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RESEARCH ARTICLE OPEN ACCESS

Bacterial Species Associate on the Body Surface of *Musca domestica* L from Various Habitats based on 16S rRNA Sequencing

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Abstract

This study aims to identify bacteria isolated from the body surface of house flies from various habitats using 16S rRNA molecular barcodes. Houseflies were isolated from forests, hospitals, traditional markets, modern markets and landfills. 25 house flies isolated in each habitat. House flies were preserved in sterile bags. Bacterial isolation was carried out using nutrient agar media in 100 mm Petri dishes. The isolates obtained were pure cultured until a single isolate was obtained. Single isolates were extracted using Geneaid's Presto TM Mini gDNA Bacteria Kit. The extracted bacterial total DNA was used as a template for amplification using primer 16s rRNA gene by PCR method. Nucleotide sequencing uses Singapore's First BASE sequencing service. The results showed that single-house fly isolates from the Fish Auction (P.L.) showed a 99.11% similarity with *Sphingobacterium faecium* [CP094931.1]. Traditional market bacterial isolates (P.T.) showed 97% similarity with *Pseudochrobactrum* sp. XF203. Hospital bacterial isolates (R.S.) showed 99.11% similarity with *S. faecium* [CP094931.1]. Bacterial isolates from residential areas (PM) showed 99% similarity with *Brucella abortus* RB51-AHVLA. Bacterial isolates from the forest (H.T.) showed 94% similarity with *Bacillus paralicheniformis* [CP043501.1]. There are associated bacteria that are used as biotechnology agents. Exploration of bacteria and even microbes associated with *M. domestica* is still extensive to be studied in the future.

Keywords: *Musca Domestica* L, Bacteria, 16S rRNA, Body Surface

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INTRODUCTION

The housefly can transfer pathogens between different environments, acting as one of the most important vectors of human disease worldwide.[1,2] The housefly (*Musca domestica*) is a widespread, synanthropic dung fly commonly found in decaying matter, refuse, human excrement, and food. They are vectors of microbes, including clinically relevant pathogens.[3,4]

Many microbial pathogens like *Salmonella typhimurium, Enterococcus faecalis* and *Klebsiella Pneumoniae*[5] in humans are transmitted by houseflies, directly or indirectly, through human food. *M. domestica* L. (Diptera: Muscidae) is a hardy commensal organism that can survive in a variety of environments.[6] *M. domestica* is found in homes, food markets, and farms, where it roams around decaying materials, garbage, excrement, and human food.[7] Flies can be found in traditional markets, fish auctions, and hospitals in the tropics. Houseflies prefer warmer (optimally 30 °C) and drier conditions but can breed slower throughout the winter, usually in livestock pens. Houseflies are found on every continent except Antarctica.[8,9]

Houseflies are closely related to microbes from the larval stage, pupa to imago. Microbiota studies on houseflies found various species of bacteria, including *Aerococcus* spp., *Staphylococcus lentus, Psychrobacter* spp., *S. sciuri, Weissella* spp., *Dietzia maris, Micrococcus* spp.[10] Furthermore, lactococci, lactobacilli, and enterococci were also found. In contrast, *Weissella* and *Chishuiella* were found in the larval and adult stages.[1,11] It was reported that several bacterial species isolated from house flies were resistant to antibiotics, namely *Acinetobacter* spp., *Stenotrophomonas* spp., and *Alcaligenes* spp.[2,5] The metagenomic analysis results indicated that *Staphylococcus, Bacillus,* and *Enterococcus* were mainly present on houseflies' surfaces at the genus level.[12] A recent study found over 100 bacterial pathogens, fungi, parasites, and viruses on or in *M. domestica* larvae and adults, some of which are potentially antimicrobial resistant.[13,14] Previous research was conducted, based on identification using 16S rRNA, several species of bacteria were found in *M. domestica* L. and *Chrysomya megacephala* L, namely *Bifidobacterium, Alcaligenes faecalis, Brucella melitensis, Sphingobacterium* spp., *Gamma proteobacterium*, and *Enterobacter asburiae*.[12]

Research has been carried out to identify bacteria in various habitats isolated from the body surface of houseflies. Single pure isolate from various habitats. The housefly sample was collected in Manado, a coastal city in the northernmost part of Sulawesi Island. However, there are still very few reports of bacterial association research on *M. domestica* in the

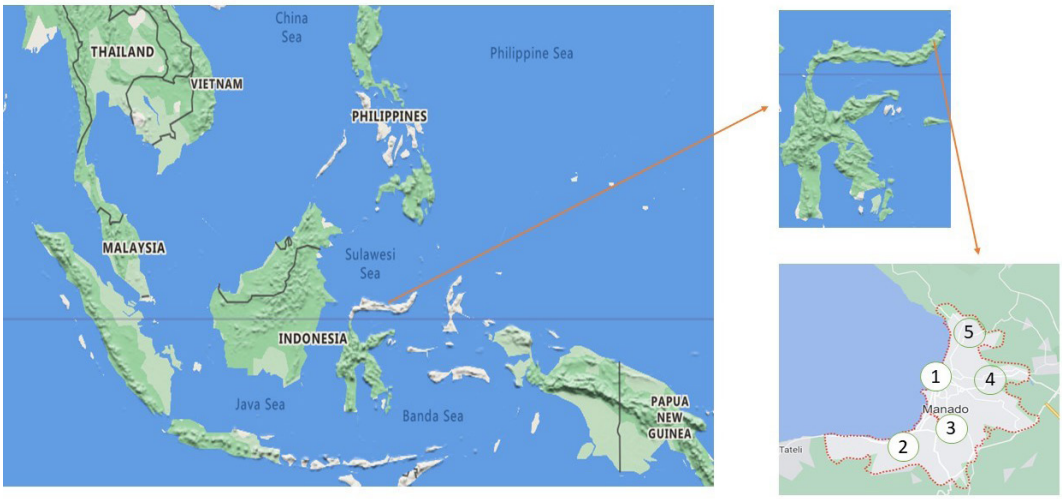

Figure 1. Housefly Sampling Locations. 1). Fish Auction, 2). Hospital, 3). Traditional Market, 4). Residential settlements, 5). Forest

tropics. Flies were isolated from fish auctions, traditional markets, modern markets, forests and hospitals. Mapping bacterial isolates associated with the abovementioned habitat is important to provide scientific data on bacteria associated with houseflies. These bacteria can potentially cause human diseases, such as typhoid fever, dysentery and diarrhoea.

MATERIALS AND METHODS

Collection of *M. domestica* L.

Houseflies were collected from several locations, namely hospitals, traditional markets, modern markets, and forests. All sample containers and nets were handled with sterile and vinyl-gloved hands (Figure 1). The collected flies were placed into 150 mL sterile containers and stored separately for each location at 25°C for 24 hours. About 25 flies per location were collected and brought to the laboratory. The house fly sample was confirmed as *M. domestica* in the laboratory using a determination method based on morphology with entomologists (Figure 2).

Bacterial Isolation

Flies were anaesthetized with filtered CO2 and transferred one by one with sterile tweezers to 100 mm Petri dishes containing nutrient agar media (Merck). Flies linger on the plates, moving over the surface while eating, walking and defecating. After 1 or 16 hours at room temperature, the flies were removed, and the dishes were incubated at 37°C overnight (~16 hours). Bacterial colonies showing morphological differences were taken and streaked on nutrient agar media. Selecting and streaking the colonies was repeated twice on new plates to obtain individually isolated colonies. Furthermore, pure cultures are cultured in nutrient broth media for DNA extraction.

Extraction, PCR, and Sequencing

Each pure culture bacterial suspension (1 x 109 cells) was used for DNA extraction. Bacterial isolate DNA was extracted using the Presto TM Mini gDNA Bacteria Kit Geneaid, following the Kit protocol. Extracted DNA templates were analyzed for purity using an Implant Nanophotometer by measuring the absorbance at A260/A280 nm. 16S rRNA gene amplification was carried out using primers: 16sA (5'CGC CTG TTT AAC AAA AAC AT 3') (Foward), 16sB2 (5'TTT AAT CCA ACA TCG AGG 3') (Reverse).[15] PCR amplification was performed with 2x MyTaq HS Red Mis Bioline PCR components 25µl; 10 pmol primer consists: 1µl forward primer, 1µl reverse primer, 2µl DNA template, and 21µl ddH_2O. On the other hand, the PCR conditions used 35x cycles with Denaturation at 94 (°C for 60 seconds), annealing at 50 (°C for 30 seconds), extension at 72 (°C for 30 seconds),

Table 1. Concentration and Purity of DNA samples extracted from isolated bacteria

No.	Origin of Samples	Conc. (ng/µl)	A260/280	A260/230	Volume (µl)
1.	Fish Auction (P.L.)	114,12	2,10	2,08	35
2.	Traditional Market (P.T.)	54,26	2,20	0,87	35
3.	Hospital (R.S.)	54.21	2,18	1,45	35
4.	Residential settlements (PM)	52,12	2,15	1,97	35
5.	Forest (H.T.)	50.10	2,12	0,76	35

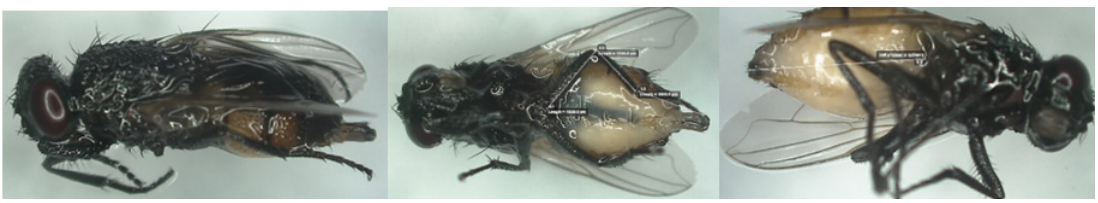

Figure 2. Example of a housefly (*M. domestica* L) specimen used as a source of bacterial isolates (Dorsal, ventral and side views), observed with a Hirox KH8700 Microscope, 200x magnification

and final extension at 72 (°C for 70 seconds). Visualization of amplicons using the 0.8% agarose gel electrophoresis method. PCR product then sent to the service company sequencing 1st BaseTM in Singapore for sequenced using the Sanger method.

Sequence Analysis

Consensus sequence determination using BioEdit. Consensus sequences were analyzed for alignment using the BLAST (Basic Local Alignment Search Tool) tool on the NCBI (National Center of Biotechnology Information) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis using Bioedit 7.2 (https://bioedit.software.informer.com/) Construction of isolate phylogeny using MEGA 11 Program (https://www.megasoftware.net/). Phylogeny construction using neighbour-joining and Minimum Evolution methods with bootstrap 1000x.

RESULTS AND DISCUSSION

DNA extraction of a single bacterial isolate from *M. domestica* L. originating from different habitats was successfully carried out. This is evidenced by the concentration of the DNA template in the range of 50 -114 ng/µl, while the purity at 2.10 to 2.20 (A260/280 (Table 1). DNA amplification using 16S rRNA primers was successfully carried out. The electrogram bands formed on each amplicon of the 16S rRNA gene were successfully generated with the PCR components and conditions applied (Figure 3). The amplicons formed were visualized at lengths of 700 to 1000 bp. Thus the amplicons were amplified over the length range of the bacterial 16S rRNA gene.

The nucleotide sequences of the sequencing products were analyzed using the Bioedit and MEGA X1 Programs. The sequencing results showed that DNA sequencing was carried out successfully, as evidenced by the chromatogram bands of each type of nucleotide that did not overlap in all samples. The forward (16SA) and reverse (16SB) sequencing results were then analyzed to determine the consensus area. Characteristics of the 16S rRNA gene consensus sequence have a length of 521 bp - 689 bp. AT percent ratio ranges from 47.02% to 56.05%. In comparison, the G.T. percent ratio ranges from 49.84% to 54.12%. The most extended consensus sequence length was in the bacterial 16S rRNA sample from the traditional market, while the

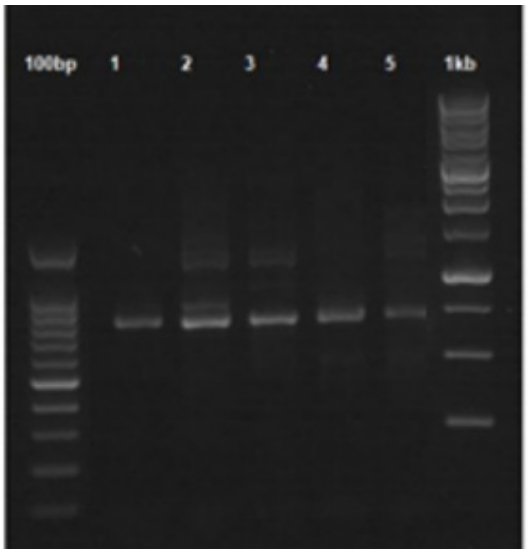

Figure 3. Amplification of the gene 16S rRNA isolates bacteria from *M. domestica.* 1). Fish Auction Isolates (P.L.), 2. Traditional Market Isolates (P.T.), 3). Hospital Isolates (R.S.), 4. Residential Isolates (PM), and 5. Forest Isolates (H.T.). 0.8% agarose gel Loaded DNA ladder per lane: 0.2 g Sample volume loaded per lane: 1µL for each 100 bp DNA Ladder (bp): 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500 100 bp DNA Ladder (ng/0.2ug): 16, 16, 14, 16, 34, 12, 11, 16, 16, 20, 29 The DNA ladder cannot be used to compare the sizes of non-linear DNA samples, such as plasmid DNA

Table 2. Characteristics of the 16S rRNA gene sequence isolates of housefly bacteria from various habitats

No.	Consensus Sample	Length (bp)	AT %	% G.T.
1.	Fish Auction (P.L.)	677	50,07	49,93
2.	Traditional Market (P.T.)	689	47,02	52,98
3.	Hospital (R.S.)	521	56,05	43,95
4.	Residential settlements (PM)	691	45,88	54,12
5.	Forest (H.T.)	678`	51,07	49,84

Table 3. The most similar sequence was the result of aligning the 16S rRNA gene of bacterial isolates using the NCBI BLAST method

No.	Consensus Sample	Species	% Identitic	Source
1.	Fish Auction (P.L.)	*Sphingobacterium faecium* [CP094931.1]	99,11	Submitted (01-APR-2022) Large Animal Clinical Sciences, Western College of Veterinary Medicine, Campus Dr, Saskatoon, SK S7N5B4, Canada
2.	Traditional Market (P.T.)	*Pseudochrobactrum* sp. XF203	97%	Submitted (30-SEP-2021) Jiangxi Normal University, College of life sciences, NO.99 of Ziyang Road, Nanchang, Jiangxi 330022, China
3.	Hospital (R.S.)	*Sphingobacterium faecium* [CP094931.1]	99,11	Submitted (01-APR-2022) Large Animal Clinical Sciences, Western College of Veterinary Medicine, Campus Dr, Saskatoon, SK S7N5B4 Canada
4.	Residential) settlements (PM)	*Brucella abortus* RB51-AHVLA	99%	Submitted (10-DEC-2019) Infectious Bacterial Diseases Research Unit, U.S. Dept. of Agriculture, Agricultural Research Service, National Animal Disease Center, 1920 Dayton Ave., Ames, IA 50010, USA
5.	Forest (HT)	*Bacillus paralicheniformis* [CP043501.1]	94%	Submitted (01-SEP-2019) Agricultural Microbiology Division, National Institute of Agricultural Sciences, 166 Nongsaengmyeong-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do 55365, Korea, Republic of

smallest was in the bacteria from the hospital (Table 2).

Consensus sequences were used for alignment at the NCBI site using the Nucleotide BLAST tool. The BLAST results show the 100 most similar sequences recorded in the NCBI genebank. P.L. bacterial isolates showed 99.11% similarity with *S. faecium* [CP094931.1]. P.T. bacterial isolates showed 97% similarity with *Pseudochrobactrum* spp. XF203. R.S. bacterial isolates showed 99.11% similarity with *S. faecium* [CP094931.1]. Bacterial isolates from PM showed 99% similarity with *Brucella abortus* RB51-AHVLA. H.T. bacterial isolates showed 94% similarity with *Bacillus paralicheniformis* [CP043501.1] (Table 3).

The phylogeny was built at the NCBI sites on each of the bacterial 16S rRNA sequences. The phylogeny construction uses a maximum of 25 sequences, similar to the BLAST results. Phylogenic Construction in NCBI automatically gives an unknown query on the 16S rRNA sequences of the proposed bacterial isolates. The results of P.L. 16S rRNA sequence phylogeny construction showed the closest phylogenetic relationship to *S. faecium*. Reconstruction of the phylogeny P.T. isolate showed the closest kinship with *Pseudochrobactrum* sp. XF203. The phylogeny reconstruction of the R.S. bacterial isolate showed the closest evolutionary relationship to *S. faecium*, the same as the P.L. bacterial isolate. Reconstruction of the PM isolate phylogeny showed the closest phylogenetic relationship to *B. abortus* RB51-AHVLA. Meanwhile, H.T. isolates phylogeny reconstruction showed the closest kinship with B. paralicheniformis [CP043501.1] (Figure 4).

The phylogeny construction of the five isolates was built using the MEGA XI program with two methods: Neighbour-joining (N.J.) and Minimum Evolution (M.E.). Two methods are used to ascertain the phylogenetic and topographical relationships of the trees formed. Reconstruction of the phylogenetic tree of five bacterial isolates using the two methods showed the same topography even though they had different calculations of the distance between nodes. The ME method shows a farther distance than the N.J. method. The results of the phylogenetic Construction of the five isolates formed two monophyletic groups. The isolate from the traditional market is in the same node as the forest isolate, while the hospital isolate is in the same node as the fish auction isolate. On the other hand, residential isolates have the closest

kinship with R.S. and Fish Auction isolates but do not form a single node (Figure 5). The results of this study show the diversity and association of bacteria in houseflies in various habitats in the city of Manado.

DISCUSSION

The 16S rRNA gene as a universal barcode gene for bacterial identification was successfully amplified in pure cultured bacterial isolates. Pure

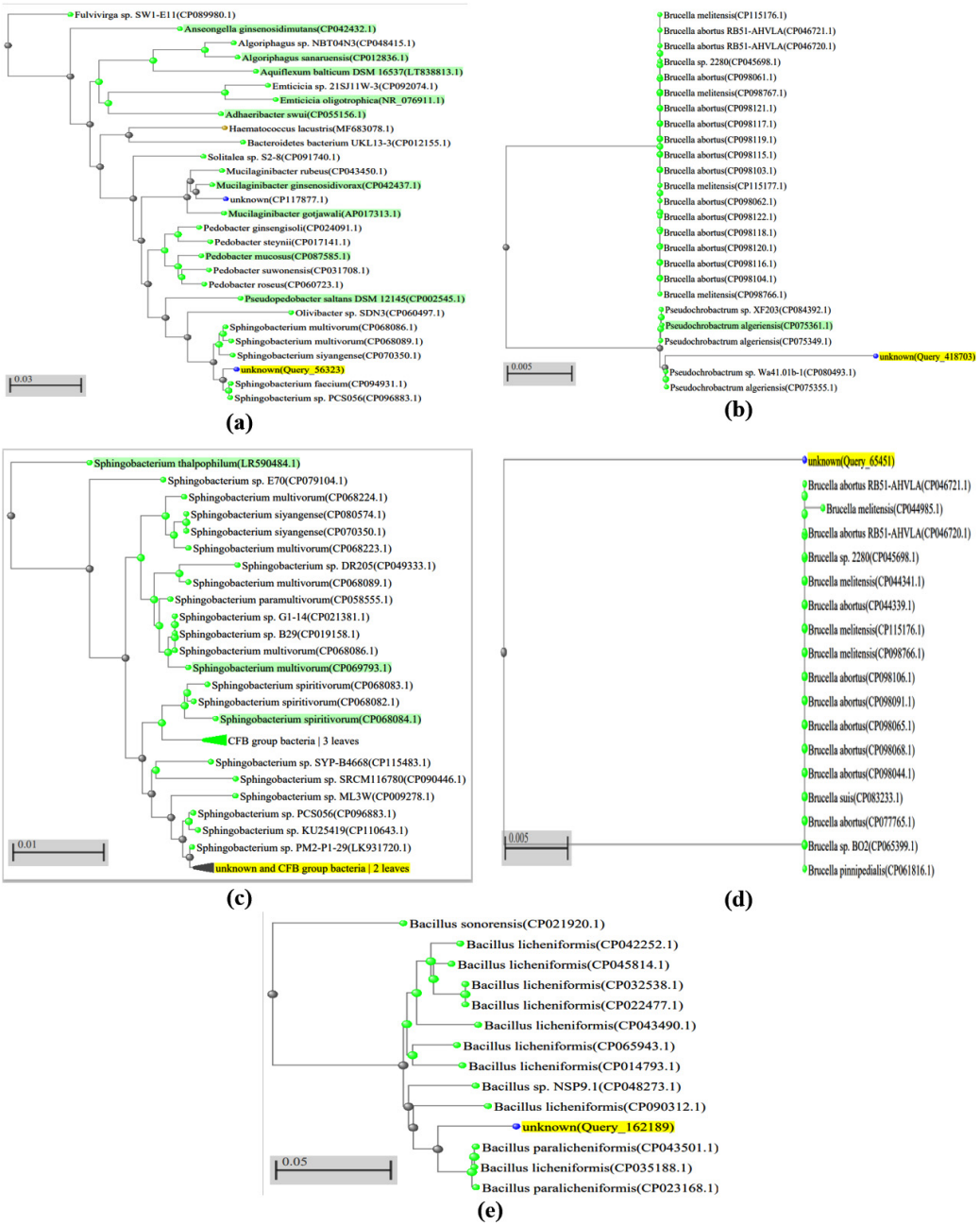

Figure 4. Phylogeny of Bacterial Isolates at the NCBI site. a. 16S rRNA P.I. b. 16S rRNA PT, c. 16S rRNA RS, d. 16S rRNA PM and e. 16S rRNA H.T

cultured bacterial isolates were isolated from multi-habitat *M. domestica* in Manado City. Based on the sequence characteristics, the sequencing results ensured that the 16S rRNA gene was successfully sequenced. Online phylogeny construction at the NCBI site was built with sequences from BLAST results showing the specific evolutionary relationships of each bacterial isolate. Phylogenic reconstruction using the neighbour-joining and Minimum Evolution methods in the MEGA XI Program confirmed bacterial isolates' position and phylogenetic relationships, strengthening the hypothesis that the diversity of bacteria associated with *M. domestica* was very high.

This study found bacteria associated with different houseflies in various habitats. However, what is interesting about the results of this study is that, based on the 16S rRNA gene, bacteria originating from the hospital have the closest phylogenetic relationship and resemblance to the same species, namely *S. faecium*. Even though the locations of the original habitat of the R.S. and P.L. samples were quite far apart. Bacterial isolates from P.T. also showed the closest relationship with bacterial isolates from H.T. In this study, geographically, the location of traditional market fly samples is indeed close to the forest. PM isolates show similarities to fish auctions and hospitals even though they are geographically far apart. This study shows that the association of bacteria with *M. domestica* is not determined by habitat alone. These results corroborate other studies which reported that *M. domestica* was an insect with the highest vectored microbial diversity.

S. faecium was reported to be associated with Delia antiqua.[13] *Sphingobacterium faecium* is a bacterium of the genus *Sphingobacterium* isolated from the manure of Bos sprunigenius taurus cows. *S. spiritivorum* is found in the digestive tract of *M. domesctia*, the larvae of *musca domestica*.[1] No scientific publications report the discovery of *S. faecium* on the body surface of *M. domestia,* so this research is the first report. *Pseudochrobactrum* spp. was reported to be found in *M. domestica* larvae.[3,10] Firmicutes are abundant as larvae and considered early colonizers, but as they mature into adults, Proteobacteria and Bacteroidetes take over.[15] *Enterobacter, Providence, Pseudomonas, Lactococcus, Klebsiella, Bacillus,* and *Acinetobacter* were among the bacteria isolated from the guts of housefly larvae.[14,16] In this study, no bacteria were found that had similar species to the group of bacteria.

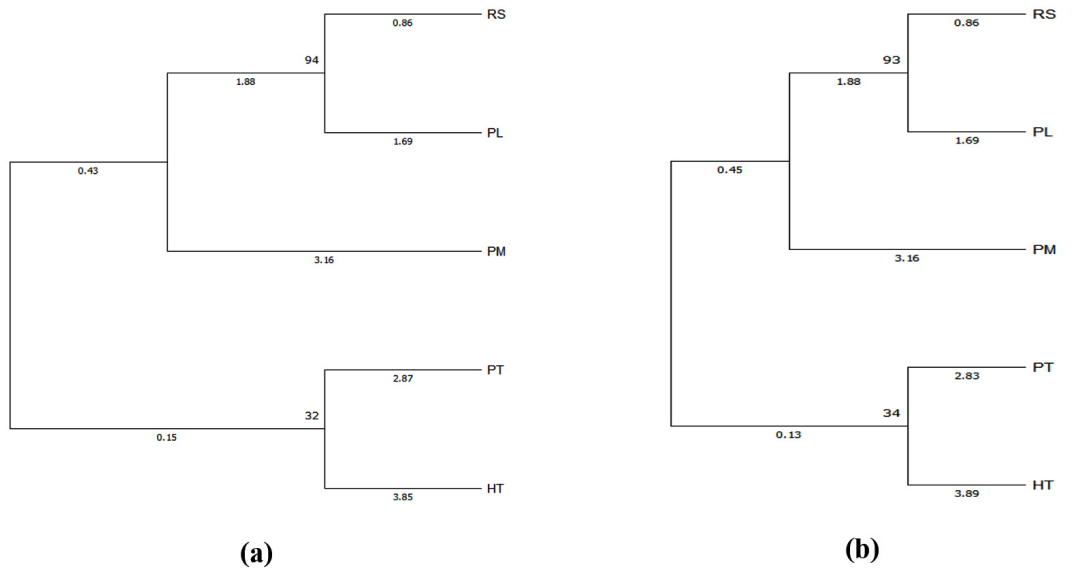

Figure 5. Reconstruction of the phylogeny of fly bacterial isolates from various locations in Manado (a). M.E. method (b). N.J. method

Brucella abortus is a Gram-negative bacterium in the *Brucella* genus, which is one of the causes of brucellosis.[17] These bacteria are rod-shaped, non-spore-forming, non-motile, and aerobic. *Brucella* bacteria that cause brucellosis can be found in various types of animals, such as cattle, goats, sheep, pigs, wild boars, hunting dogs, deer, bison and camels. Not many studies have reported *B. abortus* found in *M. domestica*. So that in the future, it can be a reference that *M. domestica* also vectors *B. abortus* to be transmitted to animals and even humans. This study also found bacterial isolates that had the closest resemblance to *B. paralicheniformis. Bacillus paralicheniformis* is a Gram-positive anaerobic motile *Bacillus* spp. Based on phylogenetic analysis, *B. paralicheniformis* is most closely related to *B. licheniformis* and *B. sonorensis*.[1,18] For decades, *B. paralicheniformis* and *B. licheniformis* have been used in biotechnology to produce enzymes, antibiotics, biochemicals, and consumer products.[18,19] Interestingly, not all harmful bacteria are associated with *M. domestica*.

CONCLUSION

Based on the 16S rRNA gene sequencing, the bacteria associated with *M. domestica* from different habitats are pretty diverse but also show associations with the same bacteria. The 16S rRNA gene selectively identified the bacterial species isolated from *M. domestica*. Not all the bacteria associated with *M. domestica* are harmful or cause disease. There are associated bacteria that are used as biotechnology agents. Exploration of bacteria and even microbes associated with *M. domestica* is still extensive to be studied in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

DVR conceptualized and supervised the study. MYS carried out the investigation and employed the technique. DVR assisted with software development. MK manage the project and performed analysis. MK conducted validation and grammatical adjustments. DVR wrote the manuscript. MYS reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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