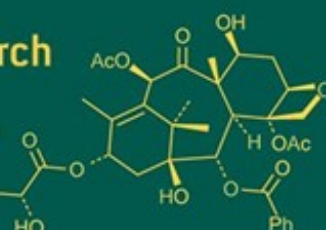
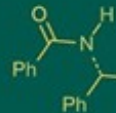


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Isolation, screening and molecular identification of chitinolytic bacteria from the gut of white grub

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ABSTRACT

Chitinolytic bacteria are potential sources of chitin-degrading enzymes with applications in agricultural and biotechnological processes. Insect gut-associated microorganisms represent a comparatively less explored source of such bacteria. The present investigation was undertaken to isolate, screen and molecularly characterize chitinolytic bacteria associated with the gut of white grub larvae collected from agricultural fields of Kolhapur district, Maharashtra, India. Gut-associated bacteria were isolated and purified using colloidal chitin agar (CCA) medium. A total of 11 morphologically distinct bacterial isolates were obtained and screened for chitinolytic potential based on clear hydrolysis-zone formation on CCA. Degradation efficiency was calculated from colony and clear-zone diameters, and two isolates showing comparatively higher chitinolytic potential were selected for molecular characterization. Among the isolates screened, Isolate 3 produced the largest clear hydrolysis zone of 23 mm with a degradation efficiency of 130.0%, while Isolate 10 produced a clear zone of 22 mm with a degradation efficiency of 120.0%. These isolates were designated HSChitin-1 and HSChitin-2, respectively. Partial 16S rRNA gene sequencing followed by sequence analysis using MEGA 11 and BLASTn comparison against the NCBI nucleotide database showed that HSChitin-1 had its highest similarity to *Sphingobacterium multivorum* strain IAM 14316, with 100% query coverage, 91.19% sequence identity and an *E*-value of 0.0. HSChitin-2 showed highest similarity to members of the genus *Stenotrophomonas*, with 100% query coverage, 93.15% sequence identity and an *E*-value of 0.0. The partial 16S rRNA gene sequences were deposited in the NCBI nucleotide database under accession numbers PZ779901 and PZ783905, respectively. The findings demonstrate that white grub gut-associated bacteria can serve as a potential source of chitinolytic microorganisms and provide promising isolates for further quantitative enzyme characterization.

Keywords: Chitinolytic bacteria, White grub, Gut-associated bacteria, Colloidal chitin agar, Degradation efficiency, 16S rRNA, *Sphingobacterium*, *Stenotrophomonas*

1. Introduction

Chitin is one of the most abundant natural polysaccharides and is widely distributed in nature, particularly as a structural component of fungal cell walls and the exoskeletons of insects and other arthropods (Yan *et al.*, 2015; Martínez-Zavala *et al.*, 2020) [2, 1]. It is a linear, unbranched polymer composed predominantly of β -(1 $\rightarrow$ 4)-linked *N*-acetyl-D-glucosamine units and contributes substantially to the structural integrity of numerous organisms (Veliz *et al.*, 2017) [3]. The continuous degradation and recycling of chitin in natural ecosystems are mediated largely by microorganisms capable of producing extracellular hydrolytic enzymes. Chitinases (EC 3.2.1.14) are glycosyl hydrolases that catalyse the hydrolysis of β -(1 $\rightarrow$ 4)-glycosidic linkages in chitin and play an important role in the conversion of this complex polymer into simpler compounds (Yan *et al.*, 2015; Elieh-Ali-Komi *et al.*, 2016) [2, 4]. Chitinolytic microorganisms, particularly bacteria and fungi, are important contributors to the natural degradation of chitin and have consequently received considerable attention as potential sources of chitinase enzymes. Chitinase-producing bacteria have been reported from diverse environments, including soil, rhizosphere and insect-associated habitats. Several bacterial genera, including *Bacillus*, *Pseudomonas*, *Streptomyces*, *Paenibacillus*, *Serratia* and *Stenotrophomonas*, have been reported to possess chitinolytic activity (Cody *et al.*, 1990; Brzezinska *et al.*, 2008; Dukare *et al.*, 2020; Kushwaha & Lalit, 2023) [6, 5, 7].

Insect-associated microorganisms represent an important and comparatively less explored source of chitinolytic bacteria. The insect gut harbours diverse microbial communities that contribute to digestion, nutrient transformation and degradation of complex organic substrates. Gut-associated microorganisms may therefore possess diverse hydrolytic capabilities and represent a potential source of bacteria with useful enzymatic properties (Akami *et al.*, 2019; Jones *et al.*, 2019) [8, 9]. Studies on scarab beetles have demonstrated consid

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erable diversity of bacteria associated with their gut and have indicated their possible contribution to the degradation and utilization of complex dietary substrates (Egert *et al.*, 2003; Andert *et al.*, 2010; Chouaia *et al.*, 2019) [11, 10, 12].

White grubs are soil-dwelling larval stages of scarab beetles (Coleoptera: Scarabaeidae) and are important agricultural pests because their larvae feed on plant roots and can cause considerable damage to several crops. Their subterranean habitat and feeding behaviour expose them to diverse microorganisms, while their digestive tract provides a favourable environment for the establishment of metabolically diverse microbial communities. These gut-associated bacteria may contribute to the breakdown of complex organic compounds and therefore represent a potential source of microorganisms with chitinolytic and other enzymatic activities.

Previous studies have demonstrated the occurrence of chitinolytic bacteria in insect-associated habitats. Karki *et al.* (2023) [13] isolated chitinase-producing bacterial isolates from the gut of pleurostict scarab beetle grubs and selected promising isolates based on their chitinolytic activity. Similarly, Chutima *et al.* (2023) recovered chitinolytic bacteria from the gut and exoskeleton of termites, while Winssy *et al.* (2024) [14] reported gut-associated bacteria from *Spodoptera frugiperda* exhibiting chitinase-producing ability. These findings indicate that insect-associated microbial communities can serve as valuable sources of chitinolytic bacteria.

Colloidal chitin agar (CCA) is commonly used for the preliminary screening of chitinolytic microorganisms. The formation of a clear hydrolysis zone surrounding bacterial colonies on CCA indicates degradation of chitin in the medium and provides a convenient qualitative measure of chitinolytic potential. The diameter of the clear zone and degradation efficiency can subsequently be used to compare isolates and select promising candidates for further characterization (Patil *et al.*, 2003) [15]. Several studies have successfully employed colloidal chitin-based media for the isolation and screening of chitinolytic bacteria from environmental and insect-associated sources (Abdullah, 2020; Dukare *et al.*, 2020; Karki *et al.*, 2023) [16, 7, 13].

Despite increasing interest in insect-associated chitinolytic microorganisms, information on chitinolytic bacteria associated with the gut of white grub larvae from the Kolhapur region of Maharashtra remains limited. Identification and characterization of such bacteria may contribute to a better understanding of the functional potential of white grub-associated microbiota and provide promising bacterial isolates for further biological and biotechnological investigations. Therefore, the present investigation was undertaken to isolate, screen and molecularly characterize chitinolytic bacteria from the gut of white grub larvae.

2. Materials and Methods

2.1 Collection of white grub larvae

White grub larvae were collected from infested agricultural fields located at Haldi (16.589267° N, 74.163116° E) and Vasagade (16.677231° N, 74.340911° E) villages of Kolhapur district, Maharashtra, India. Larvae collected from Haldi were associated with

sugarcane, whereas those collected from Vasagade were associated with groundnut. The collected larvae were carefully transferred to sterile containers and transported to the Entomology Laboratory, R.C.S.M. Government College of Agriculture, Kolhapur, for further processing and isolation of gut-associated bacteria.

2.2 Dissection and isolation of gut-associated bacteria

The collected white grub larvae were processed under aseptic conditions for isolation of gut-associated bacteria. Before dissection, the larvae were surface sterilized by immersing them in 70% ethanol for 1 min, followed by three successive washes with sterile distilled water to remove surface-associated microorganisms.

The surface-sterilized larvae were mounted on a paraffin wax dissecting plate using sterile pins. A mid-ventral incision was carefully made using sterile fine scissors and forceps, and the alimentary canal was exposed. Visible fat bodies and adhering tissues were carefully removed under aseptic conditions. Sterile saline was gently flushed into the body cavity to facilitate separation of the internal organs. The alimentary canal, particularly the fermentation chamber/hindgut region, was carefully excised and transferred aseptically into a sterile Eppendorf tube containing 0.85% sodium chloride (NaCl) solution.

The gut tissue was macerated using a sterile micropestle to obtain a homogeneous suspension. Ten-fold serial dilutions were prepared using sterile 0.85% NaCl solution. Seven sterile dilution tubes, each containing 9.0 mL of 0.85% NaCl, were arranged sequentially. One millilitre of the gut homogenate was transferred into the first tube to obtain the 10⁻¹ dilution, followed by serial transfer of 1 mL from each dilution into the subsequent tubes up to 10⁻⁷.

Aliquots of 1 mL from the 10⁻⁵, 10⁻⁶ and 10⁻⁷ dilutions were spread aseptically onto sterile colloidal chitin agar (CCA) plates. The inoculated plates were incubated in an inverted position at 30 ± 2 °C for 48–72 h. Bacterial colonies showing distinct morphological characteristics and clear zones on CCA were selected and purified by repeated streaking on fresh CCA plates. Pure cultures were maintained for subsequent screening.

2.3 Screening of bacterial isolates for chitinolytic potential

The purified bacterial isolates were screened for chitinolytic potential using colloidal chitin agar (CCA) medium. Each bacterial isolate was inoculated separately onto CCA plates and incubated at 28 ± 2 °C for 72 h. Chitin degradation was indicated by the formation of a distinct clear hydrolysis zone surrounding the bacterial colony.

After incubation, the colony diameter and clear hydrolysis-zone diameter were measured in millimetres using a ruler. All 11 bacterial isolates were evaluated based on their ability to produce clear zones on CCA. Isolates producing comparatively larger clear zones were considered promising chitinolytic isolates.

2.4 Determination of chitin degradation efficiency

The chitinolytic potential of the bacterial isolates was assessed based on clear hydrolysis-zone formation on CCA. Degradation efficiency (DE) was calculated from the measured colony diameter and clear-zone diameter using the formula described by Qureshi *et al.* (2012):

$$DE(\%) = \frac{\text{Clear zone diameter} - \text{Colony diameter}}{\text{Colony diameter}} \times 100$$

where,

DE= Degradation efficiency

The calculated DE values were used as a comparative screening index for selection of promising isolates. They were not considered a direct

quantitative measurement of the amount of chitin degraded or enzyme activity.

Table 1. Screening of bacterial isolates for chitinolytic potential on colloidal chitin agar

Isolate	Colony diameter (mm)	Clear-zone diameter (mm)	Degradation efficiency (%)
Isolate 1	10	18	80.0
Isolate 2	11	19	72.7
Isolate 3	10	23	130.0
Isolate 4	12	21	75.0
Isolate 5	10	17	70.0
Isolate 6	11	19	72.7
Isolate 7	10	18	80.0
Isolate 8	12	20	66.7
Isolate 9	10	20	100.0
Isolate 10	10	22	120.0
Isolate 11	11	20	81.8

Note: $DE (\%) = \frac{\text{Diameter of clear zone} - \text{Diameter of colony}}{\text{Diameter of colony}} \times 100$.

2.5 Selection of promising bacterial isolates

Based on preliminary screening on CCA and the calculated degradation efficiency, two bacterial isolates showing comparatively higher chitinolytic potential were selected for further characterization. Isolate 3, which produced a clear hydrolysis zone of 23 mm and recorded the highest degradation efficiency of 130.0%, and Isolate 10, which produced a clear hydrolysis zone of 22 mm with a degradation efficiency of 120.0%, were selected. These isolates were designated HSChitin-1 and HSChitin-2, respectively.

2.6 Sequence analysis and molecular characterization

The two selected bacterial isolates, HSChitin-1 and HSChitin-2, were subjected to partial 16S rRNA gene sequencing. Raw nucleotide sequencing data generated through Sanger sequencing were obtained as chromatogram files in .ab1 format and processed for sequence quality assessment.

The chromatograms were imported into Molecular Evolutionary Genetics Analysis (MEGA) software version 11 for visual inspection and sequence editing. Low-quality base calls were manually trimmed to obtain reliable nucleotide sequences. Following quality trimming, the finalized sequences were exported in FASTA format.

The resulting sequences were compared against the NCBI nucleotide database using the BLASTn algorithm. BLASTn results were evaluated with emphasis on sequence identity, query coverage and *E*-value. The closest database matches were considered for taxonomic characterization of the selected isolates.

The 16S rRNA gene sequencing was carried out with assistance from Agharkar Research Institute, Pune, while subsequent sequence analysis and taxonomic interpretation were performed with assistance from the concerned senior researcher.

2.7 Sequence accession numbers

The partial 16S rRNA gene sequence of HSChitin-1 was deposited in the NCBI nucleotide database under accession number **PZ779901**. The partial 16S rRNA gene sequence of HSChitin-2 was deposited under accession number **PZ783905**.

3. Results

3.1 Isolation of gut-associated bacteria

Following dissection, serial dilution, plating and purification of gut-associated bacterial colonies, a total of 11 morphologically distinct bacterial isolates were recovered. The purified isolates were subsequently screened for chitinolytic potential using CCA.

3.2 Screening of bacterial isolates for chitinolytic potential

All 11 bacterial isolates produced distinct clear hydrolysis zones on CCA, indicating their ability to degrade colloidal chitin under the experimental conditions. Considerable variation was observed among the isolates in colony diameter, clear-zone diameter and calculated degradation efficiency (Table 1).

The clear-zone diameter ranged from 17 to 23 mm, whereas the calculated degradation efficiency ranged from 66.7 to 130.0%. Isolate 3 produced the largest clear hydrolysis zone of 23 mm and recorded the highest DE of 130.0%. Isolate 10 produced the second-largest clear zone of 22 mm, with a DE of 120.0%. Isolate 9 also showed comparatively high DE of 100.0%, with a clear-zone diameter of 20 mm.

Based on the comparatively higher clear-zone formation and degradation efficiency, Isolate 3 and Isolate 10 were selected as the two promising isolates and designated HSChitin-1 and HSChitin-2, respectively.

Figure 1. Chitinolytic activity of selected bacterial isolates on CCA

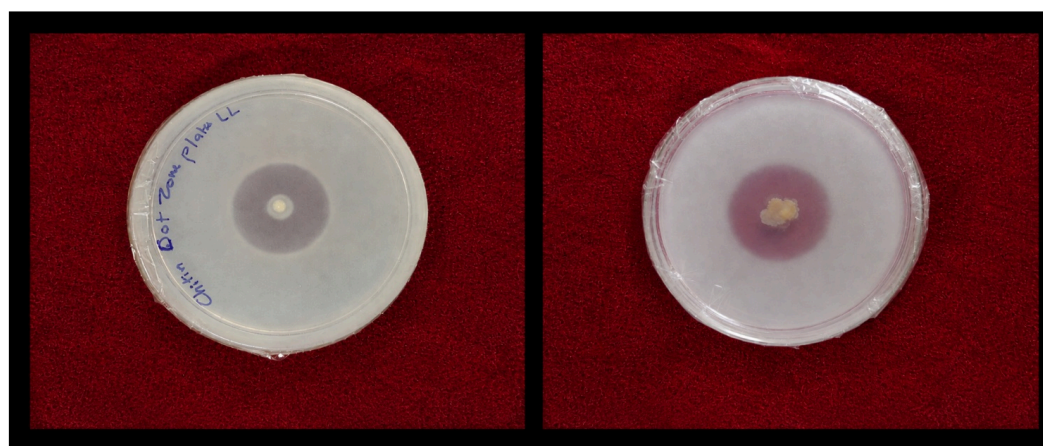


Figure 1. Clear hydrolysis zones produced by the selected bacterial isolates on colloidal chitin agar: (A) HSChitin-1 (Isolate 3) and (B) HSChitin-2 (Isolate 10). Clear zones surrounding the colonies indicate chitinolytic potential.

3.3 Molecular characterization of selected isolates

The two selected isolates were subjected to partial 16S rRNA gene sequencing and BLASTn analysis.

HSChitin-1

The partial 16S rRNA sequence of HSChitin-1 was 813 bp in length and was deposited under accession number PZ779901. BLASTn analysis showed its highest similarity to *Sphingobacterium multivorum* strain IAM 14316 (NR_040953.1), with 100% query coverage, 91.19% sequence identity and an *E*-value of 0.0. Because the sequence identity was 91.19%, the result is interpreted conservatively as the closest/highest BLASTn match to *S. multivorum* rather than defin-

itive species-level identification solely on the basis of the partial 16S rRNA sequence.

HSChitin-2

The partial 16S rRNA sequence of HSChitin-2 was 409 bp in length and was deposited under accession number PZ783905. BLASTn analysis showed highest similarity to members of the genus *Stenotrophomonas*, with 100% query coverage, 93.15% sequence identity and an *E*-value of 0.0. The top-ranked match shown in the BLAST result was *Stenotrophomonas nematodica* strain W5 (NR_181111.1); however, several other *Stenotrophomonas* species showed similar identity and coverage values. Therefore, HSChitin-2 was conservatively assigned as *Stenotrophomonas* sp.

Table 2. BLASTn results of selected chitinolytic bacterial isolates

Isolate	Sequence length (bp)	Highest/closest BLASTn match	Reference accession	Query coverage	Sequence identity	<i>E</i> -value	NCBI accession
HSChitin-1	813	<i>Sphingobacterium multivorum</i> strain IAM 14316	NR_040953.1	100%	91.19%	0.0	PZ779901
HSChitin-2	409	<i>Stenotrophomonas</i> sp.	NR_181111.1*	100%	93.15%	0.0	PZ783905

Note: NR_181111.1 corresponds to the top-ranked *Stenotrophomonas nematodica* strain W5 match in the supplied BLASTn result. Multiple *Stenotrophomonas* taxa showed comparable BLAST statistics; therefore, HSChitin-2 is reported conservatively as *Stenotrophomonas* sp.

4. Discussion

The present investigation demonstrated that the gut of white grub larvae harbours culturable bacterial isolates with chitinolytic potential. A total of 11 bacterial isolates were recovered from dissected gut material and subsequently screened using CCA. The occurrence of chitinolytic bacteria in insect-associated habitats has also been reported by several researchers, indicating that insect gut microbiota can serve as a source of microorganisms with hydrolytic capabilities. Akami *et al.* (2019) [8] and Jones *et al.* (2019) [9] reported diverse functional roles of insect-associated microorganisms in digestion and nutrient transformation. The recovery of chitinolytic bacteria in the present study further indicates the potential of white grub-associated gut microbiota as a source of bacteria capable of utilizing chitin-containing substrates.

CCA provided an effective preliminary method for screening the bacterial isolates for chitinolytic potential. The formation of clear hydrolysis zones around bacterial colonies indicated degradation of colloidal chitin present in the medium. Patil *et al.* (2003) [15] reported halo formation on colloidal chitin agar as a useful qualitative indicator of chitinase-producing microorganisms. However, hydrolysis-zone size can be influenced by several factors, including extracellular enzyme production, diffusion through the agar, bacterial growth and incubation conditions. Therefore, the clear-zone measurements and degradation-efficiency values in the present study were used primarily as comparative screening indices rather than direct quantitative measurements of enzyme production. Similar screening approaches have been employed for chitinolytic bacteria from environmental and insect-associated sources (Abdullah, 2020; Dukare *et al.*, 2020; Karki *et al.*, 2023) [16, 7, 13].

Considerable variation was observed among the 11 isolates in their ability to produce clear hydrolysis zones. Isolate 3 produced the largest clear zone of 23 mm and recorded the highest DE of 130.0%, whereas Isolate 10 produced a 22 mm clear zone with a DE of 120.0%. These isolates were consequently selected for molecular characterization. Such variation among bacterial isolates may reflect differences in extracellular enzyme production, secretion and diffusion under the conditions used for screening. Similar variation in chitinolytic potential among bacterial isolates has been reported in previous studies (Abdullah, 2020; Karki *et al.*, 2023) [16, 13].

The recovery of chitinolytic bacteria from white grub gut material is particularly relevant because insect-associated microbial communities may possess metabolic capabilities associated with utilization of com-

plex substrates. Scarab beetle larvae have been reported to harbour diverse gut-associated microbial communities involved in metabolic processes and nutrient transformation (Egert *et al.*, 2003; Andert *et al.*, 2010; Chouaia *et al.*, 2019) [11, 10, 12]. Karki *et al.* (2023) [13] also demonstrated the occurrence of chitinolytic bacterial isolates in the gut of pleurostict scarab beetle grubs. The present findings further support the potential of white grub-associated microbiota as a source of chitinolytic microorganisms.

Molecular characterization provided further information on the taxonomic affiliations of the two selected isolates. HSChitin-1 showed its highest BLASTn similarity to *Sphingobacterium multivorum* strain IAM 14316, with 100% query coverage and 91.19% sequence identity. Although the database match was assigned to *S. multivorum*, the relatively low sequence identity indicates that the result should be interpreted cautiously and not treated as definitive species-level identification based solely on the partial 16S rRNA sequence. Nevertheless, the finding is of interest because the selected isolate demonstrated clear hydrolysis of colloidal chitin under the experimental conditions. HSChitin-2 showed its highest similarity to members of the genus *Stenotrophomonas*, with 100% query coverage and 93.15% sequence identity. Several *Stenotrophomonas* species appeared among the high-ranking BLASTn matches, preventing reliable species-level assignment from the available partial sequence. The isolate was therefore conservatively reported as *Stenotrophomonas* sp. The occurrence of a *Stenotrophomonas* isolate showing chitinolytic potential is consistent with previous reports of chitinolytic activity among members of this genus.

The finding associated with HSChitin-1 is also noteworthy because reports specifically describing chitinolytic activity of *Sphingobacterium multivorum* are comparatively limited. Members of *Sphingobacterium* have been associated with degradation of complex organic substrates and hydrolytic activities. In the present investigation, the isolate showing the closest BLASTn match to *S. multivorum* produced a distinct hydrolysis zone on CCA, indicating chitinolytic potential under the experimental conditions. This observation should be interpreted as a characteristic of the particular isolate recovered in the present study rather than as evidence that all strains of *S. multivorum* are chitinolytic.

The present investigation was based on preliminary CCA screening and partial 16S rRNA-based characterization. The calculated degradation efficiency was useful for comparing isolates and selecting candidates, but it does not represent a direct quantitative estimate of chitinase activity or the amount of substrate degraded. Therefore, further studies involving quantitative chitinase assays, enzyme activity mea-

surements, optimization of culture conditions, biochemical characterization and evaluation of enzyme stability are required to establish the chitinase-producing ability and application potential of the selected isolates.

Overall, the recovery of 11 culturable bacterial isolates and the selection of two isolates showing comparatively strong hydrolysis-zone formation demonstrate the potential of white grub gut-associated bacteria as a source of chitinolytic microorganisms. The isolates HSChitin-1 and HSChitin-2 provide candidates for further biochemical and molecular investigation.

5. Conclusion

The present investigation demonstrated that the gut of white grub larvae harbours culturable bacteria with chitinolytic potential. A total of 11 bacterial isolates were recovered and screened on colloidal chitin agar. Among these, Isolate 3 and Isolate 10 showed the highest degradation efficiencies of 130.0% and 120.0%, respectively, and were designated HSChitin-1 and HSChitin-2.

Partial 16S rRNA gene sequencing followed by BLASTn analysis showed that HSChitin-1 had its highest similarity to *Sphingobacterium multivorum* strain IAM 14316, whereas HSChitin-2 showed highest similarity to members of the genus *Stenotrophomonas*. The corresponding sequences were deposited in the NCBI nucleotide database under accession numbers PZ779901 and PZ783905, respectively.

The findings highlight white grub gut-associated bacteria as a potential source of chitinolytic microorganisms. However, because the present study was based primarily on qualitative CCA screening and partial 16S rRNA analysis, further quantitative chitinase assays, optimization of culture conditions and biochemical characterization are required to confirm enzyme production and determine the application potential of the selected isolates.

6. Acknowledgements

The authors are grateful to the R.C.S.M. Government College of Agriculture, Kolhapur, for providing laboratory facilities and necessary support for the present investigation. The authors sincerely acknowledge the assistance of Agharkar Research Institute, Pune, in 16S rRNA gene sequencing and the support received during sequence analysis and molecular characterization.

7. Authors' contributions

Amruta Abaso Pawar: Conducted the laboratory experiments, recorded observations, performed statistical analysis and prepared the first draft of the manuscript.

Dr. A. S. Bagde: Designed the study, Supervised the research work, interpretation of results and critical revision of the manuscript. All remaining authors read and approved the final manuscript.

8. Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The partial 16S rRNA gene sequences generated in this study are available in the NCBI nucleotide database under accession numbers PZ779901 and PZ783905.

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