize citrate. Thus, the green color of the medium remains the same after incubation over 48 hours.

3.7.2.6 Urease test

In this test, it was performed to determine the ability of bacteria to hydrolyze urea with the presence of enzyme urease that rapidly degrade urea into carbon dioxide and ammonia. Among Enterobacteriaceae, bacteria from genus *Proteus* (especially *P. mirabilis*) are a rapid hydrolyzer of urea. This is shown when a urease-producing bacterium hydrolyze urea in a medium, causing accumulation of ammonia that made the

medium more alkaline. This causes pH changes of phenol red that turns the color of medium from yellow to bright pink.