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RESEARCH ARTICLE

Isolation and Characterisation of Bacteria and Fungus from the Intestine of Sea Cucumber *Acaudina molpadioides*

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Abstract *Acaudina molpadioides* or locally known as 'beronok' is a high valued sea cucumber that is widely distributed in the muddy shores in the west coast Peninsular Malaysia and being consumed by the local people as traditional healthy delicacies. They are made into dishes and are usually consumed raw as 'kerabu'. The aim of this study was to isolate and characterise the bacteria and fungus from the intestine of *A. molpadioides* using standard method for biochemical tests, safety assessments and molecular identification. A total of 100 samples were collected randomly from Pulau Langkawi, Malaysia and 1642 isolates were obtained from the intestine. Biochemical tests, safety evaluation and molecular identification were performed. Six strains (AM8h, AM47e, AM59a, AM67d, AM80d, and AM84d1) were selected for characterisation as they showed distinct morphology and from the biochemical tests. Further molecular identification showed the strains were identified as *Priestia megaterium*, *Carnobacterium maltaromaticum*, *Bacillus tropicus*, *Staphylococcus saprophyticus*, *Bacillus cereus*, and *Yarrowia lipolytica* (GenBank accession number: MZ947169, MZ934727, MZ947170, MZ934728, MZ934726, and MZ956769). The results indicated that both bacteria and fungus were presence in the intestine of *A. molpadioides*, hence there is a need for adequate measures in consuming this sea cucumber raw.

Keywords: *Acaudina molpadioides*, antimicrobial activity, bacteria, fungi, sea cucumber.

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Introduction

Sea cucumbers have been used traditionally as tonic food and medicinal resource in China, Japan, Korea, and south eastern Asian countries [1]. *Acaudina molpadioides*, known as sea potato or 'beronok' is a species of sea cucumber of order Molpadida and family Caudinidae [2]. This species inhabits in mud and it is a common species in the muddy shores in the west coast Peninsular Malaysia [3]. The environments are complex and submitted to extreme condition. They obtain their food by ingesting the marine sediment that contains microorganisms, meiofauna, decaying organic debris, inorganic components, and dissolved organic matter. This situation lead to survival adaptation and production of secondary bioactive metabolites which are lack in other organisms [4, 5]. *A. molpadioides* is consumed by the locals at Pulau Langkawi either raw (as salad) or cooked for their general health benefits due to the presence of bioactive compounds in their body wall such as triterpene glycosides, chondroitin sulphates, and sterols. *A. molpadioides* has been proven rich in vitamins and minerals [3].

The most important organ in animals is intestinal tract [6]. It is the site of food digestion, creates numerous substances that transmit messages to other parts of the body, and is crucial in fighting pathogens and controlling the body's water balance. Large number of microbes inhabit the intestine, exists symbiotically and contribute to material metabolism, growth and development, vitamin synthesis, immune responses and host resistance to disease [7, 8]. Intestine microbiota are currently mostly understood in relation to verterbrates, particularly mammals. Corals and sponges are the marine species with the most studied microbiota but other marine invertebrates have received less attention [9].

There is a scarcity of information about the microorganisms found in the intestine of *A. molpadioides*. The habitat of this species encoils the question on the type of microorganisms exist and the safety towards the consumers. Hence, this study aimed to isolate and characterise the microbes from the intestine of this marine invertebrate.

Materials and Methods

Sample collection

A total of 100 sea cucumber *A. molpadioides* samples were collected randomly from Pulau Langkawi, Malaysia (6° 21' 41.2" N, 99° 42' 38.3" E). The intestines of the *A. molpadioides* were eviscerated and stored in clean ice box containing ice cubes before transported to the laboratory for immediate processing.

Isolation and selection

The intestine was sliced open under sterile condition to reveal the interior part. The exudate was swabbed out and cultured on tryptic soy agar (TSA). The culture was incubated at 30°C for 24 hours. Colony formed on the culture was inoculated several times on TSA agar to get the pure culture. The pure cultures were stored in 2 conditions: slant agar and 30% glycerol stock prior to use [10].

Morphological identification

The microorganisms were subjected to external morphological and microscopic analyses. Colony observations were made after obtaining pure culture. The observations included colour, size, form, elevation, margin and opacity of colony were made visually. Gram stains was performed to identify the cell characteristic. Crystal violet, iodine, decolorizer and safranin were used to stain the cells in order to distinguish the types of staining [11].

Biochemical tests

Six biochemical tests were performed: catalase, indole, methyl red (MR), Voges-Proskauer (VP), citrate, and carbohydrate tests. The catalase test was conducted by dropping a drop of 15% hydrogen peroxide onto the colony. The positive result of the test was indicated by effervescence characteristic [12]. The indole test was performed by inoculating the isolates in tryptophan broth and incubated at 30°C for 24 hours. An amount of 0.3 mL of Kovac's reagent was added into the broth. Positive indole was indicated by the presence of red ring [13]. The colonies were also cultured into MR-VP broth and incubated at 30°C for 24 hours. For MR test, two drops of methyl red reagent were dropped into the culture and the positive test was indicated by the formation of red solution. Meanwhile, for VP test, a total of 15 drops of alpha-naphthol were added into the culture and followed by 5 drops of potassium hydroxide. Red cherry colour indicated positive result of VP test [14]. The colonies were cultured on Simmon citrate agar and incubated at 30°C for 24 hours for citrate test. The positive results were shown by the change of agar colour from green to blue [15]. Whereas, for the carbohydrate test, the media containing glucose and lactose were prepared in different tubes. MR was added as indicator and Durham tubes were inserted in an inverted position in each tube. The preparation was autoclaved at 115°C for 15 minutes. The colonies were cultured into the media and incubated at 30°C for 24 hours [16].

Safety assessments

Further safety assessments were carried out involving hemolysis test and antibiotic assay. Blood agar enriched with 5% sheep blood was prepared for the hemolysis test. The isolates were grown on blood agar and incubated at 30°C for 24 hours. The characteristic of the agar was observed after fermentation [17]. Using disc diffusion method, the sensitivity of the isolates towards antibiotics were performed. The four types of antibiotics: tetracycline (10 µg),

streptomycin (10 µg), ampicillin (10 µg), and meropenem (10 µg). The results were classified as sensitive, "S" (≥ 21mm); intermediate, "I" (16–20mm); and resistant, "R" (≤ 15mm) [18].

Molecular identification

The isolates were cultured in tryptic soy broth (TSB) at 30°C for 24 hours. The DNA extraction was performed according to the instructions of manufacturer [19]. The DNA was used as template in polymerase chain reaction (PCR). The reaction consisted of 12.5 µL Taq PCR Mastermix, 1.0 µL of universal primers (785F 5'-GGATTAGATACCCTGGTA-3'and 907R 5'-CCGTCAATTCMTTTRAGTTT-3' for bacteria; ITS1 and ITS4 primers for fungi), 1.5 µL DNA template and 9.0 µL nuclease free water. The mixture was denatured for 1 min at 94°C, then subjected to 40 cycles of 95°C for 30 s, 50°C for 1 min, and 72°C for 30 s. A final cycle was run for 7 minutes at 72°C and then cooled to 25°C for 30 seconds. The PCR product was observed by 1.5% (w/v) TAE agarose gel electrophoresis at 100V for 60 min with negative control (TAE buffer) and positive control (*Enterococcus thailandicus* SH11iii and *Penicillium simplicissimum* SC12a), then purified and sequenced. The sequence was compared to those kept in the GenBank database maintained by the National Center for Biotechnology Information (NCBI).

Data analysis

Data analysis was done using Statistical Package for the Social Science (SPSS) version 21 for this study. The data analysis was done descriptively and presented as percentages, means, and standard deviations.

Results

Sample collection

The anatomical of *A. molpadioides* was studied (see Figure 1) and appeared as smooth, brownish, slippery skin and elongated sausage-like body. The adult body size was in the range of 10 cm to 20 cm long. Short feeding tentacles surrounded the mouth which can be pulled back inside the body. The body wall was soft and it hardened when being cut (see Figure 2). The intestine could be distinguished by examining the structure close to the anus. From the observation, the intestine contained mud as this species inhabits the muddy shore.

Figure 1. *Acaudina molpadioides*

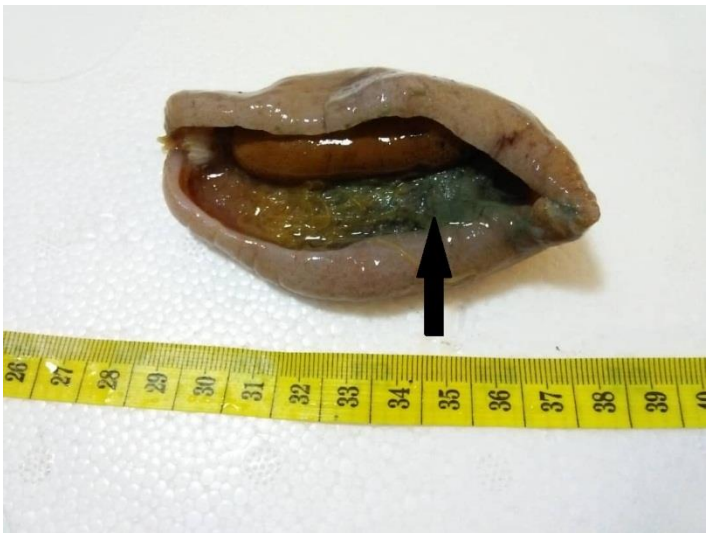

Figure 2. Internal organ of *Acaudina molpadioides* with hardened body wall. Arrow shows the intestine

Isolation and selection

The colonies were characterised based on their morphology and isolated onto TSA plate for pure culture storage. A total of 1642 colonies were isolated from 100 *A. molpadioides* and 56.8% (n=933) were Gram positive bacteria.

Morphological identification

Table 1 shows the characteristics of colony (colour, size, form, elevation, margin and opacity) and the shape of individual bacteria isolated from the intestine of *A. molpadioides*. The colour of colonies was seen as white, yellowish, creamy white and pinkish. The size of colonies was ranged from less than 0.5 mm to 2.5 mm. Most of the isolates were circular form. A total of 152 (9.26%) colonies were in irregular form and 12 colonies were seen as filamentous form. Various colonies elevation were seen and majority were convex and raised elevations. Eight isolates were seen as crateriform and 19 were umbonate. Most of the isolates appeared as entire margin with opaque colony. Others with filiform, undulate, and curled margin. Out of 98.54% (1618 isolates) were bacilli-shaped bacteria (Figure 3). Meanwhile, 1.11% (18 isolates) were found as cocci-shaped (Figure 4) and 6 isolates were coccibacillus.

Table 1. Characteristics of colony and shape of selected microorganisms isolated from *A. molpadioides*

Isolate	Colony						Shape
	Colour	Size (mm)	Form	Elevation	Margin	Opacity	
AM8h	Yellowish	1.0 – 1.5	Circular	Convex	Entire	Opaque	Bacillus
AM47e	White	0.5 – 1.0	Circular	Convex	Entire	Opaque	Bacillus
AM59a	White	0.5 – 1.0	Irregular	Raised	Filiform	Translucent	Bacillus
AM67d	Creamy white	0.5 – 1.0	Circular	Convex	Entire	Opaque	Coccus
AM80h	White	1.0 – 1.5	Circular	Flat	Entire	Opaque	Bacillus
AM84d1	Creamy white	2.0 – 2.5	Circular	Umbonate	Curled	Opaque	Coccobacillus

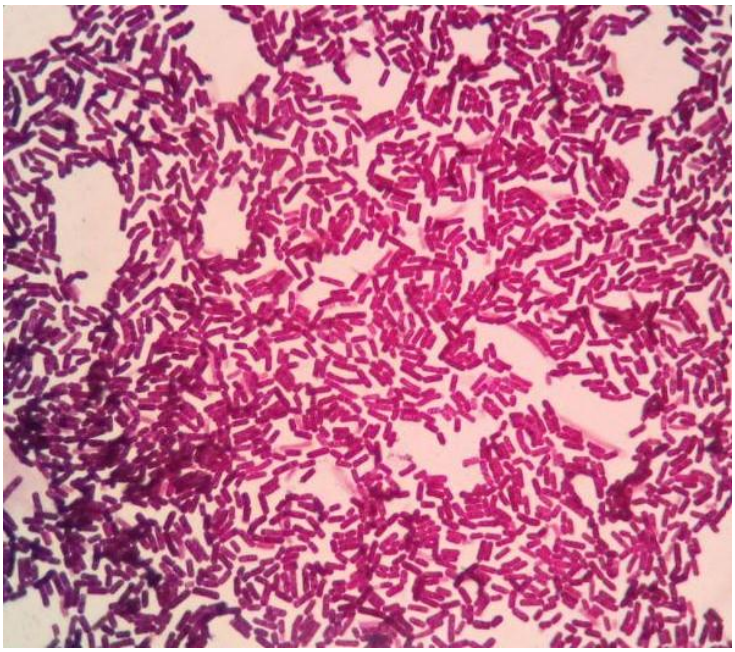

Figure 3. Gram positive bacilli isolate (1000× magnification)

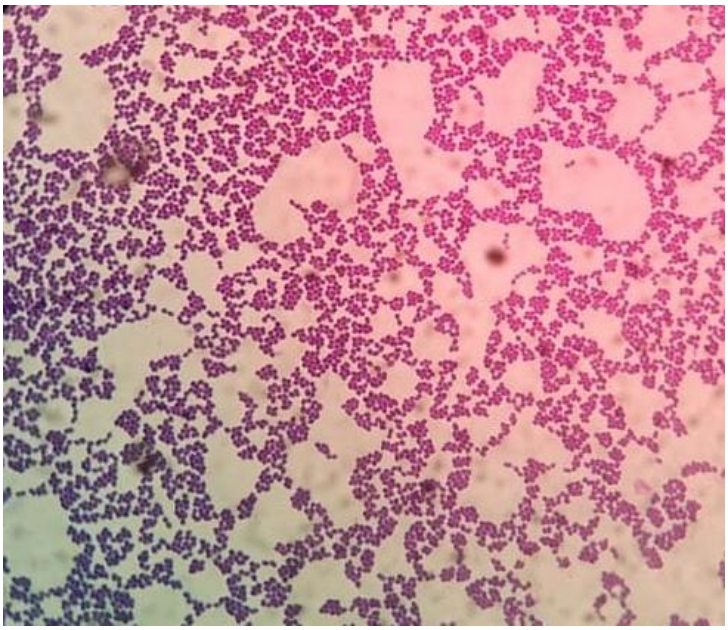

Figure 4. Gram positive cocci isolate (1000× magnification)

Biochemical tests

Catalase activity was not detected in 983 of 1642 isolates. For indole test, a total of 258 (15.7%) isolates gave positive result. Meanwhile, 88% (n=1445) isolates were found to be MR positive, 182 (11%) isolates were positive to VP test, and 283 (17.2%) isolates were positive to citrate test. Table 2 shows the biochemical tests of selected isolates from the intestine of *A. molpadioides*. A total of 660 isolates from *A. molpadioides* gave positive result towards both lactose and glucose fermentation. Only 19 (1.2 %) isolates were positive to lactose but negative to glucose. Fermentation with negative lactose but positive glucose was obtained the most from *A. molpadioides*, with 837 (51 %) isolates. A total of 82 (5 %) isolates were negative to both types of sugar. In all, 38 (2.3 %) isolates were attained fermenting lactose and

glucose with production of gas. Six isolates were observed with negative lactose fermentation, positive glucose fermentation with formation of gas.

Table 2. Biochemical tests of selected isolates isolated from *A. molpadioides*

Isolate	Catalase Test	IMViC Test				Carbohydrate Test	
		Indole	MR	VP	Citrate	Lactose	Glucose
AM8h	−	−	−	−	+	+	+
AM47e	−	−	−	−	−	+	+
AM59a	−	−	+	−	−	+	+
AM67d	−	−	−	−	−	+	+
AM80h	−	−	+	−	−	−	+
AM84d1	−	−	+	−	−	+	+

* + = positive, − = negative, MR = methyl red, VP = Voges-Proskauer

Safety evaluation

From the 1642 isolates, 52% isolates were α –hemolysis. Meanwhile, β- and γ-hemolysis shared the same ratio that was 24%. Isolates from the *A. molpadioides* intestine showed that α-hemolysis was the most prevalence. Hemolysis test was conducted to evaluate the hemocompatibility of erythrocytes by determining the hemolytic properties of microorganisms. This property enables them to break down the epithelial layer of the host cells and may cause invasive diseases in host [20]. Absence of hemolytic activity is one of the important safety precautions for microorganisms.

A total of 259 isolates were selected for the antimicrobial assay according to morphology, biochemical tests and hemolysis test. From the number, about 57% (147 isolates) were sensitive to tetracycline and 92% (238 isolates) were sensitive to meropenem. Herein, most of the bacteria were sensitive to both drugs disregard their mechanism of action. A total of 55% (142 isolates) were classified as intermediate to streptomycin. Ampicillin was not the drug of interest due to 63% (163 isolates) which was more than half of isolates that resistant to the drug. Three isolates namely AM56d, AM57d and AM72b were obtained to resist all four antibiotics.

Descriptive statistics accordance with diameter of inhibition zone of isolates from intestine of *A. molpadioides* and the type of antibiotics were performed. A one-way ANOVA was conducted to compare the types of antibiotic: tetracycline (10 µg), streptomycin (10 µg), ampicillin (10 µg) and meropenem (10 µg) with the diameter of inhibition zone. Results from ANOVA test suggests that there is a significant difference between the antibiotic groups [$F(3, 1032) = 180.536$, $P < 0.05$]. A further Tukey's post hoc test revealed the diameter of streptomycin (17.8 ± 5.1 mm), ampicillin (14.5 ± 9.8 mm), and meropenem (28.9 ± 7.0 mm) is significantly lower than tetracycline (20.6 ± 6.8 mm). Whereas the diameter of ampicillin (14.5 ± 9.8 mm), tetracycline (20.6 ± 6.8 mm), and streptomycin (17.8 ± 5.1 mm) was obtained significantly lower than meropenem (28.9 ± 7.0 mm). Tetracycline (20.6 ± 6.8 mm), streptomycin (17.8 ± 5.1 mm), and meropenem (28.9 ± 7.0 mm) were significantly lower than ampicillin (14.5 ± 9.8 mm). Tetracycline (20.6 ± 6.8 mm), ampicillin (14.5 ± 9.8 mm) and meropenem (28.9 ± 7.0 mm) was significantly lower than streptomycin (17.8 ± 5.1 mm). Table 3 shows the type of hemolysis and antimicrobial activity of the six selected isolates: AM8h, AM47e, AM59a, AM67d, AM80h, and AM84d1 towards tetracycline (10 µg), streptomycin (10 µg), ampicillin (10 µg) and meropenem (10 µg).

Table 3. Types of hemolysis, diameter of inhibition zone and susceptibility of bacteria from intestine of *A. molpadioides* to tetracycline (10 µg), streptomycin (10 µg), ampicillin (10 µg) and meropenem (10 µg)

Isolate	Hemolysis test	Tetracycline (10 µg)		Streptomycin (10 µg)		Ampicillin (10 µg)		Meropenem (10 µg)	
		Diameter (mm)	Susceptibility	Diameter (mm)	Susceptibility	Diameter (mm)	Susceptibility	Diameter (mm)	Susceptibility
AM8h	γ	22.17 ± 0.76	S	24.70 ± 1.16	S	33.93 ± 1.49	S	41.44 ± 1.14	S
AM47e	γ	25.83 ± 0.76	S	12.02 ± 1.36	R	30.87 ± 0.69	S	31.38 ± 2.08	S
AM59a	γ	20.00 ± 0.00	S	22.59 ± 0.95	S	19.09 ± 1.73	I	43.26 ± 1.38	S

Isolate	Hemolysis test	Tetracycline (10 µg)		Streptomycin (10 µg)		Ampicillin (10 µg)		Meropenem (10 µg)	
		Diameter (mm)	Suscep tibility	Diameter (mm)	Suscep tibility	Diameter (mm)	Suscep tibility	Diameter (mm)	Suscep tibility
AM67d	γ	21.70 ± 1.25	S	21.53 ± 1.14	S	15.67 ± 0.49	I	31.47 ± 0.32	S
AM80h	α	20.83 ± 0.29	S	11.98 ± 0.76	R	17.89 ± 3.08	I	44.75 ± 2.04	S
AM84d 1	γ	17.00 ± 0.00	I	-	R	-	R	23.33 ± 0.58	S

* Diameter ± SD; Susceptibility: S = Sensitive, I = Intermediate, R = Resistant; - no inhibition zone.

Molecular identification

Six isolates namely AM8h, AM47e, AM59a, AM67d, AM80h, and AM84d1 were selected for molecular identification according to their morphology, biochemical tests and safety assessment. The PCR products were detected by gel electrophoresis. The bands were spotted at around 1500bp. The PCR product for AM84d1 was not detected during gel electrophoresis and was repeated using ITS1 and ITS4 as primer. The band was clearly seen at 250 – 500bp. The sequences were compared using The Basic Local Alignment Search Tool (BLAST) to identify the sequence similarity. Table 4 shows the DNA identification of the bacteria obtained from *A. molpadioides* using BLAST comparisons. This validated BLAST search was submitted successfully to GenBank under the accession number MZ947169 (*Priestia megaterium*), MZ934727 (*Carnobacterium maltaromaticum*), MZ947170 (*Bacillus tropicus*), MZ934728 (*Staphylococcus saprophyticus*), MZ934726 (*Bacillus cereus*), and MZ956769 (*Yarrowia lipolytica*). Figure 5 shows the maximum likelihood (ML) tree obtained from the combination of 16s rRNA sequences of species obtained from the five selected bacteria strains using Molecular Evolutionary Genetics Analysis (MEGA) 11, Multiple Sequence Alignment.

Table 4. DNA identification of microorganisms from *A. molpadioides* based in similarities of sequences in BLAST

Isolate		Reference Bacteria			
Code	GenBank Accession Number	Similarity (%)	GenBank Accession Number	Species name	Reference
AM8h	MZ947169	100	CP045272	*Priestia megaterium*	Nagar *et al*., 2021.
AM47e	MZ934727	99.93	CP045040	*Carnobacterium maltaromaticum*	-
AM59a	MZ947170	100	MT611943	*Bacillus tropicus*	Paul *et al*., 2021.
AM67d	MZ934728	100	CP054831	*Staphylococcus saprophyticus*	Vendl *et al*., 2021.
AM80h	MZ934726	100	MT611946	*Bacillus cereus*	Paul *et al*., 2021.
AM84d1	MZ956769	100	CP028459	*Yarrowia lipolytica*	Lubuta, 2018

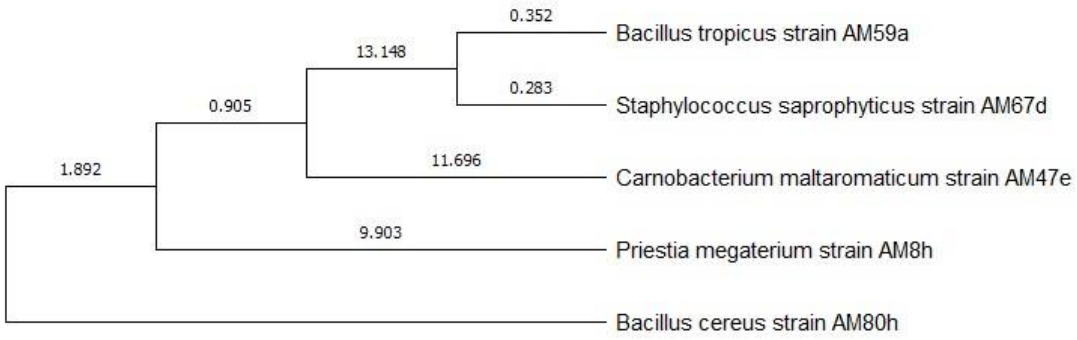

Figure 5. Maximum likelihood (ML) tree obtained from the combination of 16s rRNA sequences of 5 bacteria strains using neighbor-joining method in Mega 11

Discussion

P. megaterium strain AM8h appeared yellowish with 1.0 – 1.5 mm diameter size colony. The colony was circular form, convex elevation, entire margin and opaque on MRS agar. The shape of the individual bacterium was bacillus. For IMViC tests, *P. megaterium* was negative to indole, MR and VP tests. It gave positive result to citrate test. It was tested positive to both lactose and glucose test. In another study, *P. megaterium* was obtained as concave, smooth and milky colony. It was seen as rod-shaped and Gram positive under LM. It did not give the same result of indole, MR and VP tests, by which it was tested negative to indole but positive to MR and VP tests. For carbohydrate test, it was in agreement with the present study, which is positive to both tests [21]. Hemolysis test indicated that *P. megaterium* was γ-hemolysis. This strain was sensitive to all the four antibiotics tested. *Priestia megaterium* was previously known as *Bacillus megaterium*. In October 2020, Gupta *et al*. [22] found a total of 17 new individual Bacillus clades of Subtilis and Cereus on conserved signature indels (CSIs). They proposed these clades as new genera with the name *Priestia* gen. nov. for Megaterium clade which consists of *B. megaterium*, *B. abyssalis*, *B. aryabhattai*, B*. endophyticus*, *B. filamentosus*, *B. koreensis* and *B. flexus* due to two CSIs in the oligoribonuclease NrnB that uniquely shared by all clade members. *P. megaterium* can be found in honey, raw meat, fish, wine, human oral cavity, sea water and most typical plants and soil [23, 24, 25]. *P. megaterium* is a vitamin B12 (cobalamin) natural producer. Due to the complexity of cobalamin molecule, it requires about 60 chemical steps and 30 enzymes for its synthesis. Bacteria such as *P. megaterium* dispenses an excellent host for cobalamin production [26]. Secretion of organic acids by *P. megaterium* providing the main basis of phosphate biofertilization. Furthermore, this species can provide reduced nitrogen to plants and there are fertilizer products being produced of this bacteria with the combination of organic matter and ammonium sulphate [27]. *P. megaterium* isolated from marine water was found to accumulate polyhudroxybutyrate (PHB). PHB, one of polyhydroxylalkanoates (PHAs) group, is a type of microbial polymer that produced by bacteria. This compound possesses biodegradability and biocompatibility which could be used in medical applications and reduce the adverse environmental effect by replacing the marketed plastics. Ability of *P. megaterium* in producing PHB has added the value in the bacteria [28].

Colony of *C. maltaromaticum* strain AM47e was seen as white with 0.5 – 1.0 mm diameter, circular form, convex elevation, entire margin and opaque on MRS agar. It was negative towards all the IMViC tests. This type of species was γ-hemolysis which does not hemolyse RBC. However, the strain was positive to lactose and glucose test. In Table 3, AM47e was resistant towards streptomycin and sensitive to tetracycline, ampicillin and meropenem.

C. maltaromaticum is a lactic acid bacterium (LAB). It can be found mostly in dairy products, meat, and fish. *C. maltaromaticum* has been applied in food industry which related to health

protection and organoleptic properties. This LAB species involve in biopreservation of food by inhibiting the growth of foodborne pathogens in cold conditions such as Listeria sp., and development of flavour in ripened cheese varieties [29]. In these applications, temperature and pH on the acidifying activity play an important role in industrial scale. Girardeau and team [30] reported that *C. maltaromaticum* must be cultivated at 20°C, pH 6 and harvested at the beginning of stationary phase to exhibit the fastest acidification activities. However, culture conditions at 30°C, pH 9.5 and harvest time between 4 – 6 h of stationary phase can be done if slower acidification activities needed. Danielski and colleague [31] reported that *C. maltaromaticum* inhibited growth of *Listeria monocytogenes* in cooked ham and did not affect the physicochemical parameters of the product during storage. In addition, *Carnobacterium* spp. ability to survive under high-pressure vacuum-packing and grow at refrigeration temperatures make them the ideal candidate as additive in preventing food spoilage, especially in meat and seafood industry [32]. Although *C. maltaromaticum* is being used in food industry and is generally recognized as safe (GRAS) by United States Food and Drug Administration (USFDA), *C. maltaromaticum* is not placed in the list of Qualified Presumption of Safe by European Food Safety Agent due to its virulence in fish [33]. A study conducted by Roh and team [34] had proved that *C. maltaromaticum* isolated from dairy products was different compared to strains isolated from diseased fish. Two virulence-related genes, wecC and xtmA were only present in strains from the diseased fish. In a different study, *C. maltaromaticum* isolated from arctic char (*Salvelinus alpinus*) and lake white fish (*Coregonus clupeaformis*) showed sensitive susceptibility towards tetracycline MIC50 (µg/µl) and resistant to ampicillin MIC50 (µg/µl) [35]. This is contradicting to *C. maltaromaticum* strain AM47e which was sensitive to ampicillin (10 µg).

AM59a was identified as *Bacillus tropicus*. The colony was observed as white, 0.5 – 1.0 mm diameter, irregular form, raised elevation, filiform margin and translucent on MRS agar. The shape of the bacterium was bacillus. On Luria-Bertani agar, the *B. tropicus* was seen as 2 – 3 mm size colony and circular shape. The species was also seen as bacillus [36]. *B. tropicus* strain AM59a was negative to indole, VP and citrate tests but positive to MR test. This is contradicting to BacDive [36] which reported *B. tropicus* was indole negative, but positive to VP and citrate. Hemolysis test showed γ-hemolysis of this strain and it was also positive to lactose and glucose test. The carbohydrate fermentation pattern is different to BacDive [36] whereas there were negative to both tests. For the antibiotic test, it was sensitive to tetracycline, streptomycin and meropenem. It is classified as intermediate susceptibility towards ampicillin. *Bacillus tropicus* was reported as an efficient microorganism to degrade low density polyethylene (LDPE) films with 10-micron thickness. In 40 days of incubation, it abled to degrade 10 % of the LDPE mass [37]. Selenium nanoparticles (SeNPs) have outstanding bioavailability and low toxicity than organic or inorganic with selenium compounds. SeNPs have been explored for their oxidative stress and inflammation mediated disorders. A research done by the team showed that *B. tropicus* possessed the capability to biosynthesize SeNPs with spherical shape and low cytotoxicity [38]. In another study, *B. tropicus* was subjected to ultraviolet (UV) irradiation, ethidium bromide (EtBr), ethyl methane sulfonate (EMS) mutagenesis, followed by cross mutation of UV irradiated strain with EMS and EtBr. It was found that mutant *B. tropicus* strains produced significantly high amount of alginase [39].

Staphylococcus saprophyticus strain AM67d was seen as creamy white, 0.5 – 1.0 mm diameter, circular form, convex elevation, entire margin and opaque on MRS agar. This strain was negative to all the IMViC tests. This species of bacteria did not hemolyse RBC which grouped under γ-hemolysis. It was positive to lactose and glucose test. Antimicrobial assay proved that *S. saprophyticus* was sensitive to tetracycline, streptomycin and meropenem; and intermediate to ampicillin. *S. saprophyticus* is a significant pathogen responsible for urinary tract infection (UTI) in sexually active young women [40]. In spite of greater successful treatment, UTI caused by *S. saprophyticus* has higher recurrent infection frequency compared to *Escherichia coli*. Acute pyelonephritis, nephrolithiasis and endocarditis are some of rare complications of *S. saprophyticus* UTI [41]. *S. saprophyticus* had been isolated from Brazilian

minas cheese, one of the most popular cheese in the country. Some reports of *S. saprophyticus* in marine environment and food derived from fresh fish attract attention to the spread of *S. saprophyticus* [42]. *S. saprophyticus* does not produce hemolysin to lyse hemoglobin [43]. A study described that *S. saprophyticus* sensitive to tetracycline (30 µg) and resistant to streptomycin (10 U) [44]. This is in agreement with the result obtained in this study which it exhibited γ-hemolysis and sensitive to tetracycline (10 µg). *S. saprophyticus* strain AM67d was sensitive to streptomycin (10 µg).

AM80h was obtained as *Bacillus cereus*. The appearance was white, 1.0 – 1.5 mm diameter, circular form, flat elevation, entire margin and opaque on MRS agar. It was negative to indole, VP, and citrate tests. Meanwhile, it was positive to MR test. *B. cereus* strain AM80h showed partial hemolysis to RBC which grouped it under α-hemolysis. It was negative to lactose test but positive to glucose test. The species reacted differently to the antibiotic drugs. It is sensitive to tetracycline, resistant to streptomycin and meropenem, and intermediate to ampicillin. *B. cereus* was seen as Gram positive with bacillus shape under LM and it is the same with the statement of Aryan [16]. For IMViC tests, it was obtained as negative to indole, VP, and citrate tests but referring to Aryan [19], the results were negative to indole and MR tests but positive to VP tests. *B. cereus* strain AM80h was tested as α-hemolysis. *B. cereus* secrets hemolysin BL which can bind erythrocytes and lyse them by forming a transmembrane pore and disrupting osmotic equilibrium [45]. *B. cereus* is positive towards hemolysis test. *B. cereus* is also associated with other several toxins including nonhemolytic enterotoxin, cytotoxin K, enterotoxin FM, potential heterotoxin hemolysin II, enterotoxin T, and emetic toxin [17, 21]. *B. cereus* was tested negative to lactose and positive to glucose [16]. *B. cereus* strain AM80h showed sensitive to tetracycline (10 µg) but the strain was intermediate susceptible to ampicillin (10 µg). *B. cereus* was susceptible to imipenem (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg), erythromycin (15 µg), kanamycin (30 µg), and cefotetan (30 µg). *B. cereus* was resistant to ampicillin (10 µg), penicillin (10 U), rifampin (5 µg), cefepime (30 µg), oxacillin (1 µg), and cephalothin (30 µg) [46]. *Bacillus cereus* is usually found in the environment. It frequently exists in soil vegetation, and also can be found in foods. *B. cereus* ables to multiply rapidly at room temperature [47]. This bacterium is responsible for intestinal illnesses, which is diarrhea. It can also emetic-type illness such as nausea, vomiting and abdominal cramp.

Colony of *Yarrowia lipolytica* strain AM84d1 was seen as creamy white with 2.0 – 2.5 mm diameter, circular form, umbonate elevation, curled margin and opaque on MRS agar. The shape was seen as coccobacillus and Gram positive under microscope. The yeast isolated from bovine subclinical mastitis were also Gram-positive [48]. This strain was tested positive for MR test and negative to indole, VP and citrate tests. It was tested as γ-hemolysis and it gave positive result to lactose and glucose tests. For antibiotic tests, it was sensitive to meropenem (10 µg), intermediate susceptibility to tetracycline (10 µg) and resistant to streptomycin (10 µg) and ampicillin (10 µg). These four antibiotics were used to treat bacterial infections. Hence, to the best of our knowledge, there was no other antibiotic test done to this yeast towards these antibiotics. Till the date, *Y. lipolytica* was tested with amphotericin B, ketoconazole, fluconazole, itraconazole, voriconazole, anidulafungin, caspofungin, micafungin, and 5-fluorocytosine. *Yarrowia lipolytica* was tested positive to lactose and glucose [54]. Consumption of xylose by *Y. lipolytica* is higher than the consumption of glucose. Herein, *Y. lipolyticus* is able to use glucose as the source of carbon and energy [50]. The report was in line with the result of glucose test in this present study. *Y. lipolytica* is a member of Ascomycota phylum and a nonpathogenic dimorphic aerobic yeast. It was formerly known as *Candida*, *Endomycopsis* or *Saccharomycopsis lipolytica* [49]. *Y. lipolytica* has been found ubiquitously in the environment and meat products, especially sausages and dairy products. In human, it may occasionally be found in feces, oropharyngeal, sputa, and skin of asymptomatic persons [51]. It is able to grow in hydrophobic environments. Due to this property, this type of yeast has the capability to an ability to metabolize fatty acids and triglycerides as carbon source. This feature has brought the species to be exploited in bioremediation of environments contaminated with

oil spill [52]. A study elaborated the production of fatty acid, ethyl esters, fatty alkanes, medium chain-length fatty acid, fatty alcohols, and triacylglycerides through mechanistic details of lipogenic phenotype, fatty acid synthase structure, activating free acids to acyl-CoAs, and decoupling nitrogen starvation from liponesis. They concluded that, *Y. lipolytica* may serve as biorefinery platform chemicals to sustain the production of diesel fuels and oleochemicals through simple chemical or biotransformation [53]. In another economical view, *Y. lipolytica* is able to grow on hydrophobic wastes and reduce the environmental pollution. In return, it produces biotechnologically valuable products which ban be used in biofuel production [54]. *Y. lipolytica* infection cases were reported in small case series. National China Hospital Invasive Fungal Surveillance Net conducted a study on invasive yeast infections. *Y. lipolytica* was identified from the samples of patients' blood. In practicing the guideline for the management of candidiasis by the Infectious Diseases Society of America, voriconazole and fluconazole was used for the systemic antifungal treatment [51].

Conclusions

A total of 1642 isolates were isolated from the intestine of *A. molpadioides* and six isolates were selected for characterization from 100 *A. molpadioides*. The isolates were identified as *Priestia megaterium*, *Carnobacterium maltaromaticum*, *Bacillus tropicus*, *Staphylococcus saprophyticus*, *Bacillus cereus*, and *Yarrowia lipolytica*. This study indicated that both bacteria and fungi can inhabit the intestine of *A. molpadioides*. *B. cereus*, *C. maltaromaticum* and *Y. lipolytica* cannot be classified as safe according to their resistance to the antibiotics and hemolytic activity. Hence, the consumption of raw *A. molpadioides* especially the intestine is not recommended.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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