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Gut bacteria isolates of *Hirudinaria manillensis* and its antibacterial activities

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ABSTRACT. Leeches have been used in traditional and modern medical practices for centuries; however, information on leech species identification, gut-associated bacterial communities, and their potential antimicrobial interactions remains limited. This study aimed to identify leech specimens and isolate and characterise their gut-associated microbiota with potential antibacterial activity. Leech samples were collected from two locations: Kemaman, Terengganu, and Jengka, Pahang, Malaysia. Species identification was conducted based on external and internal morphological characteristics. Gut bacteria were isolated and differentiated according to colony morphology and cellular characteristics. The antibacterial activity of the isolates was evaluated using cross-streaking and disk diffusion assays against selected pathogenic bacteria, namely *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. Morphological analysis confirmed that all leech specimens belonged to the dark buffalo leech, *Hirudinaria manillensis*. A total of three bacterial isolates were successfully obtained from the leech gut, of which two showed observable zones of inhibition against the tested bacteria. Overall, this study provides baseline information on the morphology of *H. manillensis* and its associated gut microbiota. The findings enhance current understanding of leech–microbe interactions and provide a basis for future studies on the antimicrobial potential of leech-associated bacteria.

Key words: Leeches, Southeast Asian freshwater, sanquivorous

1. INTRODUCTION

The first antibiotic recovery was from *Penicillium notatum*, which secreted Penicillin. Until then, many more classes of antibiotics have been reported; some from natural sources and others are synthetic. This compound has been proven to treat infectious diseases and help to improve global human health. The antibiotic revolution is a breakthrough technology, but inappropriate use has led to serious medical challenges. The emergence of resistant pathogens due to the prolonged use of this antibiotic is undeniable. Antibiotics are becoming useless and are collapsing the human health system, including treatment failure, prolonged illness, and increased mortality. This situation creates a serious gap in treatment options and highlights the need for a new antibiotic with effective modes of action against resistant pathogens. Therefore, exploring natural sources, particularly symbiotic bacteria, could be beneficial for identifying new antimicrobial compounds.

Microorganisms can be found almost everywhere, and their survival often depends on competition, symbiosis, and parasitism. To adapt to this interaction, microorganisms develop several survival strategists including genetic exchange, biofilm formation, antimicrobial production, and metabolic cooperation. Many classes of antibiotics are listed, such as streptomycin, tetracycline, gentamicin, penicillin, cephalosporins, and peptide antibiotics by *Bacillus*

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sp. There are secondary metabolites of their producer. These kinds of compounds are commercially feasible and sustainable. Their mass production can be controlled and relatively easily purified. A distinct class of antimicrobial is a bacteriocin whose production is encoded in its genes (Solis-Balandra & Sanchez-Salas, 2024; Sidhu & Nehra, 2021). Bioengineering will benefit from that while manipulating their genes, their production can be enhanced to improve stability and their mode of action to a more specific target of the pathogen. To date, even though many antibiotics are available, searching for newly discovered is crucial. A few new antibiotics have been reported, such as Saarvernin (Kaur et al., 2025) and Lariocidin (Silver, 2025). However, despite these novel antibiotic discoveries, antibiotic development continues to fall behind the rapid rise of antimicrobial resistance.

One promising environment for discovering antimicrobial-producing microorganism is the gut microbiome. The gut microbiome is a complex ecosystem that provides an ecological niche for many microorganisms. Within this well-structured community complex lies intense competition for nutrients and space among them. Microorganisms evolve, producing antimicrobial compounds either to kill or inhibit their competitors' growth. Besides, the gut microbiome plays a pivotal role in the host's health. Gut microbiome supports digestive function, competitor to pathogens, and plays a significant role in regulating the immune system. The gut microbiome produces a metabolic product that indirectly becomes signal compound that boosts complex interactions with other residents and shapes both innate and adaptive immunity of the host (Ullah et al., 2024; Xue et al., 2020; Kamada et al., 2013).

Due to its biological importance, gut microbiome has been widely studied in both There are tremendous studies of vertebrates and invertebrates. One of the earliest studies on invertebrate gut microbiomes involved seven deep-water invertebrates and was conducted by Dilmore & Hood (1986), which was recorded as the first published paper in the Scopus database. Later, studies on vertebrate gut microbiomes, such as fish gut microbiomes, were reported by Olsen et al. (2002). A lot more researchers are deep into vertebrate microbiomes and have reported more massive data on it, as de Jonge et al. (2022) cover 54 Mammalian species, Alberdi et al. (2021) 24 species, and Xie et al. (2024) reported 113 vertebrate species. Even though the vast diversity of organisms is invertebrate, data on the invertebrate gut microbiome is still limited (Petersen & Osvatic, 2018). Yun et al. (2014) reported 218 species of invertebrate gut microbiome in their single study, but most of the available studies still focus on specific host. This indicates that invertebrate gut microbiome remains less explored, and yet the feasibility of finding novel antimicrobial bacteria is high.

Among invertebrates, leeches are an interesting host for gut microbiome studies because they inhabit aquatic environment such as ponds, marshes, and slow-flowing rivers. These habitats expose leeches to diverse bacteria and microorganisms. In response to this environment leeches maintain a specialized gut microbiota that is critical to the health and ecological adaptation (Phillips et al., 2020). These microbiotas have gained interest in studies over the past few years in the gut of leeches. This is given the fact that there is an increased level of antibiotic resistance in the world. Many of the old antibiotics have been failing or ineffective, and people are in search of alternative sources of antibiotics or other antimicrobial agents. There is also a possibility that bacteria that inhabit the avascular gut of leeches would be a tremendous source of exciting new drugs because we understand little about these bacteria

(Davido et al., 2025). Therefore, exploring leech gut bacteria may lead to the discovery of relevant microbes with potential applications in developing new treatment for resistant infections.

Before investigating the antimicrobial potential of leech gut. Bacteria, accurate identification of leech species is essential. Differences in feeding behavior, habitat, and physiology can influence the composition of their gut microbiota. Variations in gut microbial communities among leech species may result in differences in antimicrobial potential and bioactive compound production. Therefore, reliable taxonomic identification of leeches is a critical first step to ensure biological and ecological relevance of the findings. Performing leech identification before bacterial isolation allows for correct association between host species and their gut-derived bacteria. This approach improves the reproducibility, comparability, and scientific validity of subsequent antimicrobial screening studies. Therefore, this study aimed to identify leeches based on external and internal morphological characteristics and to isolate and characterise gut microbiota associated with potential antimicrobial activities.

2. METHODOLOGY

Leeches were collected from 2 locations in October 2025, one at the Kampung Mak Lagam (4°11'20.0"N 103°22'58.3"E, 4°11'38.9"N 103°23'12.0"E), Kamaman, Terengganu and one at UiTM Campus Jengka (3°45'26.0"N 102°34'06.0"E). Leech specimens were collected and transported to the laboratory in glass jars with a small pinhole puncture to allow air circulation, where they were carefully examined and identified. Leeches underwent an isolation period to empty their guts before bacterial isolation.

2.1. Identification of leech species

Each leech was identified based on morphological characteristics including body coloration, dorsal patterns, and size. Species identification was based on established taxonomic keys available from earlier journal studies (Zulhisyam et al., 2014; Chong et al., 2014).

2.2. Gut content extraction

The gut microbiota components of leeches were examined using a standardized gut extraction process. The live leeches were soaked in 90% ethanol for five minutes to sterilize the outer surface and to anesthetize the leech. After that, the leeches were placed on a sterile dissection tray. During aseptic conditions, sterile scissors were used to make a longitudinal incision along the ventral midline. The sterile forceps were used to properly expose and remove the stomach tissue, and only the gut content needed to be collected. The gut contents were transferred into sterile microcentrifuge tubes and mixed with 1 ml of saline solution. The mixture was stirred until homogeneous, then stored in the refrigerator to make a stock.

2.3. Bacterial isolation and characterization

A basic microbiological technique for separating pure bacterial colonies from a mixed microbial population like the intestinal contents of leeches, is the broth solution, spreading and streaking plate method. The gut extracts are distributed on a nutrient agar, which improves the likelihood of obtaining isolated colonies. The bacterial isolates were further purified until pure cultures were obtained. These pure colonies are undergoing microbiological analysis, including morphological characterization and Gram stain to distinguish among isolates.

2.4. Preparation of Antibacterial Compound Solution

The experiment aimed to induce antibacterial agents in isolated bacterial cultures against the tested bacteria *Escherichia coli, Bacillus subtilus* and *Staphylococcus aureus*. Co-cultures were prepared by culturing isolated bacteria with one of the three tested bacteria in a conical flask containing 50 ml of nutrient broth. The co-culture medium was incubated at 35°C for 48 hours. Then, the broth was centrifuged at 8000 rpm for 20 minutes to obtain cell-free supernatant of co-culture (CFS-Co). CFS-Co was tested for its antibacterial activities against the same bacteria to which it was exposed while preparing CFS-Co. CFS-*E. coli* was prepared using *E. coli*, CFS-*S. aureus* was prepared using *S. aureus*, and CFS-B. *subtilis* was prepared using *B. subtilis*.

2.5. Determination of Potential Antimicrobial Activities

Antibacterial screening was performed to assess the inhibitory capacity of the bacterial isolates derived from the leech gut against the suggested test bacteria. Two methods to determine antimicrobial activities applied are cross-streak and disc diffusion techniques. The cross-streak method enables immediate identification of antagonistic interactions between isolates and test bacteria through the observation of growth inhibition within the intersection zone (Suman & Yugandhar, 2021).

The second method is Kirby-Bauer disk diffusion. This approach is commonly used to study the potential applications of bacterial extracts in preventing the growth of selected microorganisms. A Nutrient Agar plate was smeared with the tested microorganisms. Using sterile forceps, the discs were placed on the agar surface, and one was an antibiotic disc, which is streptomycin as a positive control, and sterile broth as a negative control. 25 µL of CFS-Co broth was placed in each disc. The plates were incubated for 18 to 24 hours in an incubator set at 37°C. The diameter of the inhibitory zones surrounding each well, measured in millimetres, is used to calculate the antibacterial activity following incubation.

3. RESULTS AND DISCUSSION

3.1. Identification of the external and internal anatomy of the leech

Based on morphological observation and dissection, the leeches from two locations were identified as *Hirudinaria manillensis.* The morphological characteristics of the specimens studied using a stereomicroscope, are presented in Figure 1 (A-H). The front view shows that the body had a granular skin texture with orange spotting along the sides. The leech specimen had 34 annuli body segments clearly visible in the dorsal view, with five pairs of anterior eye spots arranged in a V-pattern, which was the major identification characteristic of the leech. The color of the leech's body is dark brownish with black line with a square pattern along the dorsal and lateral sides and dark green on ventral side. The lateral view of the leech highlighted the body shape and the arrangement of the sensory papillae, which are characteristic of buffalo leeches found in this region, while the close-up view presented the granular texture of the skin and the presence of mucus pores, which are characteristic of the outer covering of this species of leech

(Jeratthitikul et al., 2020). Figure 1 (F, G) displays that the posterior sucker, which is about double the size of the small anterior sucker, is consistent with *H. manillensis* from Southeast Asia, as described by Tubtimon et al. (2014). The oral region of the mouth with the usual three jaws with sharp conical teeth, consistent with ultrastructural details from similar populations (Bam et al., 2025). The length of leeches is approximately 120 mm, which indicates the adult phase. The measured body length of around 120 mm indicates that the sample is a mature adult, as buffalo leeches *Hirudinaria manillensis* and similar species are often documented to reach lengths of 3–10 cm when completely developed in their natural habitats (Chuang et al., 2022). Based on observation in Figure 1 (H), leeches have both male and female reproductive organs in a single individual. In relation to *Hirudo* species, the male and female gonopores are outwardly distinct, divided by a sequence of annuli, illustrating a consistent anatomical configuration of reproductive pores utilized in taxonomic classification (Arias et al., 2021).

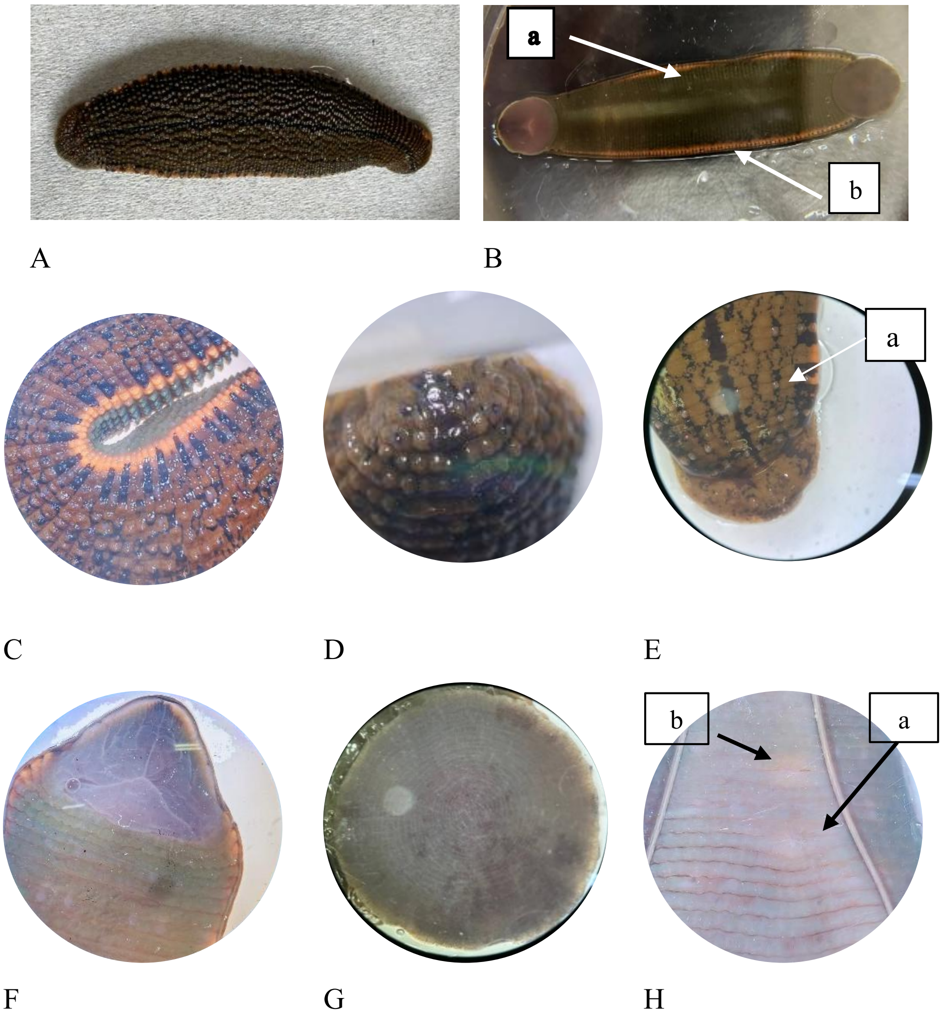

Figure 1. Morphological characteristics *Hirudinaria manillensis*: (A) Front view with granular surface skin texture ; (B) Dorsal view: 34 annuli body segments (a), Orange lateral spotting (b); (C) Black stripe pattern at ventral side; (D) D 5 pairs of eyes; (E) Mucus secretion glands visible along body margin; (F) anterior sucker; (G) posterior sucker; (H) Reproductive organ (Gonad): Female (a); Male (b).

As shown in Figure 2, the arrangement of the internal organs clearly showed the typical strong muscle structure as well as segmentation of the *Hirudinaria* species as per previous studies by (Guan et al., 2020). Internal examination of the leech disclosed reproductive and digesting systems typical of these kinds of leeches. Based on Figure 2, the specimen has paired testis sacs, signifying a hermaphroditic reproductive system, a characteristic prevalent among members of the class Hirudinea (Phillips et al., 2020; Hosseini et al., 2024). Additionally, testes sacs were estimated to be 10 pairs in an arranged manner. The size of testes sacs is small with shape of a red bean in structure which it connected to the crop. Next, the crop is like vein structure that is located at the center of the body, and it connected to caeca. However, the crop can only be seen if the leech consumes blood because it will fill in like a balloon, but it became colorless if the leech does not consume blood (Karasartova et al., 2025).

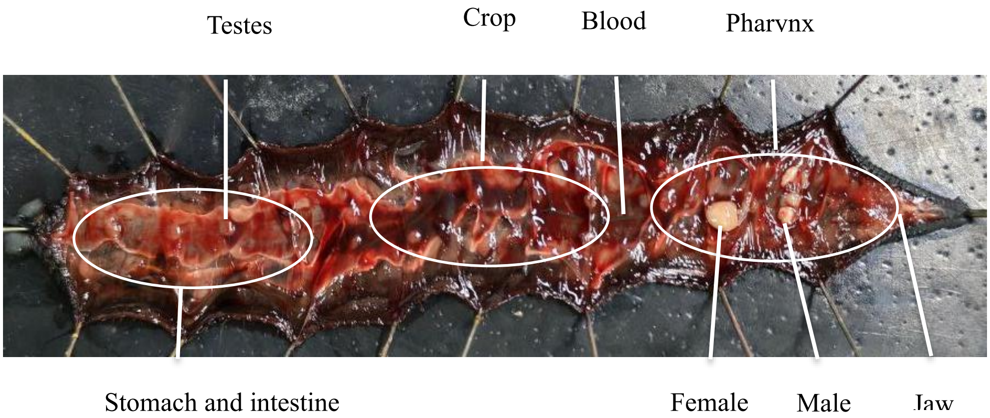

Figure 2. Internal organs of *Hirudinaria manillensis*

3.2. Isolation and characterization of bacterial gut microbiota

Three bacteria with distinguishable colony morphologies were successfully isolated. A 2 out of three isolates are Gram-positive. Isolate 1 appears to be Gram-negative with a relatively short rod, whereas isolates 2 and 3 are Gram-positive rods. The first isolation was the white colonies, which had irregular shapes with undulated margins, convex elevation, and smooth surface texture. The second isolation was clear colonies, a circular shape with entire margins, raised elevation, and smooth surface texture. The third isolate was white colonies, circular form, umbonate elevation, with smooth surface texture. Table 1 summarizes the characteristics of isolate 1, isolate 2 and isolate 2.

Table 1. The characteristics of three isolated bacteria.

Characteristics		Isolate 1	Isolate 2	Isolate 3
Colony morphology	Color	White color and translucent	Clear color and transparent	White color and translucent
	Margin	undulate	entire	entire
	Elevation	convex	raised	umbonate
	Form	irregular	circular	circular
Cell morphology		Relatively short rod	rod	rod
Gram stain		Gram negative	Gram positive	Gram positive

3.3. Antibacterial activity

Positive antibacterial activity appears in both antibacterial screening methods that employ the potential of those isolated bacteria produce antibacterial compounds.

Preliminary screening

Figure 3 presents the results of the cross-streaking assay. The bacterial isolates showed consistent growth under the established culture conditions, indicating their ability to survive in the assay medium. Observable inhibition zones were detected around selected leech-associated bacterial isolates, as indicated by the arrows, suggesting potential antibacterial activity against the tested bacteria. These findings indicate that some gut bacterial isolates from *Hirudinaria manillensis* may produce antimicrobial compounds capable of inhibiting bacterial growth.

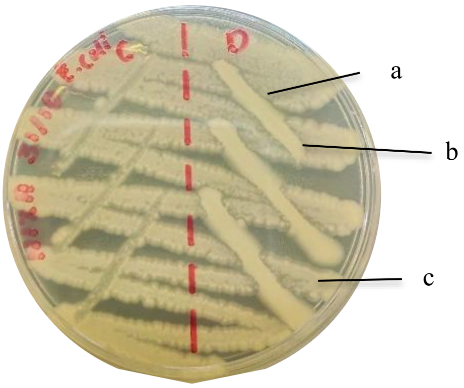

Figure 3. Cross streak: Inhibition zone of potential antibacterialaction (a), Isolated bacteria (b), *E. coli* (c).

Table 2 presents the one-way ANOVA results showing the effect of different leech gut bacterial isolates on antibacterial activity against three tested bacteria. Antibacterial activity was evaluated based on the diameter of the inhibition zone (mm). The analysis was performed to determine whether significant differences existed among the isolates in their ability to inhibit the growth of the tested bacteria. CFS-Isolate 1 have antimicrobial activity against *S. aureus* (13.50 ± 6.67) the large zone of inhibition followed by *E. coli* (10.75 ± 1.00) and *B. subtilis* (9.67 ± 1.00). While for CFS-isolate 2, the antimicrobial activity only positive against *B. subtilis* (9.67 ± 1.00) and *E. coli* (6.89 ± 1.54) and but not for *Staphylococcus aureus*. CFS-isolate 3, the antimicrobial activity only positive against *S. aureus* (6.67 ± 0.82). One-Way ANOVA was run separately for each different tested bacteria and the analysis was performed at $\alpha = 0.05$ with $N = 3$. Statistical analysis revealed that there is a significant different exist among sample against all three tested bacteria. Tested against *B. subtilus*, Tukey's HSD test revealed significant differences between CFS-Isolate 1, CFS-Isolate 2, compared to the Negative control and Positive control (p=0.01). While for *E. coli*, Tukey's HSD post hoc test revealed significant differences between CFS-Isolate 1 to the negative control (p=0.015). Whereas for *S. aureus*, Tukey's HSD test revealed significant differences between CFS-Isolate 1 and the negative control (p=0.015). However, in all Tukey's test, CFS-co culture differed significantly from positive controls. The observed inhibition suggests that antimicrobial metabolites produced during co-cultivation of Isolate 1 and 2 are responsible for inhibiting the growth E. coli, *B. subtilis* and S. aureus. Co-culture systems have been shown to produce extracellular antimicrobial metabolites in inhibiting S. *aureus* and other Gram-positive bacteria (Sun et al., 2022). CFS-Isolate 1 is the most promising candidate to be explored and the feasibility of this isolate producing antibacterial compounds as its secondary metabolites is high.

Table 2. One-Way ANOVA of mean zone of inhibition (mm) of Supernatant Cell Free Supernatant (CFS) against the same co-culture bacteria.

Sample	Tested Bacteria		
	B. subtilis	*E. coli*	*S. aureus*
CFS-Isolate 1	9.67 ± 1.00^{b}	10.75 ± 1.00^{b}	13.50 ± 6.67^{b}
CFS-Isolate 2	9.67 ± 1.00^{b}	6.89 ± 1.54^{c}	6.00 ± 0.00^{c}
CFS-Isolate 3	6.00±0.00^{c}	6.00±0.00^{c}	6.67±0.82^{c}
Streptomycin (Positive control)	21.34 ± 4.71^{a}	20.89 ± 3.43^{a}	21.00 ± 0.00^{a}
Sterile Broth (Negative control)	6.00 ± 0.00^{b}	6.00 ± 0.00^{b}	6.00 ± 0.00^{c}

Means sharing the same superscript letter are not significantly different (Tukey's HSD, $p < 0.05$).

CONCLUSION

The findings of this study confirmed the leech specimens as *Hirudinaria manillensis* based on external morphological characteristics, including body shape, segmentation, colour pattern, and other anatomical features observed under laboratory conditions. Internal anatomical examination further supported the identification by revealing characteristic features commonly associated with hirudinean leeches. Three distinct bacterial isolates were successfully obtained from the leech gut and subjected to preliminary cross-streak screening. Among these isolates, two showed potential antimicrobial activity by inhibiting the growth of the tested bacteria. Isolate 1, identified as a Gram-negative rod-shaped bacterium, exhibited the most promising inhibitory activity. Overall, this study provides preliminary information on the morphological characteristics of *H. manillensis* and the properties of its associated gut bacteria. These findings contribute to a better understanding of leech–microbe interactions and provide a foundation for future studies on the antimicrobial potential of leech-associated microbiota.

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AUTHOR CONTRIBUTIONS

Siti Suhaila conceived and designed the study. Fairuzniza Nur Ain and Muhammad Azfarull Hariqh performed the experiments and collected the data. All authors carried out the statistical analysis and drafted the manuscript. Siti Suhaila contributed to the revision and approved the final version.

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DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this manuscript, the author(s) used ChatGPT (OpenAI) to assist with language editing, including grammar and clarity improvement. The author(s) carefully reviewed and revised the content as necessary and took full responsibility for the final version of the publication.

DATA AVAILABILITY

Not applicable.

COMPETING INTEREST

The authors declare that there are no competing interests.

COMPLIANCE OF ETHICAL STANDARDS

Not applicable.

SUPPLEMENTARY MATERIAL

No supplementary material is associated with this article.

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