

Gram-positive Bacteria with Commercial Potential from the Gastrointestines of *Holothuria (Mertensiothuria) Leucospilota (Timun Laut)* and *Stichopus Horrens (Gamat)* from Malaysian Waters

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ABSTRACT

A few studies on the presence of microbes and their association in sea cucumbers can be found to date, especially in *Apostichopus japonicus*. However, such studies on Malaysian sea cucumbers and in the gastrointestines of the echinoderms are still lacking. Therefore, the aims of this study are to isolate and identify associated bacteria in the gastrointestines of two common species of Malaysian sea cucumbers i.e. *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835), the most dominant *sea cucumber* species in Malaysia, and *Stichopus horrens* Selenka, 1867, the commercial *gamat* species. A number of six genera of Gram-positive bacteria representing the order Bacillales and the order Micrococcales i.e. *Bacillus*, *Brevibacillus*, *Lysinibacillus*, *Staphylococcus*, *Dermacoccus* and *Micrococcus* are isolated from the gastrointestines of *H. leucospilota*, as suggested by the phylogenetic trees of partial 16S rRNA gene sequences. Meanwhile, three genera of Gram-positive bacteria that represent the order Bacillales i.e. *Bacillus*, *Brevibacillus* and *Lysinibacillus* are isolated from the gastrointestines of *S. horrens*. Interestingly, 60% of the bacterial species have been known to contain commercial potentials mainly as antibiotic producers, and only bacteria with commercial potentials were present in the gastrointestines of *S. horrens*. In contrast to that, bacteria that could be pathogenic were also present in the gastrointestines of *H. leucospilota*. The presence of all bacteria that have been

known to contain commercial potentials in the gastrointestines of *S. horrens* and more diverse microbial population in *H. leucospilota* could be due to the higher level of antimicrobial properties in the gastrointestines of *S. horrens*. However,

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experiments on antibacterial properties of the isolates have to be done and those proven to contain commercial potential can be exploited towards the development of industrial applications in Malaysia.

Keywords: 16S rRNA gene, bacteria, gastrointestines, *Holothuria leucospilota*, *Stichopus horrens*

INTRODUCTION

Sea cucumber (Phylum Echinodermata: Class Holothuroidea) is a renowned echinoderm in Malaysia due to its *gamat* species e.g. *Stichopus horrens* Selenka, 1867 or dragonfish and *Stichopus herrmanni* Semper, 1868 or curryfish that are well known for their medicinal value (Kamarudin et al., 2017; Kamarudin et al., 2015; Hashim, 2011). At least 52 sea cucumber species inhabit Malaysian waters (Kamarudin et al., 2015, Hashim, 2011; Kamarudin et al., 2010a, 2010b; Sim et al., 2009; Kamarudin et al., 2009; Zulfigar et al., 2008; Zaidnuddin, 2002). In fact, Malaysia is geographically part of the Coral Triangle recognised as the global centre of marine biodiversity. *Timun laut*, *gamat*, *trepang*, *balat*, *bat*, *brunok/beronok* and *hoi sum* or *hai shen* are the popular local names in Malaysia. This marine-dwelling soft animal is consumed as food e.g. as a popular Chinese delicacy during the Chinese New Year celebration, and is exploited as traditional and modern medicines.

Analgesic effects, anti-anaphylaxis effects, therapeutic effects and antioxidant properties are among the medicinal properties discovered in Malaysian sea

cucumbers (e.g. Hashim, 2011; Osama et al., 2009; Khartini et al., 2003; Fredalina et al., 1999; Ridzwan et al., 1995). The coelomic fluid of *S. herrmanni* has been shown to cause a vasorelaxation effect on rat coronary arteries (Hashim, 2011; Tan et al., 2005) and antioxidant activities have been detected in the coelomic fluid of Malaysian sea cucumbers (Hashim, 2011; Hawa et al., 1999). The medicinal properties of Malaysian sea cucumbers are speculated to have a correlation with the existence and contribution of some microbes including pigment-producing strains associated with them. The functions of plant pigments were classified into five main groups by Wissgott and Bortlik (1996) i.e. (1) to convert light energy during photosynthesis in the presence of chromophore; (2) to serve as a communication medium between plants and animals; (3) to cause detoxification of reactive oxygen species i.e. antioxidation; (4) to play a role in response to stress; and (5) to be responsible for unknown functions of pigments. The microbial pigments are believed to hold all or some of the functions.

S. horrens is the most popular *gamat* species in Malaysia. Known as dragonfish in English, it is locally known as *gamat emas* in Malaysia. *Gamat* is the most well accepted Malaysian sea cucumber among Malaysians. However, it is actually a specific local name for all species of the family Stichopodidae that are well known to have medicinal properties. *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835), a non-*gamat* species, is the most

dominant species in Malaysia (Kamarudin et al., 2015; 2011). It is known as white threadfish in English or *bat puntil*. It is a long and black tubular sea cucumber often with a reddish body. This local species may have microbes that help it to adapt to numerous conditions.

Farouk et al. (2007) isolated 30 bacterial strains from *Holothuria (Halodeima) atra* Jaeger, 1833 or lollyfish from Malaysian waters. Antimicrobial activity associated with Malaysian sea cucumbers was also found (Farouk et al., 2007; Kaswandi et al., 2007; Ridzwan et al., 2003). A number of seven strains recorded moderate antibacterial activity against *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (Farouk et al., 2007). Despite the fact that there are 142 research papers on Malaysian sea cucumbers until the year 2011 (Kamarudin, 2011), there is still a lack of studies on the presence and association of microbes in Malaysian sea cucumbers, specifically in the gastrointestines. Hence, this study primarily aimed to isolate and identify bacteria associated with the gastrointestines of two common species of Malaysian sea cucumbers i.e. *H. leucospilota*, the most dominant sea cucumber species in Malaysia, and *S. horrens*, the commercial *gamat* species using the non-protein-coding region 16S ribosomal RNA gene. *S. horrens* and *H. leucospilota* (Brandt 1835) or white threadfish from Pangkor Island, Perak Darul Ridzuan, Malaysia were used in this study for isolation and identification

of microbes inhabiting the gastrointestines of both species. The gastrointestinal part was chosen to represent the internal parts of the species. It is a long and coiled alimentary canal inside the sea cucumber. Both species can be found under rocks or on the sandy sea floor of Malaysian waters. In fact, the use of the 16S rRNA gene marker for bacterial species identification is as important as the morphological approach. Both approaches always complement each other in order to achieve more accurate identification. Therefore, 16S rRNA gene sequencing was incorporated in this study for bacterial species identification.

MATERIALS AND METHOD

Study Site

The specimen collection of *H. leucospilota* and *S. horrens* was conducted at Teluk Nipah and Pangkor Laut, Pangkor Island, Perak Darul Ridzuan, Malaysia, respectively (Figure 1). Each species was collected in triplicate. The samplings took place from 8 to 9 November, 2011 and were done during low tide. There were no fixed or standard sampling hours, and the Global Positioning System (GPS) was used to read the position of the sampling site (not shown specifically). The live and fresh specimens were stored in clean ice boxes containing ice cubes or sea water during sampling. Prior to transporting the specimens from the study sites to the laboratory that took approximately seven hours, the specimens were frozen and stored in sterilised storage boxes.

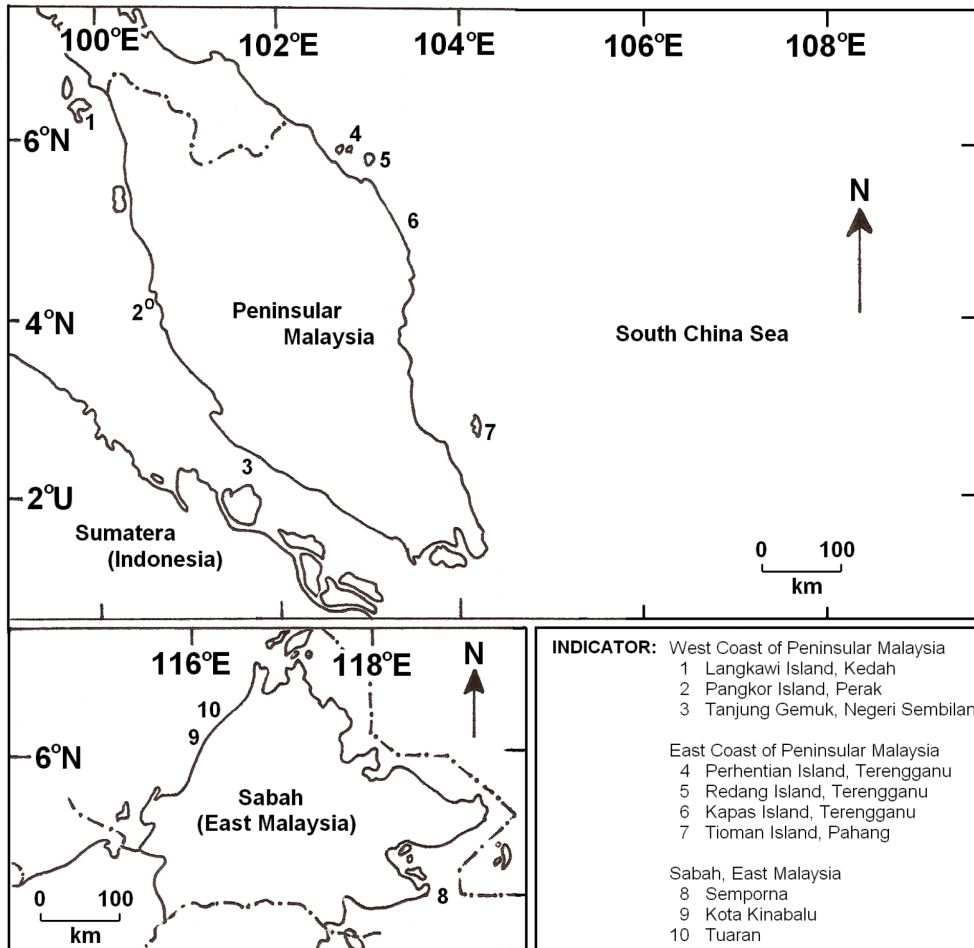


Figure 1. Specimens of *Holothuria (Mertensiothuria) leucospilota* and *Stichopus horrens* used in this study were collected from Pangkor Island, Perak Darul Ridzuan, Malaysia (sampling site 2). Adapted from Kamarudin et al. (2009)

Culture Media and Cultivation

A sterile blade was used to cut a small piece of tissue from each of the intestines of the specimens. The middle part of each intestine was selected for the dissection. The tissue was placed on Tryptone Glucose Yeast Extract Agar (TGYES, Fluka analytical, Sigma-Aldrich; ingredients: casein enzymic hydrolysate - 5 g/L, yeast extract - 3 g/L, glucose - 1 g/L, and agar

- 15 g/L) with pH 7.19. Incubation was done at 37°C. After overnight incubation (16-18 h), microbial colonies with different morphologies were each streaked and restreaked onto new TGYES plates until single colonies were observed. A microbial culture collection (in nutrient broth; ingredients: peptone - 5 g/L, yeast extract - 3 g/L, and glucose - 1 g/L) was designed with a 96-well plate and stored

in a freezer until further use. Nutrient agar (ingredients: peptone - 5 g/L, yeast extract - 3 g/L, glucose - 1 g/L, and agar - 15 g/L) with pH 6.8 was used for strain activation from the microbial culture collection. All facilities used for the isolation and culturing were in clean condition and each surface was sterilised prior to every use in order to minimise any potential of contamination.

Total Genomic DNA Extraction

Geneaid Genomic DNA Mini Kit (Blood/Cultured Cell) was used for the total genomic DNA (tgDNA) extraction. Electrophoresis was then used for determination of estimated yields of tgDNA, the quantity and quality, on 1% agarose gel with ethidium bromide as gel stain.

Polymerase Chain Reaction

Two universal primers of the 16S rRNA gene were used for the standard thermal cycle amplification (i.e. Polymerase Chain Reaction (PCR)). The expected length of PCR products was approximately 1.5 kilo base pairs (kb).

PB36 (forward) – 5' – AGR GTT TGA
TCM TGG CTC AG – 3' (20 bases)
PB38 (reverse) – 5' – GKT ACC TTG
TTA CGA CTT – 3' (18 bases)

PCR was performed using the Eppendorf Mastercycler gradient, which is a thermal cycler in 50 µL reaction volume i.e. 33.75 µL of sterilised dH₂O, 5.0 µL of 10X PCR reaction buffer, 3.0 µL of magnesium chloride

(25 mM), 2.5 µL of each universal primer (5 µM), 1.0 µL of dNTP mix (10 mM), 2.0 µL of the DNA extract and 0.25 µL of 5 u/µL *Taq* DNA polymerase. The cycle parameters were 5 min at 95°C for initial denaturation, 45 s at 95°C for denaturation, 90 s at an optimised temperature (i.e. 55°C) for annealing, 1 min 30 s at 72°C (60 s/kb; 29 cycles) for extension, 7 min at 72°C for final extension and then hold at 4°C. Electrophoresis was then used for determination of estimated yields of PCR products, the quantity and quality, on 1% agarose gel with ethidium bromide as gel stain.

PCR Products Purification and DNA Sequencing

Direct purification of the PCR products was done using the Geneaid Gel/PCR DNA Fragments Extraction Kit. Purified PCR products were sent for DNA sequencing in suspension form. DNA sequencing was done at First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor Darul Ehsan, Malaysia.

Phylogenetic Analyses

The results of fluorescence-based DNA sequence analyses received from First BASE Laboratories Sdn Bhd were displayed using the Chromas Lite (version 2.01) programme (Copyright © 1998-2005 Technelysium Pty Ltd). The ClustalX (version 2.1) programme (Thompson et al., 1997) was used to run a multiple sequence alignment for forward reaction sequences and subsequently aligned

by observation. The reconstruction of phylogenetic trees using the Neighbour-Joining method (Saitou & Nei, 1987) and Maximum Likelihood method (Figure 2 & Figure 3) was subsequently done using the

Molecular Evolutionary Genetics Analysis 5 software (MEGA5) (Tamura et al., 2011). The reconstructed phylogenetic trees were later displayed and edited using TreeView (Win32) version 1.6.6 by Page (1996).

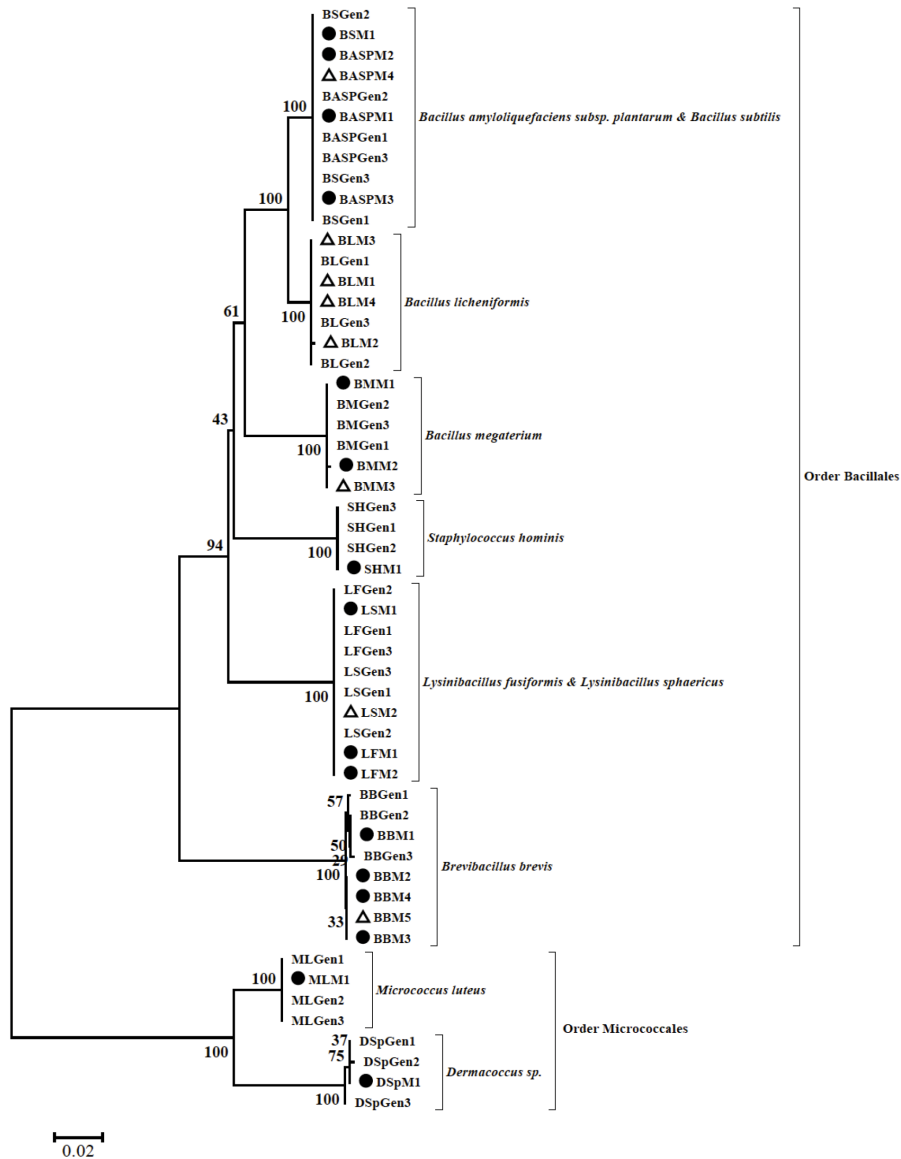


Figure 2. The evolutionary history of 16S rRNA gene sequences of bacteria associated with the gastrointestines of *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835) and *Stichopus horrens* Selenka, 1867 from Pangkor Island, Perak Darul Ridzuan, Malaysia was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Note: Dark round shape – from *H. leucospilota*; White triangle shape – from *S. horrens*; refer to Table 1 for more details

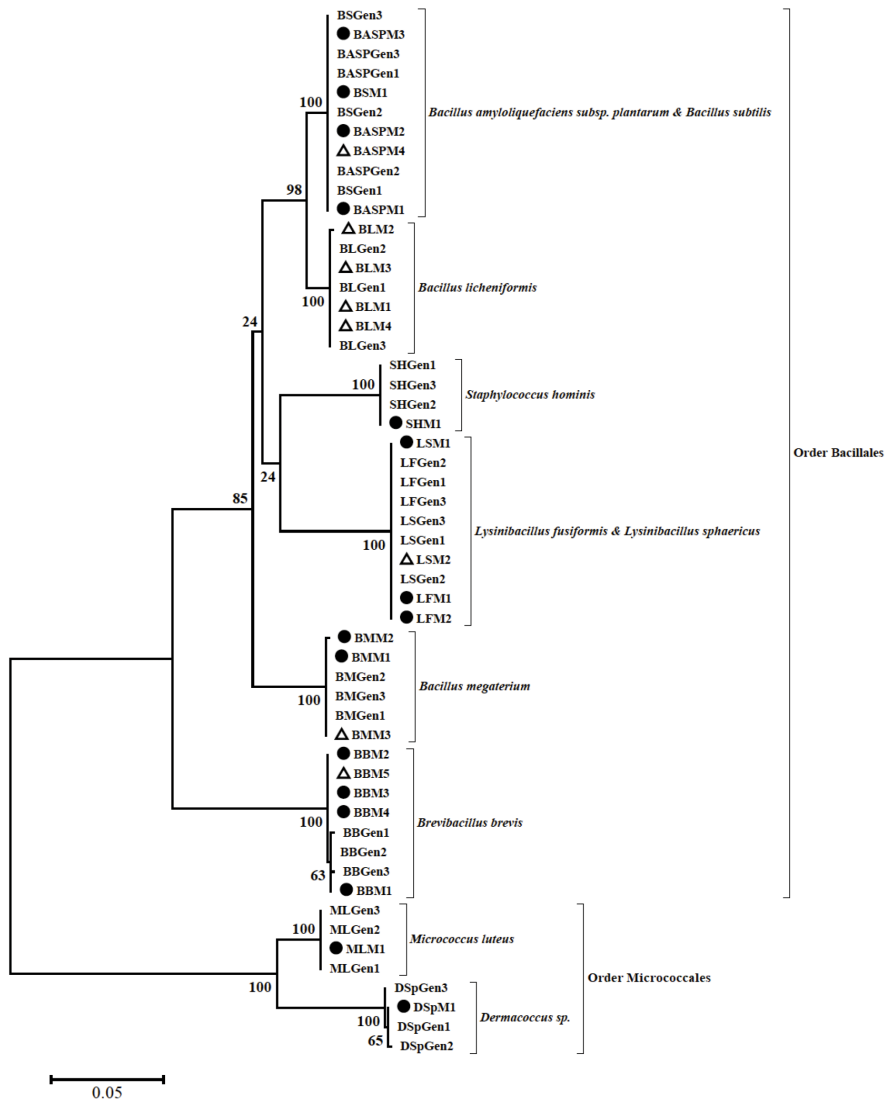


Figure 3. The evolutionary history of 16S rRNA gene sequences of bacteria associated with the intestines of *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835) and *Stichopus horrens* Selenka, 1867 from Pangkor Island, Perak Darul Ridzuan, Malaysia was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Note: Dark round shape – from *H. leucospilota*; White triangle shape – from *S. horrens*; refer to Table 1 for more details

RESULTS AND DISCUSSION

A number of 24 partial 16S rRNA gene sequences of the isolated bacteria have been registered with the GenBank,

National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine (GenBank Accession No.: JX286677 - JX286700, Table 1). The

partial gene sequences were accepted by the GenBank on 10 July, 2012 and released in the online databank on 26 August, 2012. In fact, constant contribution of nucleotide sequences to the GenBank including nucleotide sequences of new

species is important as an effort to enable various users around the world to obtain a better Basic Local Alignment Search Tool programme for nucleotide (blastn) results due to higher probability for the closest corresponding matches.

Table 1

List of Gram-positive bacteria associated with the gastrointestines of *Holothuria* (Mertensiothuria) *leucospilota* (Brandt, 1835) and *Stichopus horrens* Selenka, 1867 from Pangkor Island, Perak Darul Ridzuan, Malaysia

Bacterial species	GenBank accession number	Sea cucumbers <i>Holothuria leucospilota</i>	<i>Stichopus horrens</i>	Remarks
Order Bacillales (8)				
- <i>Bacillus amyloliquefaciens</i> subsp. <i>Plantarum</i> (BASPM1-BASPM4)	JX286677 JX286678 JX286679 JX286693	x	x	*Important source of alpha-amylase and protease for industrial applications
- <i>Bacillus megaterium</i> (BMM1-BMM3)	JX286680 JX286681 JX286698	x	x	*Antibiotics producer i.e. megacin
- <i>Lysinibacillus sphaericus</i> (LSM1-LSM2)	JX286690 JX286700	x	x	*Important organism to study because it can be used as an insecticidal toxin that controls mosquito growth
- <i>Brevibacillus brevis</i> (BBM1-BBM5)	JX286683 JX286684 JX286685 JX286686 JX286699	x	x	*Antibiotics producer i.e. gramicidin and tyrocidin
- <i>Bacillus licheniformis</i> (BLM4)	JX286694 JX286695 JX286696 JX286697		x	*Polypeptide antibiotics producer i.e. bacitracin
- <i>Staphylococcus hominis</i> (SHM1)	JX286692	x		Commensal of the skin
- <i>Lysinibacillus fusiformis</i> (LFM1-LFM2)	JX286688 JX286689	x		Unknown pathogenicity
- <i>Bacillus subtilis</i> (BSM1)	JX286682	x		*Antibiotics producer i.e. subtiline; may accumulate metal ions (aluminium, cadmium, iron and zinc) non-enzymically by adsorption to their cell surfaces and this can be of importance in waste treatment and natural environments
Order Micrococcales (2)				
- <i>Micrococcus luteus</i> (MLM1)	JX286691	x		Part of the normal flora of the mammalian skin
- <i>Dermacoccus</i> sp. (DSpM1)	JX286687	x		Undetermined species

The phylogenetic analyses involved 54 nucleotide sequences including 30 corresponding sequences from the BLAST results. Elimination was done in all positions containing gaps and missing data. As a result, the final dataset contained a total of 776 positions. Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets. For the reconstruction of the Neighbour-Joining tree (Figure 2), the optimal tree with the sum of branch length equals to 0.49601184 is shown. The distance-based tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The Tamura-Nei method (Tamura & Nei, 1993) was incorporated to compute the evolutionary distances and the distances are in the units of the number of base substitutions per site. Meanwhile, the Maximum Likelihood tree with the highest log likelihood (-2926.6407) is shown in Figure 3. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise the BIONJ method with Maximum Composite Likelihood (MCL) distance matrix was used. The branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The reconstruction of the Maximum Likelihood tree was based on

the Hasegawa-Kishino-Yano model (1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Six genera of Gram-positive bacteria from the order Bacillales (four genera) and order Micrococcales (two genera) i.e. *Bacillus*, *Brevibacillus*, *Lysinibacillus*, *Staphylococcus*, *Dermacoccus* and *Micrococcus* were isolated from the gastrointestines of *H. leucospilota* as inferred using the Neighbour-Joining method (Figure 2) and Maximum Likelihood method (Figure 3) based on partial 16S rRNA gene sequences. Lukman et al. (2014) isolated five bacterial genera from the coelomic fluid of *H. leucospilota* i.e. *Bacillus*, *Exiguobacterium*, *Pseudomonas*, *Stenotrophomonas* and *Vibrio*. In addition, Kamarudin et al. (2013) isolated an orange-pigment-producing *Staphylococcus kloosii* from the respiratory tree of *H. leucospilota* from Teluk Nipah, Pangkor Island, Perak, Malaysia. In terms of the gastrointestines of *S. horrens*, three genera of Gram-positive bacteria from order Bacillales i.e. *Bacillus*, *Brevibacillus* and *Lysinibacillus* were isolated. Three genera of gram-positive bacteria from the Micrococcaceae family i.e. *Kytococcus*, *Micrococcus* and either *Kocuria* or *Rothia* were isolated from the coelomic fluid of *Stichopus chloronotus* or the greenfish, one of the *gamat* species in Malaysia (Lukman et al., 2014). The order Bacillales formed the major group of eight bacterial species followed by the order Micrococcales with two bacterial species

(Figure 2 & Figure 3). Morphologically, the Gram-staining technique stains Gram-positive bacteria with a dark blue or violet colour.

In terms of genus composition of the bacterial community in the gastrointestines of *H. leucospilota* and *S. horrens* (Figure 4), the genus *Bacillus* was the largest group and it was present in both species along with the genus *Brevibacillus* and genus *Lysinibacillus*. Li et al. (2016) reported that the genus *Bacillus* was the main enzyme-producing microflora in the gut of *Apostichopus japonicus*. Interestingly, 60% of the associated bacteria have been acknowledged to hold commercial potential, specifically as antibiotic producers (Table 1). Only bacteria with commercial potentials were present in the gastrointestines of *S. horrens*. On the contrary, unknown potentials of bacteria that could be pathogenic were also present in the gastrointestines of *H. leucospilota*. In other words, more diverse microbial population was observed in the gastrointestines of *H. leucospilota*. Omran and Allam (2013) isolated five human Gram-negative pathogenic bacteria i.e. *Esherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* sp. and *Shigella* sp.; and a yeast *Candida albicans* from *Holothuria polii* collected from the Mediterranean Sea. However, experiments on the antibacterial properties have to be done in the future in order to confirm the presence of commercial potential among the isolates. In addition, a high level of antimicrobial properties in the

gastrointestines of *S. horrens* could be one of the contributing factors for the presence of all bacteria with commercial potential in the gastrointestines of *S. horrens*. According to Lukman et al. (2014), a number of environmental factors could contribute to the less diverse microbial population in *Stichopus chloronotus* from Tioman Island, Pahang Darul Makmur, Malaysia compared to *H. leucospilota* from Dayang Bunting Island, Yan, Kedah Darul Aman, Malaysia e.g. the feeding behaviour of *H. leucospilota*, the higher level of antimicrobial properties of coelomic fluid in *S. chloronotus* and the penetration of light surrounding the habitats of both species.

In order to facilitate and provide better insight into the bacterial communities in the gastrointestines of *gamat* species and *timun laut* species of Malaysian sea cucumbers, more studies with additional specimens of *S. horrens* and *H. leucospilota* from wide-ranging geographical locations and the use of different molecular techniques accompanied by morphological approaches for bacterial species identification are suggested. Besides that, the bacterial isolation methods by Gao et al. (2017) can be considered in future in order to isolate as much bacterial species as possible and also to further minimise the potential of any contamination. The current outcomes also suggested that the two sea cucumber species could be the new sources of bacteria with known commercial potential that can be exploited for the development of industrial applications in Malaysia.

Bacteria with Commercial Potential in Two Sea Cucumber Species

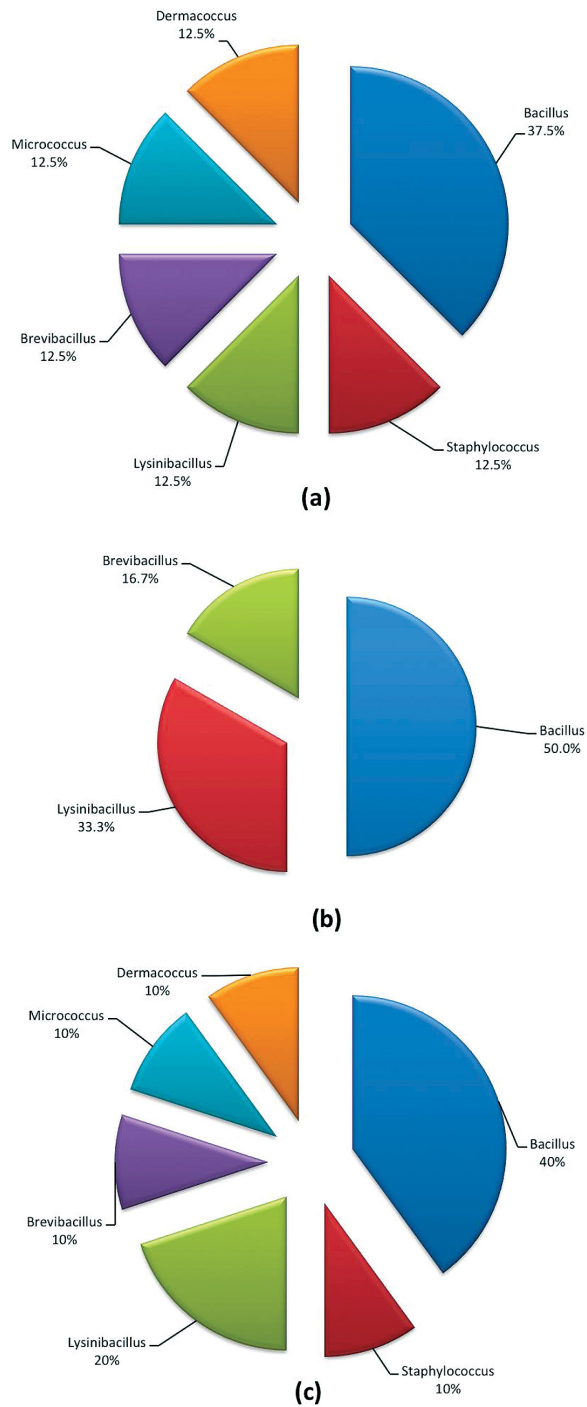


Figure 4. Genus composition of the bacterial community in the gastrointestines of *Holothuria (Mertensiothuria) leucopilota* (Brandt, 1835) and *Stichopus horrens* Selenka, 1867 from Pangkor Island, Perak Darul Ridzuan, Malaysia identified by sequence analysis of 16S rRNA gene. (a) - composition in the gastrointestines of *H. leucopilota*, (b) - composition in the gastrointestines of *S. horrens*, (c) - total composition for both Malaysian species

CONCLUSION

The Neighbour-Joining tree and the Maximum Likelihood tree that were used based on partial 16S rRNA gene sequences suggested the presence of six genera of Gram-positive bacteria from the order Bacillales and the order Micrococcales i.e. *Bacillus*, *Brevibacillus*, *Lysinibacillus*, *Staphylococcus*, *Dermacoccus*, and *Micrococcus* in the gastrointestines of *H. leucospilota*. Besides that, three genera of Gram-positive bacteria from the order Bacillales i.e. *Bacillus*, *Brevibacillus* and *Lysinibacillus* were isolated from the gastrointestines of *S. horrens*. Interestingly, 60% of the bacterial species have been known to contain commercial potential mainly as antibiotic producers. Moreover, only bacteria with commercial potential were present in the gastrointestines of *S. horrens*. However, unknown potential of bacteria that could be pathogenic was also present in the gastrointestines of *H. leucospilota*. A total number of 24 partial 16S rRNA gene sequences have been registered with the GenBank, NCBI, U.S. National Library of Medicine (GenBank Accession No.: JX286677 - JX286700).

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