Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Unveiling the Cultivation-Dependent Gut Bacterial Microbiota in the Feral Stingless Bee, *Tetragonula iridipennis* Smith (Hymenoptera: Apidae)

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Article History

Edited by
Evandro Nascimento Silva, UEFS, Brazil
Received 23 July 2024
Initial acceptance 25 November 2024
Final acceptance 19 March 2025
Publication date 11 July 2025

Keywords
Social insect, Stingless bee gut bacteria, 16S rRNA gene sequencing, *Bacillus* sp., *Clostridium* sp., *Citrobacter* sp.

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Abstract

The feral stingless bee, *Tetragonula* iridipennis, is a vital pollinator in diverse ecosystems and produces valuable honey with unique characteristics. This study aimed to explore the gut microbiota of *T. iridipennis* by identifying and characterizing its bacterial communities and examining the interactions between gut bacteria and pathogens. Total Plate counts (TPC) were assessed after a day of incubation on nutrient agar at 37 °C, revealing the highest TPC from SL1 (7.00 ± 0.03 log cfu/ml) and the lowest from SL3 (6.79 ± 0.04 log cfu/ml). Eleven bacterial isolates were recovered and classified through morphological characterization and Gram staining. All bacterial isolates were subject to antimicrobial assessment against infective bacteria. The isolates SB1, SB4, SBT2, and SBM1 showed better antagonistic effect (12.67 ± 2.08; 11.33 ± 1.53; 10.67 ± 4.16 and 9.67 ± 1.53 mm) among the eleven isolates against the pathogen *Staphylococcus aureus,* respectively. These selected isolates were taken into molecular characterization through 16S rRNA gene sequencing. The amplified 1500 base pair fragments showed more than 98% similarity with known sequences in the GenBank database. The isolates were identified as *Bacillus subtilis* (SB1), *Bacillus tropicus* (SB4), *Clostridium tunisiense* (SBT2), and *Citrobacter freundii* (SBM1). The phylogenetic analysis using MEGA 11 and BLASTn revealed that *B. subtilis* SB(CM)-1 had 77% similarity with *B. subtilis* C785F of South Korea, *B. tropicus* SB(OD)-1 had 69% similarity with *B. tropicus* SA39 from Pakistan, *C. tunisiense* SB(TK)-2 had 100% similarity with *C. tunisiense* from Japan, and *Citrobacter freundii* SCF(CM)-2 had 100% similarity with *Citrobacter* sp. from Brazil.

Introduction

Microbes play a vital role in the inclusive growth and development of insects. An array of microbial populations inhabits the gastrointestinal tract of an insect, which may be either coexisting mutually with the host or in an obligatory relationship (Gupta & Nair, 2020). The symbiotic interaction between the organism and bacteria has coevolved to assist the host in various facets, like digestion, nutrient absorption, and fighting invading pathogens. Gut microbial products are key factors for health in the immune system against pathogens (Feldhaar, 2011; Kwong & Moran, 2016), and also the endosymbionts are essential for the host in the supply of essential nutrients, aids in digestion and reproduction,

and production of pheromones (Singh et al., 2021). The innate gut bacteria of the arthropods cleanse the detrimental secondary metabolites and defend the host from colonization by pathogens (Dillon et al., 2010). It is recognized that the gut bacterial endosymbionts are associated with the overall ecological suitability and biological eminence of their host, i.e., the insects (Cai et al., 2018).

Stingless bees have the peculiar characteristics of other bees. However, as the name suggests, the absence of a functional sting makes them less harmful to humans and animals. The tribe Meliponini represents a monophyletic group of eusocial insects that belongs to a larger group of corbiculate bees (Hymenoptera: Apidae) (Romiguier et al., 2016). The reproductive system of daughter colonies of stingless bees is allied to their parent colony for a period to stabilize symbiosis between the microbes and bees across generations (Kwong et al., 2017). This transmission of beneficial microbes from mother to daughter colony was performed during the swarming, as the worker bees transport the nesting resources and deposit the food from parent to daughter colony (Wisselink et al., 2020). Furthermore, they are resistant to pathogens that are prone to honey bees (Delfinado-Baker et al., 1989). Numerous studies have investigated the microbiota of honey bees, utilizing both culture-dependent and culture-independent techniques to examine microbial diversity and quantify microorganisms (Ellegaard & Engel, 2019; Mohammad et al., 2020).

The microbiota associated with stingless bees includes bacteria, yeasts, viruses and filamentous fungi and however yeasts and bacteria are metabolically active and assists in enzyme production, organic acid production and sugar fermentation, also aids in initiating the biochemical changes in the nectar and pollen collected by stingless bees through microbial transformation which may provide the nutritional benefits to the young ones and adult bees (Nglaimat et al., 2019). In honey bees, lactic acid bacteria have probiotic properties and also can inhibit the growth of pathogenic bacteria, *Paenibacillus larvae* (American foul brood), and *Streptomyces* isolated from the nests of *Trigona laeviceps* and *Trigona fuscobalteata* inhibit the causative agent of European foul brood, *Melisococcus plutonis* (Forsgren et al., 2010; Kroiss et al., 2010). The microbial community in stingless bees is less studied than in other bees (Vasquez et al., 2012). Moreover, the major bacterial genus isolated from varieties of stingless bee species is *Bacillus.* For instance, the bacteria associated with the *Melipona panamica* were *Bacillus alvei* and *Bacillus circulans.* Besides, other genera, including *Streptomyces, Clostridium, Fructobacillus, Staphylococcus, Enterobacter,* and *Pseudomonas,* have been reported to be found associated with stingless bees (Leonhardt & Kaltenpoth, 2014; Yaacob et al., 2018). Stingless bees are known for producing honey with unique physicochemical properties and antimicrobial activity, primarily influenced by their associated microbial communities. Previous investigations have highlighted the bacterial diversity in honey produced by stingless bees, emphasizing the role of these microbes in fermentation, preservation, and potential health benefits (Anderson et al., 2013). In the present study, we aimed to isolate and characterize the bacteria from the gastrointestinal tract of the stingless bee, *Tetragonula iridipennis,* and to explore the antimicrobial properties of the bacterial microbiota and their similarity relationship with the bacteria isolated from various locations around the world. The study on the characteristics of the gut bacterial microbiota aims to bridge the gap in understanding how these bacterial communities might relate to those found in honey, providing a foundation for future probiotic application in health, the immune system, and their colony productivity.

Materials and Methods

Sample collection and isolation of gut bacterial isolates

The healthy foraging worker samples of the feral stingless bee, *Tetragonula iridipennis*, were collected from two districts (Dindigul and Cuddalore) of Tamil Nadu, India, with two locations for each district during the honey flow period of February to March 2023. Each location was named and denoted by the code (Sampling Location -SL 1 to SL 4) (Fig 1). From each location, adult bees (n = 30) were collected and surface sterilized using 95% ethanol. The entire digestive system of the stingless bees was aseptically dissected by pinning the mesosoma of bee on lateral surface with sterile needle followed by dissecting the lateral surface between tergum and sternum and the entire alimentary canal was pulled off gently by using sterile micro forceps (Coleman et al., 2007; Anjum et al., 2018). The dissected guts were transferred to phosphate buffer saline (PBS) and immediately stored at -20 °C for experimental studies. The gut samples of the stingless bee (n = 30) from each location were macerated using a micro pestle by adding 100 µl PBS. Two different dilutions (10^{-3} and 10^{-4}) of this homogenate were made, and 10 µl aliquots of each sample were aseptically inoculated into Nutrient Agar (NA) using the spread plate technique, with three replications performed for each dilution and incubated at 37 °C for 24 hours. Morphologically distinct cultures were selected and further subcultured to obtain pure bacterial colonies.

Antimicrobial activity assessment

It was assessed using an agar well-diffusion method with slight modifications (Yilmaz et al., 2006). Three bacterial genera of human pathogens were selected to test against bacteria isolated from stingless bees, aiming to assess their antagonistic effects and highlight their potential for future probiotic applications. The pathogenic bacterial strains were obtained from MTCC (Microbial Type Culture Collection and Gene Bank, Chandigarh, India), such as *Staphylococcus aureus* MTCC96, *Erwinia* sp. MTCC2760 and *Salmonella enterica* MTCC733 were spread onto the NA medium containing a petri plate, using an 8 mm diameter cork borer, wells were punched, and 100 µl of 24-hour-old active bacterial cultures were seeded into each well, and Tetracycline (20 µg/ml)

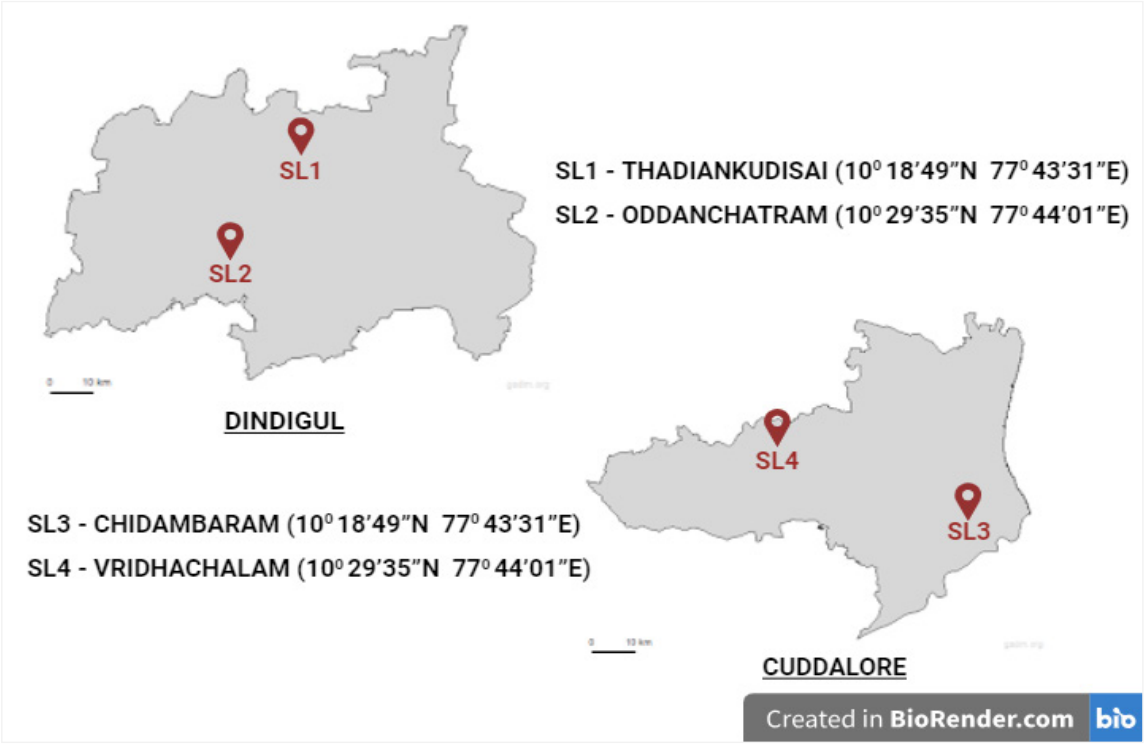

Fig 1. Geographical coordinates of feral stingless bee sampling locations from districts of Tamil Nadu.

was used as a positive control. Later, the diameter of the clear zone was measured after the incubation period at 37 °C for 24 hours.

Genomic DNA extraction of bacterial isolates

For DNA isolation, the phenol-chloroform method was followed with some modifications for extraction (Wright et al., 2017). The well-grown distinct bacterial culture (1 ml) was pipetted out and transferred into a 2 ml microcentrifuge tube, then centrifuged at 8000 rpm for 10 min, and the supernatant was discarded. Resuspended the pellet with 560 µl of TE (Tris-EDTA) buffer and 30 µl of SDS (Sodium dodecyl sulfate) and gently mixed by adding 100 µl of 5M NaCl and 80 µl of CTAB and incubated at 65 ºC for 10 min. An equal volume (700 µl) of PCI (Phenol: Chloroform: Isoamyl alcohol) was added and centrifuged at 12000 rpm for 15 min. The upper phase was transferred to a sterile 2 ml microcentrifuge tube, and an equal volume of ice-cold isopropanol was added and incubated at -20 °C for one hour, then centrifuged at 12000 rpm for 10 min. The supernatant was decanted and pellet washed with 500 µl of 70% ethanol and centrifuged at 12000 rpm for 5 min. Finally, the pellet was air dried at room temperature for 30 minutes, and then 40 µl of double-distilled water was stored at 4 °C for further use.

PCR amplification and DNA sequencing

For molecular characterization, the 16S rRNA gene of isolated bacterial cultures was amplified by using universal bacterial primers 27F (5'-GAGTTTGMTCCTGGCTCA-3') and 1492R (5'-TACGGYTACCTTACGACT-3'). The PCR cycle consisted of an initial denaturation step at 94 ºC for 2 min followed by 32 cycles of denaturation (94 °C for 30 sec), annealing (55 °C for 30 sec), and extension (72 ºC for 2 min). The final extension was kept at 72 °C for 10 min. The PCR bands were visualized under UV-Trans illuminator and documented using a Gel documentation unit (BIOZEN ZENITH). The DNA samples with an amplicon size of 1.5 kbp (kilobases) were sent for Sanger sequencing to Par Life Science and Research / HiMedia Laboratories Pvt. Ltd., Chennai. At the National Centre for Biotechnology Information, the resultant FASTA sequences were used to perform a homology search using the BLASTn programme (Basic Local Alignment Search Tool nucleotide) in NCBI/ BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest bacterial neighbours.

Phylogenetic analysis

The closely related bacterial species were retrieved from the NCBI GenBank. The multiple alignment of nucleotide gene sequences was performed using the MEGA 7.0 software and Clustal W program (Tamura et al., 2004; Kumar et al., 2016). For phylogenetic tree construction, the neighbour-joining method with the p-distance method was followed, and with 1000 replications, the robustness of individual branches was estimated by bootstrapping (Tamura et al., 2021).

Statistical analysis

Data analysis was performed using R Studio (Chambers, 2008) with the 'stats' package for one-way ANOVA, followed by Tukey's ad hoc test to assess the significance among the CFU/ml in stingless bee gut bacterial microbes of different localities.

Results

Isolation, screening, and selection of gut bacterial isolates

The total plate count estimates the total cultivable bacteria present in the sample. After 24 hrs of incubation at 37 ºC, the TPC was counted for each sample. Results revealed that the count varied for different sampling locations (Table 1). The highest TPC of gut bacteria was recorded from SL1 (7.00 ± 0.03 log cfu/ml), followed by SL4 (6.97 ± 0.04 log cfu/ml) and SL2 (6.88 ± 0.04 log cfu/ml), whereas the lowest total plate count was recorded from SL 3 (6.79 ± 0.04 log cfu/ml). The 11 morphologically distinct bacterial cultures recovered from the gut of *Tetragonula iridipennis* were taken for further study. As scrutinized by morphological characterization and Gram staining technique, seven isolates (SB1, SB2, SB4, SBT2, SBT3, SBG2, SBM2) were Gram-positive bacteria, whereas four isolates (SB3, SBT1, SBG1, SBM1) were Gram-negative bacteria (Table 2) (Fig 2).

Table 1. Number of Total Plate Count (TPC) from *Tetragonula iridipennis* gut bacteriome

Sampling location	*Total Plate Count (Log CFU/ml) ± SD	
	Dilution 10^{-3}	Dilution 10^{-4}
SL1	5.72 ± 0.51	7.00 ± 0.03
SL2	5.92 ± 0.04	6.88 ± 0.04
SL3	5.84 ± 0.04	6.79 ± 0.04
SL4	5.99 ± 0.06	6.97 ± 0.04

*Total plate count is the mean value of four replication of each sampling location. The one-way ANOVA test showed non-significant differences for both the dilutions 10^{-3} ($p = 0.36$) and 10^{-4} ($p = 0.54$) from different locations.

Determination of antimicrobial activity against pathogenic bacteria

The antimicrobial activity of 11 bacterial isolates was evaluated using agar well diffusion assay to evaluate their antagonistic activity against pathogenic bacteria such as Staphylococcus aureus, Erwinia sp., and Salmonella enterica. Among the 11 isolates, seven bacterial isolates had antibacterial activity against at least one pathogenic bacteria. Four bacterial isolates (SB1, SB4, SBT2 and SBM1) exhibited excellent antagonistic effect (12.67 ± 2.08;

Table 2. Morphological characterization of gut bacterial isolates of the stingless bee, *T. iridipennis.*

Isolate	Form	*Size (mm)	Elevation	Margin	Opacity	Texture	Colour	Gram test	shape
SB1	Irregular	S	Slightly raised	Entire	Slightly translucent	Moist	Creamy white	+	Rod shaped
SB2	Irregular	M	Flat	Entire	Translucent	Moist	White	+	filamentous
SB3	Irregular	M	Convex	Entire	Opaque	Dry	Yellowish white	_	Rod shaped
SB4	Circular	S	Raised	Entire	Opaque	Moist	Creamy white	+	Rod shaped
SBT1	Irregular	M	Flat	Entire	Opaque	Viscid	White	_	Rod shaped
SBT2	Circular	S	Raised	Entire	Opaque	Moist	Creamy white	+	Rod shaped
SBT3	Circular	S	Slightly raised	Entire	Opaque	Moist	Pinkish white	+	Rod shaped
SBG1	Irregular	S	Convex	Entire	Translucent	Dry	White	_	Rod shaped
SBG2	Irregular	M	Slightly raised	Entire	Opaque	Moist	White	+	Filamentous
SBM1	Circular	M	Raised	Entire	Opaque	Moist	Yellowish-white	_	Rod shaped
SBM2	Irregular	M	Flat	Entire	Opaque	Viscid	white	+	Rod shaped

*Size- S- small (2-3mm); M- medium (4-5mm); L- large (>5mm)

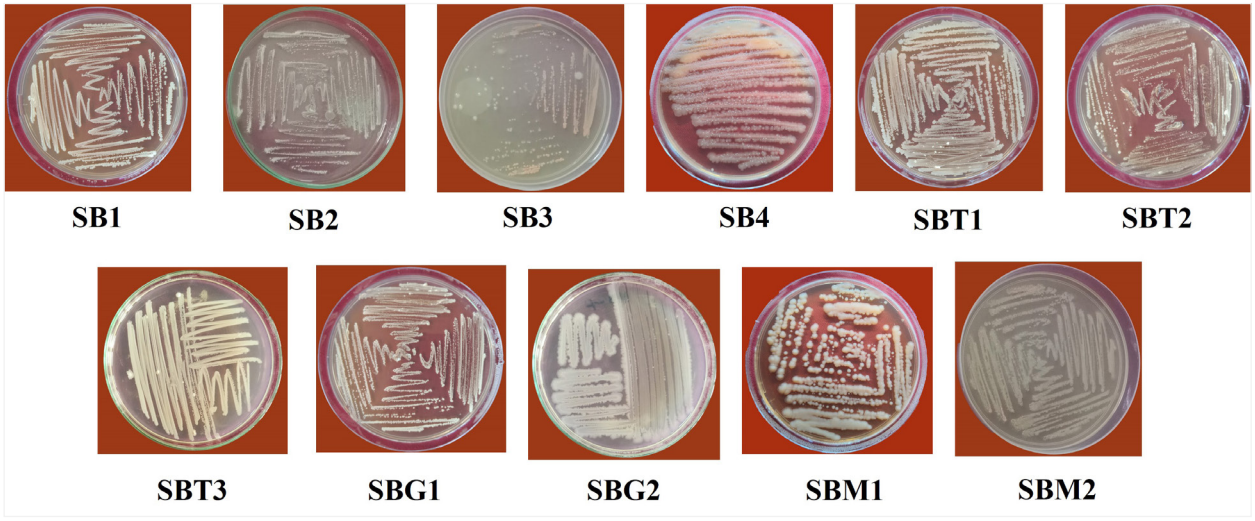

Fig 2. Bacterial isolates from the gut of stingless bee, *T. iridipennis.*

11.33 ± 1.53; 10.67 ± 4.16 and 9.67 ±1.53 mm) against the pathogenic bacteria (*S. aureus*) and the remaining bacterial isolates exhibited remarkable but lower antagonistic effects in comparison to the four isolates (Table 3) (Fig 3). The four well performing bacterial isolates were nominated for further molecular characterization.

Table 3. Antagonistic activities of bacterial isolates against three different pathogenic bacteria.

Bacterial isolates	Zone of inhibition against pathogenic bacteria (mm)*		
	Staphylococcus aureus	*Erwinia* sp.	*Salmonella enterica*
SB1	12.67 ± 2.08	12.33 ± 2.52	15.67 ± 1.53
SB2	5.67 ± 0.08	5.33 ± 1.53	4.67 ± 1.53
SB3	NI	NI	NI
SB4	11.33 ± 1.53	7.67 ± 1.53	11.33 ± 1.53
SBT1	NI	NI	NI
SBT2	10.67 ± 4.16	10.67 ± 2.52	5.67 ± 1.15
SBT3	9.33 ± 1.53	NI	5.33 ± 4.73
SBG1	NI	6.33 ± 1.15	NI
SBG2	NI	NI	NI
SBM1	9.67 ± 1.53	5.33 ± 1.53	8.33 ± 2.31
SBM2	NI	NI	NI
Tetracycline (c)	14.67 ± 2.52	13.67 ± 1.53	12.67 ± 1.53

*Each value is a mean of three replications followed by standard deviation (mean ± SD), NI – No Inhibition zone. (c)- Tetracycline as control.

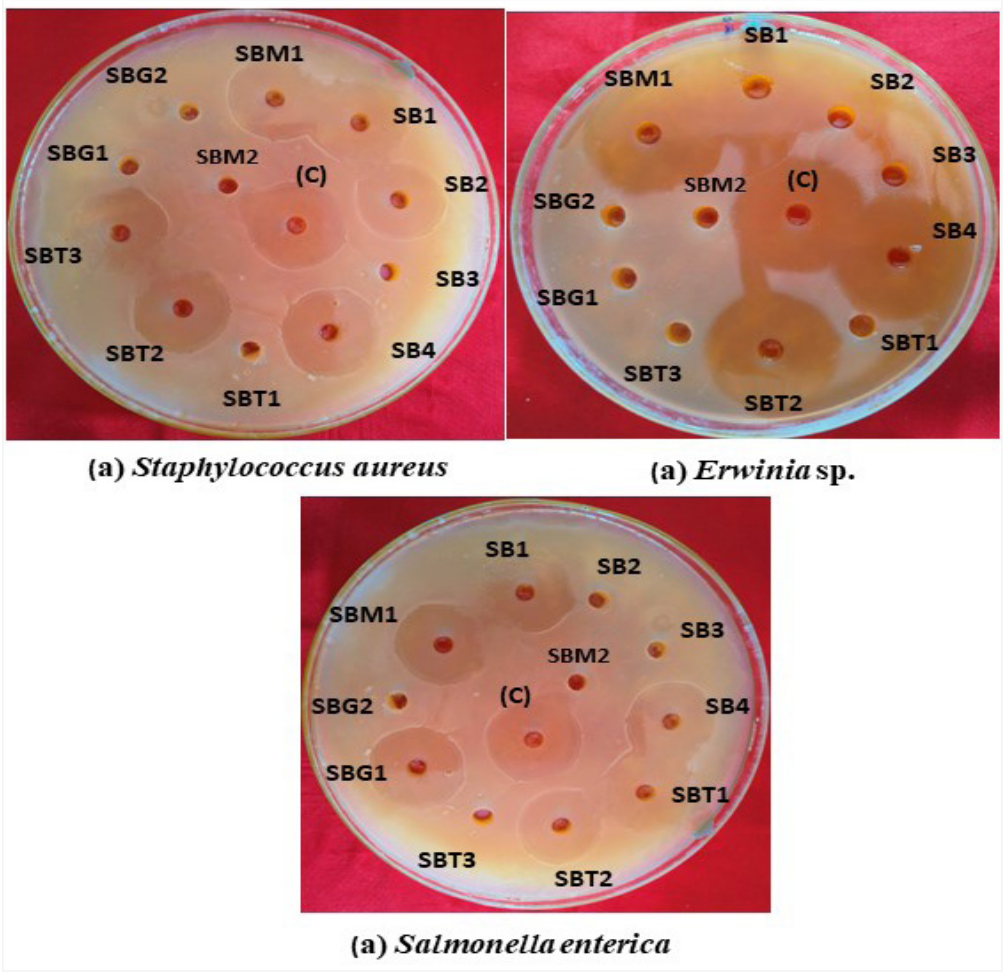

Fig 3. Inhibition zones of bacterial isolates against the pathogenic bacteria (a) *Staphylococcus aureus;* (b) *Erwinia sp.;* (c) *Salmonella enterica.*

Identification and phylogenetic analysis of gut bacterial isolates based on 16S rRNA gene sequencing

A 1.5 kbp nucleotide fragment of 16S rRNA gene was amplified from the genomic DNA for molecular identification of selected bacterial isolates. The PCR (Polymerase Chain Reaction) amplified fragments were compared with the sequence deposited in the NCBI GenBank database to determine the similarity of the closest relative organism. However, the gene sequence (16S rRNA) showed more than 98% similarity to the closest species. Based on the gene sequence, targeting the 16S rRNA gene resulted in the identification of the three isolates of Firmicutes, which were (SB1) *Bacillus subtilis,* (SB4) *Bacillus tropicus,* and (SBT2) *Clostridium tunisiense,* whereas one isolate of Proteobacteria was (SBM1) *Citrobacter freundii.* The BLASTn analysis of the isolated bacterial sequence with the reference sequence matching percentage and their query coverage were mentioned to depict the sequence similarity among the subsequent accession numbers of bacteria from GenBank (Table 4).

Phylogenetic analysis of selected bacterial isolates

A phylogenetic tree was constructed based on the bacterial universal primers of 27 F and 1492 R in MEGA 11. All sequences generated were deposited into the NCBI GenBank database. The phylogenetic tree was branched with four clades and one outgroup (*Mycobacterium tuberculosis* NR 102810.2). *Clostridium tunisiense* strain SB(TK)-2 (PP059610.1) sequence data revealed maximum similarity of 100% with *C. tunisiense* (AB600546.1) from Japan. The sequence of *Citrobacter freundii* strain SCF(CM)-2 (OR681488) have maximum similarity of 100% with *Citrobacter* sp. strain BR13841 (MK156437) from Brazil, the *Bacillus subtilis* strain SB(CM)-1 (OR569673.1) sequence show the maximum similarity of 77% with the *B. subtilis* strain C785F (KX950748.1) from South Korea and *B. tropicus* strain SB(OD)-1 (PP053372.1) shows similarity of 69% with *B. tropicus* strain SA39 (MK467594.1) from Pakistan (Fig 4).

Table 4. Comparison of gene sequence (16S rRNA) of gut bacterial isolates of *Tetragonula iridipennis* with 16S rRNA gene sequence in GenBank.

Gene Sequence					
Isolates	****Number of nucleotides (bp)**	**Accession number**	**Closest phylogenetic relative**	**Score**	**Identity (%)**
*SB1	896	OR569673	*Bacillus subtilis* strain SB(CM)-1	1655	100
	976	MN855574	*Bacillus subtilis* strain KUY 2019	1648	99.89
	1025	KX950748	*Bacillus subtilis* strain C_785F	1648	99.89
	997	KJ865748	*Bacillus subtilis* strain OUR13	1648	99.89
*SB4	868	PP053372	*Bacillus tropicus* strain SB(OD)-1	1604	100
	1057	MK467594	*Bacillus tropicus* strain SA39	1604	100
	1414	MK318251	*Bacillus paramycoides* strain NGB-SF327	1598	99.88
	1133	OP680476	*Bacillus albus* strain AMZZ17	1598	99.88
*SBT2	1159	PP059610	*Clostridium tunisiense* strain SB(TK)-2	2141	100
	1475	DQ479415	*Clostridium* sp. CYP5	2124	99.74
	1497	NR_145903	*Clostridium punense* strain BLPYG-8	2061	98.79
	1470	AB600546	*Clostridium tunisiense*	2061	98.79
*SBM1	913	OR681488	*Citrobacter freundii* strain SCF(CM)-2	1687	100
	1422	OR256216	*Citrobacter freundii* strain MBCTB01	1640	99.13
	1402	MT903209	*Citrobacter murliniae* strain Seq_ban6-VP-D25-67	1640	99.23
	1398	MK156437	*Citrobacter* sp. strain BR13841	1639	99.12
Outgroup	1532	NR_102810	*Mycobacterium tuberculosis* H37Rv	-	-

*Bacterial gene sequence of this study. From GenBank, accession number of each isolate was acquired. **Number of gene nucleotides (base pairs) by combination of 27F and 1492R bacterial primers. The matching score with the closest phylogenetic relative has 0.0E value.

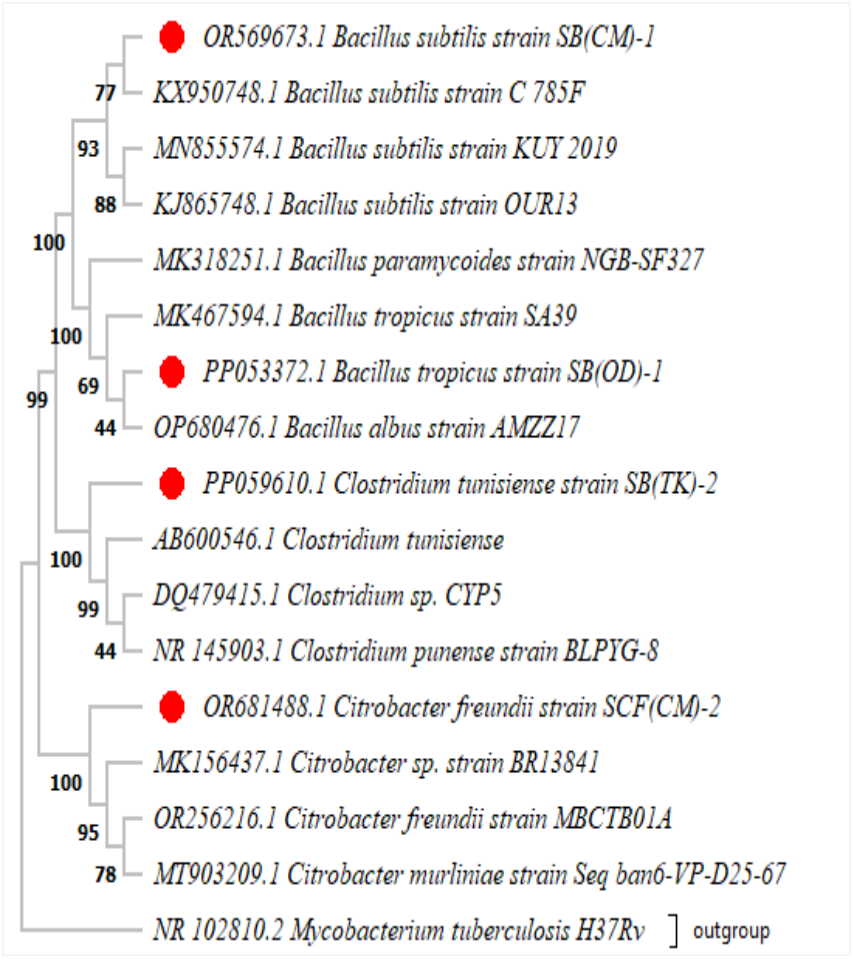

Fig 4. Phylogenetic trees of selected isolates and the strains in this study are pointed in red.

Discussion

In this study, the investigation on the bacteria in the stingless bee gastrointestinal tract is likely to be due to the absorption of food nutrients and to prevent environmental contamination. The bacterial count from the honey of stingless bees have ranged from 0.0 to $8.0x10^3 \pm 1.06x10^3$ cfu/ml (Ngalimat et al., 2019), which strongly agrees with the current study that the maximum total plate count of gut bacterial microflora was recorded as 5.99 ± 0.06 log cfu/ml at the dilution factor of 10^{-3}. The analysis of colony-forming units showed no significant differences in bacterial load among the regions ($p > 0.05$). This might be because the gut microbiota of stingless bees in these regions would be relatively similar regarding the environment and the bees' floral diet. The isolated bacteria from the gut were compared with those commonly found in honey, as the gut microbiota of stingless bees is often influenced by their diet and environment, including honey production. Although honey was not directly tested in this study, the association is supported by previous research linking the bee gut bacteria to roles in honey fermentation and antimicrobial activity (Anderson et al., 2013).

The stingless bee honey is found to have the microflora of gram-positive bacterial isolates such as *Bacillus vallismortis* and *Staphylococcus lentus* and gram-negative bacteria of *Pantoe* sp. (Divya et al., 2020), which was pretty consistent with the present findings that the gram-positive bacterial isolates from the gut were *Bacillus subtilis, Bacillus tropicus,* and *Clostridium tunisiense,* whereas the gram-negative bacteria were *Citrobacter freundii.* It is also reliable with the finding that *Citrobacter freundii* was found to be in the gut of *A. cerana indica* (Disayathanoowat et al., 2012), *Bacillus* sp. in the honey of *Heterotrigona itama* (Zulkhairi et al., 2020), and *Clostridium* sp. in the honey of *Tetragonisca angustula* (Pucciarelli et al., 2014).

Previous studies have shown that the gut microbiota of *Apis mellifera* is dominated by three phyla: Firmicutes, Actinobacteria, and Proteobacteria (Li et al., 2012; Prabhakar et al., 2013). Similarly, *Apis cerana indica* has been found to harbor a common group of bacteria, primarily from the Gamma-proteobacteria phylum (Disayathanoowat et al., 2012). Recent investigations on stingless bees have also reported the presence of dominant bacterial groups such as Proteobacteria, Firmicutes, and Actinobacteria in their gut microbiota. This suggests that specific phyla may be consistently prevalent in the gut microbiota of different bee species, regardless of whether they are honey bees or stingless bees.

The subsequent purpose of this study is to identify the gut bacterial isolate that exhibits antimicrobial activity against

pathogenic bacteria. The successful selection of bacteria that produce antimicrobial substances has been reported from stingless bee honey (Pajor et al., 2018). For example, Zulkhairi Amin et al (2020) reported that *Bacillus amyloliquefaciens* and *Bacillus subtilis* have excellent antagonistic effects on different pathogenic bacteria such as *Staphylococcus aureus, Salmonella thyphimurium, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. In line with this study, *B. subtilis* and *B. tropicus* showed more inhibition zones against the pathogenic bacteria of *S. aureus, P. aeruginosa,* and *Salmonella enterica.* In contrast, the *C. tunisiense* and *Citrobacter freundii* showed a lower antagonistic effect. Similar to the findings of Grover et al. (2009), *B. subtilis* has a broad spectrum of antimicrobial activities over diverse pathogenic bacteria and fungi. The rationale for testing these pathogenic bacteria lies in their significance to human and animal health, and their potential presence in environments where stingless bees may encounter contaminants. While bees may not directly contact these pathogens, their gut microbiota could protect against various microorganisms, supporting colony health and productivity. Additionally, gut bacteria may aid in nutrient absorption and environmental contamination prevention, as shown in studies on other bee species (Anderson et al., 2013). These identified bacteria, with their antagonistic effect against human pathogens, could have significant applications in various fields, such as antimicrobial therapies, probiotics, and prebiotics.

Conclusion

Our results revealed the presence of bacterial microflora in the gut of the stingless bee, *Tetragonula iridipennis,* having potent antimicrobial properties in several isolates. Morphological and molecular characterization confirmed the key bacterial species' identities and genetic relationships, such as *Bacillus subtilis, Bacillus tropicus, Clostridium tunisiense,* and *Citrobacter freundii.* These findings contribute to the growing body of knowledge on the gut bacteriome of stingless bees. Further studies could explore their potential use in improving the health and productivity of stingless bee colonies and their possible benefits for human and animal health.

Acknowledgments

We acknowledge the locals who helped in locating the feral stingless bee colonies.

Authors' Contribution

BSV: Conceptualization, methodology, and writing-original draft.
JJ, RN, MRS, and KK: Supervision, data curation, writing-review & editing.
MJ and KS: Validation, supervision, writing-review & editing.
PS: Formal analysis and supervision.
All authors have gone through and approved the manuscript.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Funding

Tamil Nadu Agricultural University supported this work under the venture capital scheme (VCS-V60 JY).

Compliance with ethical standards

This article does not contain any studies with human participants performed by any authors.

Conflict of interest

The authors declare that they have no conflicts of interest.

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