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# Isolation and characterization of *Carnobacterium maltaromaticum* from the intestine of sea cucumber *Acaudina molpadioides*

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## ABSTRACT

**Aims:** *Acaudina molpadioides* is a highly valued sea cucumber that is distributed on the muddy shores on the west coast of Peninsular Malaysia and is considered a local delicacy. *Carnobacterium maltaromaticum* is a type of lactic acid bacteria commonly found in meat, fish and dairy products and is usually isolated from the intestine of aquatic animals. It is scarcely reported from the intestine of *A. molpadioides*. This species is known to be pathogenic in fish but unknown in humans. The aim of this study was to isolate and characterize *C. maltaromaticum* from the intestine of *A. molpadioides*.
**Methodology and results:** Using standard methods, the strains were tested for their biochemical and carbohydrate properties, antibiotic sensitivity tests, temperature sensitivity tests and molecular identification. A total of 1642 isolates were obtained, out of which three strains were chosen as they were catalase-negative, Gram-positive bacilli, negative to IMViC tests, γ-hemolysis, and positive to lactose and glucose tests. Molecular identification was made to strain AM47e and 16S rRNA genes sequence showed 99.93% similar to *C. maltaromaticum*. The sequence was submitted to GenBank as *Carnobacterium maltaromaticum* (Accession No: MZ 934727). The optimum growth temperature for the strains was 37°C and the antibiotic susceptibility showed they were sensitive to tetracycline, ampicillin and meropenem.
**Conclusion, significance and impact of study:** In conclusion, *C. maltaromaticum* can be isolated from the intestine of *A. molpadioides* with the potential of the probiotic applications and less potential vulnerability for consumers.

*Keywords: Acaudiana molpadioides*, *Carnobacterium maltaromaticum*, lactic acid bacteria, sea cucumber

## INTRODUCTION

*Acaudina molpadioides* is known as sea potato or 'bronok' is a species of sea cucumber of the order Molpadida and the family Caudinidae (Khotimchenko, 2018). It is a common species in the muddy shores on the west coast of Peninsular Malaysia (Choo *et al*., 2016). *Acaudina molpadioides* is consumed by the locals at Pulau Langkawi for their general health benefits due to the presence of bioactive compounds in their body walls, such as triterpene glycosides, chondroitin sulphates and sterols. *Acaudina molpadioides* has been shown to be rich in vitamins and minerals, too (Choo *et al*., 2016).

Lactic acid bacteria (LAB) are known for their benefits to humanity. They are extensively distributed in carbohydrate-rich environments and typically discovered in decayed plants and animal matter (Mokoena, 2017). They are also typically found to reside in the gastrointestinal tract of animals (Li *et al*., 2020). LAB refers to Gram-positive bacteria lack of catalase activity, non-spore-forming, non-motile and acid-tolerant. They are distinguished by their morphology, capacity to ferment carbohydrates, carbon dioxide production, growth at various temperatures and resistance to high salt concentrations (Lambuk *et al*., 2022).

*Carnobacterium maltaromaticum* (formerly known as *Carnobacterium piscicola*) is a species of LAB that is abundant in dairy, fish and meat products (Danielski *et al*., 2020). This species is frequently isolated from a range of cold and temperate environments. *Carnobacterium maltaromaticum* is known to cause disease in fish, particularly Australian salmonids, striped bass, channel catfish, carp and rainbow trout. However, in humans, the pathogenicity of this bacteria remains obscure (Roh *et al*., 2020; Pastorino, *et al*., 2021). On the other hand, *C. maltaromaticum* is being exploited for its benefit. With the

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implementation of technology, the latter is being used in food biopreservation and food development (Puentes *et al*., 2021). These findings prompted the authors to evaluate the safety of *C. maltaromaticum* from the intestine of *A. molpadioides* to humans. Hence, the goal of this study was to isolate and characterize *C. maltaromaticum* from the intestine of *A. molpadioides*.

## MATERIALS AND METHODS

### Sample collection

A total of 100 *A. molpadioides* were collected from Pulau Langkawi, Kedah (6°20'60" N, 99°48'0.01" E). The intestines of the organism were separated from their body walls and stored in a clean ice box containing ice cubes before being brought to the laboratory for immediate processing.

### Isolation and selection

Under the sterile condition, the intestine was cut to expose the inner part. The exudate was swabbed out, cultured on De Man, Rogosa, and Sharpe (MRS) agar, and incubated at 30°C for 24 h. Colonies from the mixed cultures were inoculated several times on MRS agar until pure, homogenous colonies were obtained. The morphology of colonies was observed and recorded.

### Biochemical tests

*Gram's staining*

Isolates were tested with Gram's staining by following standard procedure: applying primary stain (crystal violet), the addition of mordant (Gram's iodine), rapid decolouration (ethanol) and counterstaining (safranin). Gram-positive isolates were preserved for further study (Aryal, 2018a).

*Catalase test*

A drop of 15% (v/v) hydrogen peroxide was dropped onto the culture. Samples with the negative result without effervescence were selected and subjected to further tests (Aryal, 2018b).

*Indole test*

The isolates were cultured into tryptophan broth and incubated at 30°C for 48 h. An amount of 0.3 mL of Kovac's reagent was added and mixed well. After 10 min, the red layer on top of the broth showed a positive result to the test (Tankeshwar, 2021).

*Methyl red (MR) and Voges-Proskauer (VP) tests*

The bacteria were cultured into MR-VP broth and incubated at 30°C for 48 h. For the MR test, 2 drops of MR were added. The red colour indicated a positive test. For the VP test, 15 drops of alpha-naphthol were added, followed by 5 drops of potassium hydroxide. The red cherry colour could be seen after 1 h and showed positive results to the test (Tankeshwar, 2021).

*Citrate test*

The isolates were cultured on Simmons citrate agar and incubated at 30°C for 24 h. Colour of the agar changed to blue, indicating a positive result of the citrate test (Tankeshwar, 2021).

*Carbohydrate tests*

Media was prepared in different tubes containing glucose and lactose. MR was added as an indicator. The media was fully filled into the Durham tube and inserted in an inverted position. The preparation was autoclaved at 115°C for 15 min. Then the isolates were cultured and incubated for 24 h at 30°C (Aryal, 2019).

*Hemolysis test*

Blood agar supplemented with 5% sheep blood was prepared. The isolates were cultured onto blood agar and incubated at 30°C for 24 h. The characteristics of the agar after fermentation was observed (Aryal, 2018c).

Isolates with the same results of morphology, IMViC, carbohydrate and hemolysis tests were grouped together for further tests.

### Antibiotic susceptibility tests

Using the disc diffusion method, the isolates were assayed for their susceptibility to bacterial growth inhibitor antibiotics: tetracycline (10 µg), streptomycin (10 µg) and cell wall inhibitor antibiotics: ampicillin (10 µg), meropenem (10 µg). The results were expressed in terms of sensitive, S (≥21 mm); intermediate, I (16-20 mm) and resistant, R (≤15 mm) (Tendecia, 2004).

### Temperature sensitivity test

Under the sterile condition, three colonies of bacteria from the mother culture plate were taken and cultured into the MRS broth and incubated for 24 h at 30°C. The turbidity of the bacteria through optical density (OD) was adjusted by adding the media until it is equivalent to the turbidity of 0.5 McFarland standard, which was between 0.08 and 0.1. The cultures were incubated in a shaking incubator at 30°C, 37°C and 45°C for 24 h. The reading of OD was taken at 600 nm (Bennani, 2017).

### Molecular identification

From overnight culture, DNA was extracted using GF-1 Bacterial DNA extraction kits and referred to the manufacturer's protocol for Gram-positive bacteria (Vivantis, n.d). The DNA was used as a template in a polymerase chain reaction (PCR). The reaction consisted

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of 12.5 µL *Taq* PCR Mastermix, universal primers (1.0 µL of 785F 5'-GGATTAGATACCCTGGTA-3' and 1.0 µL 907R 5'-CCGTCAATTCMTTTRAG TTT-3') (Manoharan *et al*., 2020), 1.5 µL DNA template and 9.0 µL nuclease-free water. In this study, universal primers were used for 16S rRNA gene amplification due to their ability to bind to a wide variety of DNA templates (Gurushankara, 2021). The mixture was set to an initial denaturation step at 94°C for 1 min, followed by 40 cycles of the following conditions: 95°C for 30 sec, 50°C for 1 min and 72°C for 30 sec, followed by incubation at 72°C for 7 min before being cooled to 25°C for 30 sec (La Duc *et al*., 2007). Aliquot of 1 µL PCR product was analysed by 1.5% (w/v) TAE agarose gel electrophoresis at 100 V for 60 min with negative control (TAE buffer) and positive control (*Enterococcus thailandicus* SH11iii). The visible band was cut and sent to 1st Base, Malaysia, for sequencing. The sequence was compared with those stored in the National Center for Biotechnology Information (NCBI) GenBank database (Lee *et al*., 2012; Lorenz, 2012).

### Data analysis

Statistical Package for the Social Sciences (SPSS) version 21 was used for data analysis in this study. The data analysis was done descriptively and results were presented in percentage, mean and standard deviation.

## RESULTS

### Sample collection

*Acaudina molpadioides* (Figure 1) had smooth, brownish, slippery skin and an elongated sausage-like body. The body wall of *A. molpadioides* was soft and became hardened when being cut (see Figure 2). The intestine could be distinguished by examining the structure close to the anus. The intestine of the animal was cut and the exudate was swabbed out and streaked on MRS agar. The colonies of bacteria appeared on the medium within 24 h.

### Isolation and selection

The pure homologous colonies were characterized based on their morphology and cultured onto an MRS agar plate for pure culture storage. A total of 1642 colonies were isolated from a total of 100 animals.

### Biochemical tests

Out of 1642 isolated colonies, 56.8% (n=932) were Gram-positive bacteria (bacillus shape) and further tested towards catalase test. *Carnobacterium maltaromaticum* was characterized as catalase negative. Thus, a total of 248 isolates from 932 isolates (27%) were negative catalase and selected for further tests. From the selected isolates of *A. molpadioides*, 211 (85%) out of 248 isolates tested negative for the indole test. Meanwhile, 28 isolates were negative for MR. A total of 223 isolates tested

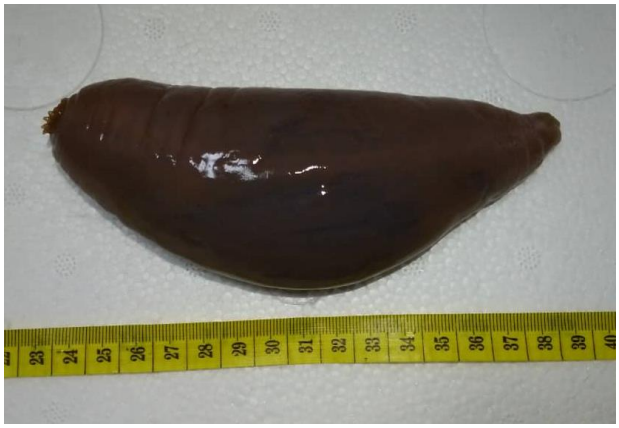

**Figure 1:** *Acaudina molpadioides.*

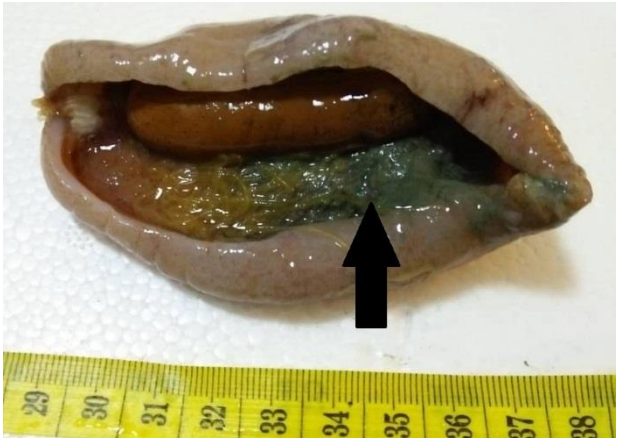

**Figure 2:** Internal organ of *Acaudina molpadioides*. Black arrow indicates the intestine.

negative for the VP test and 41 were negative to the citrate test. From the observation, most of the isolates were negative for indole, VP and citrate test. In the contrary, the majority of them were positive for the MR test. A total of 97 isolates from *A. molpadioides* gave positive results in both lactose and glucose fermentation. Only three isolates were positive for lactose but negative to glucose. Fermentation with negative lactose but positive glucose was obtained the most from *A. molpadioides* which was 128 isolates. Thirteen isolates were negative for both types of sugar. In all, six isolates were attained by fermenting lactose and glucose with the production of gas. One was observed with negative lactose fermentation and positive glucose fermentation with the formation of gas. Table 1 shows the results of biochemical tests for the presumptive *C. maltaromaticum* strain AM47e, AM54d and AM80d. There were three types of hemolysis: α-hemolysis which was identified by the greenish-grey colour of the colony, β-hemolysis was characterized as clear agar surrounding the colony and γ-hemolysis with no changes to the agar could be seen. The majority, that was 52% of bacteria isolated from *A.*

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**Table 1:** Results of biochemical tests for presumptive *C. maltaromaticum* strain AM47e, AM54d and AM80d.

| Biochemical tests | AM47e | AM54d | AM80d |
|---|---|---|---|
| Catalase | - | - | - |
| Indole | - | - | - |
| Methyl red | - | - | - |
| Voges-Proskauer | - | - | - |
| Citrate | - | - | - |
| Lactose | + | + | + |
| Glucose | + | + | + |
| Hemolysis | γ | γ | γ |

*+: Positive; -: Negative.

*molpadioides* grouped in α-hemolysis. Meanwhile, β- and γ-hemolysis shared the same ratio, which was 24%. Three presumptive isolates, AM47e, AM54d and AM80d previously selected, were seen as γ-hemolysis (see Table 1).

**Antibiotic susceptibility**

Inhibition zone diameters were measured and expressed in terms of sensitive, S (>20 mm); intermediate, I (16-20 mm) and resistant, R (<16 mm) (Tendecia, 2004). Table 2 shows the antimicrobial activity of AM47e, AM54d and AM80d towards tetracycline (10 µg), streptomycin (10 µg), ampicillin (10 µg) and meropenem (10 µg). The strains showed susceptibility towards tetracycline, ampicillin and meropenem but were resistant to streptomycin.

**Temperature sensitivity test**

The three selected presumptive *C. maltaromaticum* showed growth towards different temperatures, 30°C, 37°C and 45°C. Table 3 shows the optical density of the strains grown at different temperatures. The strains showed an optimal growth at a temperature of 37°C which gave the highest OD (0.5705 ± 0.023) for AM47e, 0.5239 ± 0.029 for AM54d and 0.4664 ± 0.059 for AM80d.

**Molecular identification**

Colonies obtained were grouped based on their morphology, biochemical tests and hemolysis activity. Representatives of each group were taken for molecular identification. From the result, AM47e was identified as *C. maltaromaticum*. Two isolates AM54d and AM80d had the same characteristics as AM47e. Figure 3 shows the results of the PCR product detected by gel electrophoresis. The band was detected with a molecular weight of around 1500 bp. The sequencing confirmed that

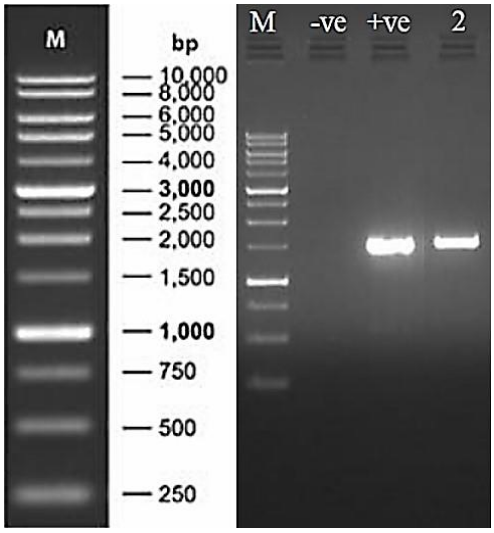


**Figure 3**: Results of PCR product detection via agarose gel electrophoresis. (M: DNA ladder 1kb (range: 250-10000 bp); -ve: Negative control; +ve: Positive control; 2: AM47e).

**Table 2:** The antibiotic susceptibility of AM47e, AM54d and AM80d strains against tetracycline (10 µg), streptomycin (10 µg), ampicillin (10 µg) and meropenem (10 µg).

| | AM47e | | AM54d | | AM80d | |
|---|---|---|---|---|---|---|
| | DI | Susceptibility | DI | Susceptibility | DI | Susceptibility |
| Tetracycline (10 µg) | 25.83 ± 0.76 | S | 29.33 ± 0.58 | S | 27. 67 ± 0.29 | S |
| Streptomycin (10 µg) | 12.02 ± 1.36 | R | 12.00 ± 1.00 | R | 15.50 ± 0.00 | R |
| Ampicillin (10 µg) | 30.87 ± 0.69 | S | 31.83 ± 0.29 | S | 27.50 ± 2.18 | S |
| Meropenem (10 µg) | 31.38 ± 2.08 | S | 29.5 ± 0.50 | S | 28.33 ± 0.58 | S |

*DI: Diameter of inhibition zone; R: Resistant; S: Sensitive. Mean ± SD.

**Table 3:** Growth (optical density) of *C. maltaromaticum* strain AM47e, AM54d and AM80d at different temperatures.

| Temperature (°C) | Mean optical density ± SD | | |
|---|---|---|---|
| | AM47e | AM54d | AM80d |
| 30 | 0.4529 ± 0.017 | 0.4773 ± 0.010 | 0.4339 ± 0.057 |
| 37 | 0.5707 ± 0.023 | 0.5239 ± 0.029 | 0.4664 ± 0.059 |
| 45 | 0.2728 ± 0.003 | 0.2787 ± 0.030 | 0.2696 ± 0.035 |

*SD: Standard deviation.

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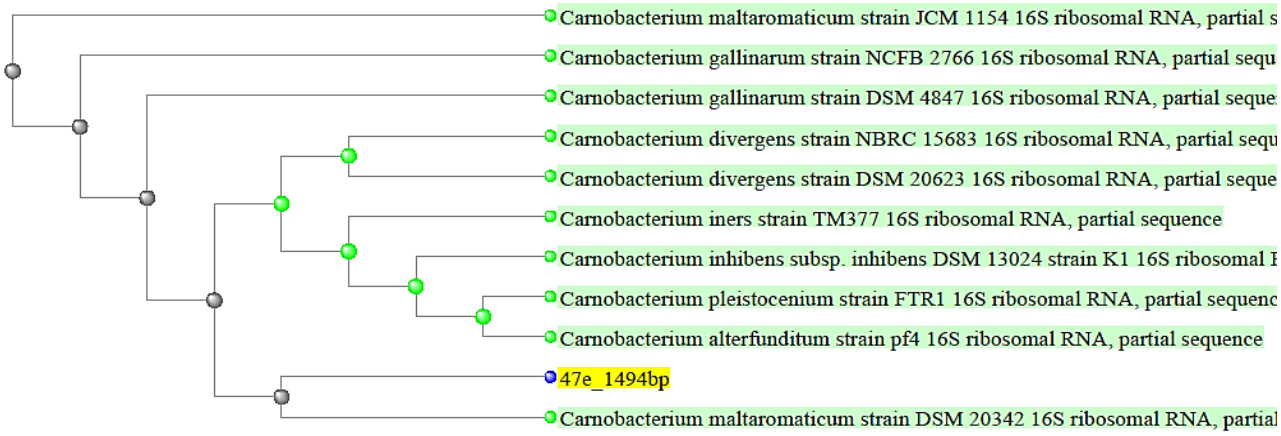


**Figure 4:** Phylogenetic tree representing isolate AM47e strain with closely related species constructed by sequencing analysis of 16s rRNA region using the Neighbor-Joining method.

the band consisted of 1494 bp. The results of the sequence were compared and identified using The Basic Local Alignment Search Tool (BLAST) to find the similarity between sequences. The sequence was 99.93% similar to *Carnobacterium maltaromaticum* with GenBank accession number CP045040. Figure 4 shows the phylogenetic tree of the isolate AM47e strain with related species. Herein, this confirmed BLAST search was successfully submitted to GenBank with accession number MZ934727.

## DISCUSSION

Colony of *C. maltaromaticum* strain AM47e, AM54d and AM80d were white with 0.5-1.0 mm diameter, circular form, convex elevation, entire margin and opaque on MRS agar. The strains were negative for catalase and IMViC (indole, MR, VP and citrate) tests. However, the strains were positive for lactose and glucose test. This result contradicted the *C. maltaromaticum* isolated from whitefish, which was revealed positive for MR and VP tests (Loch *et al*., 2008). A similar positive VP test was observed from *C. maltaromaticum* isolated from chilled meats and seafood (Laursen *et al*., 2005). Biochemical features do not reflect the whole extent of the species genomic accurately. Phenotypic properties also can be unstable at times and expression can be affected by changes in environmental factors such as temperature, growth substrate and pH levels (Janda and Abbott, 2002). Of note, this species was identified as γ-hemolysis, which did not hemolyse erythrocytes. This agrees with the claim by Afzal *et al*. (2010) that *C. maltaromaticum* has never been implicated in human infection and only one case had been reported in the medical literature that isolated from human pus.

The strains obtained were taken to antibiotic susceptibility tests. The testing aimed to detect possible drug resistance in *C. maltaromaticum* and to assure susceptibility to tetracycline, streptomycin, ampicillin and meropenem for the strain. Referring to the results in Table 3, *C. maltaromaticum* was sensitive to tetracycline (10 µg), ampicillin (10 µg) and meropenem (10 µg) but resistant to streptomycin (10 µg). This contradicted the *C. maltaromaticum* strain sm-2 from Arctic migratory salmonids, which indicated that the *C. maltaromaticum* was ampicillin and streptomycin-resistant (Moniz *et al*., 2021). Other findings suggest that *C. maltaromaticum* isolated from arctic char (*Salvelinus alpinus*) and lake white fish (*Coregonus clupeaformis*) was susceptible to tetracycline $MIC_{50}$ (µg/µL) and yet resistant to ampicillin $MIC_{50}$ (µg/µL) (Moniz *et al*., 2021). Herein, the diverse environment could lead to different susceptibility of bacteria towards different antibiotics. Gene transfer between *C. maltaromaticum* and other species in the same environment may occur, resulting in distinct antibiotic resistance.

In this study, molecular identification was made by targeting the 16S rRNA. The prokaryotic ribosome consists of a large subunit (with 5S rRNA and 23S rRNA) and a small subunit (with 16S rRNA). Ribosomal RNA (rRNA) genes have been used as a source of phylogenetic information (Kim *et al*., 2019). The 16S RNA genes have become a standard in bacteria taxonomic classification due to rapid sequenced, easier to conduct and containing enough phylogenetic information (Caporaso *et al*., 2011). Analysis using 16S rRNA is important for reliability, quality assurance and organisms safety which is able to identify the species of isolates accurately. The function of 16S rRNA gene over time has been consistent and sufficient for informative purposes (Muryany *et al*., 2017).

*Carnobacterium maltaromaticum* is known as mesophilic bacteria, which is able to grow in the range of 11°C to 45°C. This study showed that the best temperature to grow *C. maltaromaticum* for the three strains was 37°C, indicated by the highest OD. This species was observed to be able to grow at 45°C. In a different study, it was reported that *C. maltaromaticum* was able to grow in meat products at temperatures as low as -1.5°C to 2°C and the maximum growth temperature,

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40°C (Leisner *et al*., 2007). However, Caillies-Grimal *et al*. (2007) discovered that 30 °C is the optimal growth temperature for *C. maltaromaticum*.

*Carnobacterium maltaromaticum* can be found mostly in dairy products, meat and fish. It has potential applications in the food industry especially related to health protection products. This LAB species is involved in the biopreservation of food by inhibiting the growth of foodborne pathogens in cold conditions, such as *Listeria* sp. and the development of flavor in ripened cheese varieties (Puentes *et al*., 2021). In these applications, the temperature and pH of the acidifying activity play an important role on an industrial scale. Girardeau and team (2019) reported that *C. maltaromaticum* must be cultivated at 20 °C, pH 6 and harvested at the beginning of the 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 are needed. Danielski and colleagues (2020) reported that *C. maltaromaticum* inhibited the 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 an additive in preventing food spoilage, especially in the meat and seafood industry (Lo and Sheth, 2021).

## CONCLUSION

Three isolates of *C. maltaromaticum* AM47e, AM54d and AM80d were isolated from a total of 100 animals of *A. molpadioides*. They were Gram-positive bacilli, negative to catalase and IMViC tests, displayed γ-hemolysis and positive to lactose and glucose tests. The isolates grow at an optimum temperature 37 °C. *Carnobacterium maltaromaticum* strains AM47e, AM54d and AM80d were resistant to streptomycin (10 µg) but sensitive to tetracycline (10 µg), ampicillin (10 µg) and meropenem (10 µg). In conclusion, *C. maltaromaticum* can be isolated from the intestine of *A. molpadioides* with the potential of the probiotic applications and less potential vulnerability of the consumers.

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## CONFLICTS OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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