Characterization Bee Bread Associated Microbials from *Tetragonula laeviceps* and Identification of *Bacillus subtilis* as Antimicrobial Agent

Antonia Caitlin Gondowardoyo [1], Ema Damayanti [2,3,*], Mastari Ayu Anggraeni [4], Tsania Taskia Nabila [5,6], Noorendra Laksamana Putra [7], Siti Muslimatun [1], Riri Rimbun Anggih Chaidir [4], Titiek Farianti Djaafar [2]

[1] School of Life Sciences, Indonesia International Institute for Life Sciences (I3L), Jakarta, Indonesia

[2] Research Center for Food Technology and Processing, National Research and Innovation Agency (BRIN), Gunungkidul, Indonesia

[3] Indonesian Biofilm Research Collaboration Center (IBRCC), Yogyakarta, Indonesia

[4] Study Program of Biotechnology, Faculty of Natural Sciences and Technology, Sumbawa University of Technology, West Nusa Tenggara, Indonesia

[5] Research Assistant, Research Center for Food Technology and Processing, BRIN, Gunungkidul, Indonesia

[6] Doctoral Student of Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

[7] Directorat of Laboratory Management, Research Facilities and Science and Technology Park, BRIN, Gunungkidul, Yogyakarta, Indonesia

ABSTRACT

Bee bread is a fermented bee product originating from bee pollen carried by bees and stored in pots in the hive, specifically by stingless bees. Bee bread serves as a food reserve for bee colonies and is naturally preserved through a fermentation process involving numerous microorganisms. As a fermented product, bee bread contains beneficial microorganisms that can be used as an antimicrobial agents against pathogenic microorganisms. This study aimed to isolate anti-pathogenic bacterial microbials from bee bread *Tetragonula laeviceps* originating from Sumbawa, Indonesia. The isolation of bee bread microbes was performed using different Agar media, including Chloramphenicol Yeast Glucose Agar (YGCA), Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Sabouraud Dextrose Agar (SDA) by using the spread plate plate method. Isolated microbes identification was carried out using Gram staining and catalase activity assay, and antimicrobial activity assay was carried out using the well diffusion method on *Candida albicans* ATCC 10231, *Escherichia coli* FNCC 0091, *Staphylococcus aureus* FNCC 0047, and *Pseudomonas aeruginosa* FNCC 0070. The isolate with the highest antibacterial activity was isolate 19. Further identification via 16S rRNA gene sequence analysis revealed that isolate 19 shares 94.91% similarity with *Bacillus subtilis* strain BCRC 10255, a finding also confirmed by morphological characteristics using scanning electron microscopy. This study highlights the potential of *B. Subtilis* 19, derived from *T. laeviceps* bee bread, as a promising antibacterial agent.

Keywords: Bee Bread, Pollen, Associated Microbials; Antibacterial Agent, Stingless Bee

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*Corresponding author: Ema Damayanti (emad001@brin.go.id)
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INTRODUCTION

Bee products have been used as alternative drugs and food applications since ancient times worldwide. Acceptable bee products not limited to propolis, honey, royal jelly, and beeswax but also tetapi juga bee pollen (Denisow & Marta Denisow-Pietrzyk, 2016). Bee pollen is collected in pellet form and shaped by the saliva of bees. In contrast, bee bread is produced when bee pollen is combined with nectar or honey and the bee's digestive enzymes, undergo lactic acid fermentation within the honeycomb during storage. This process imparts a more acidic taste to the bee bread (Rodica Mărgăoan et al., 2019). Bee-collected pollen is rich in various nutritious components, such as proteins, carbohydrates, lipids, dietary fibres, and bioactive micronutrients. Owing to its high-quality composition, bee pollen is increasingly recognized as a functional food, prompting research to explore and confirm its therapeutic potential in medical and food-related applications (Ghouizi et al., 2023) Therefore, since bee bread is a product of natural fermentation, it has some beneficial effects, especially from its microbiological agents and bioactive compounds.

Lactobacillaceae family in bee bread have obtained from different regions of Anatolia (Mediterranean region), such as Bacteroiaceae, Oscillospiraceae (Hungateiclostridiaceae), and Prevotellaceae (Ilıkkan & Samples, 2023). Yeast species also found in bee bread, such as *Starmerella meliponinorum, Rhodotorula mucilaginosa, Saccharomyces cerevisiae*, and *Papiliorama flavescens* (Kieliszek et al., 2018). Within bacteria and yeast, species of lactic acid bacteria (LAB) are also abundant in bee bread, as have reported in previous study (Taşdelen & Arslan, 2025). These studies demonstrated that LAB obtained from bee bread, such as *Apilactobacillus kunkeii, Limosilactobacillus fermentum*, *Lactococcus lactis*, *Wesella cibaria, Leuconostoc mesenteroides*, and *Lactiplantibacillus plantarum*, have strong antibacterial activity against Gram postive such as *Bacillus cereus* (10.15 – 26.58 mm) and *Staphyloccus aureus* (6.8 – 19.78 mm) and Gram negative bacteria such as *Escherichia coli* (11.75 – 27.02 mm). Another study also reported that LAB from bee bread, especially *Lactobacillus musa* SGMT17, *L. crustorum* SGMT19, *L. crustorum* SGMT20, *L. mindensis* SGMT22, and *Leuconostoc mesenteroides* U39, have antibacterial activity against pathogenic bacteria such as *Eschericia coli* (2.3 – 5.0 mm), *Staphylococcus aureus* (2.0 – 6.3 mm), *Bacillus cereus* (3.0 – 4.5 mm), *Klebsiella pneumonia* (1.7 – 4.0 mm), *Salmonella thypimurium* (2.3-5.0 mm), and *Pseudomonas aeruginosa* (3.5 – 6.7 mm) (Mohammad et al., 2021).

However, the nutritional value of pollen varies greatly depending on the floral species and geographic origin (Alshallash et al., 2023; Rodica Mărgăoan et al., 2019). Most bee bread research has been conducted in different regions of the world, where differences in flower and bee species affect the diversity of microorganisms present in bee bread. Previous research identified *Lacticaseibacillus rhamnosus* (BBAT2) and *Fructobacillus fructosus* (BBST20) in the bee bread of the stingless bee *Tetragonula laeviceps* in Sumbawa (Damayanti et al., 2025). In addition to LAB in bee bread, other potentially significant microbes warrant further investigation. Consequently, this study aimed to isolate, identify, and characterize additional microbial species with potential antibacterial properties against pathogens. This study aimed to enhance the diversity of microbes derived from bee bread produced by stingless bees, particularly *T. laeviceps*, from Sumbawa, Indonesia.

MATERIALS AND METHODS

Bee Bread Samples

Bee bread samples were obtained from a stingless bee hive of *Tetragonula laeviceps* (Sumbawa, West Nusa Tenggara, Indonesia). Bee bread was taken directly from the beehives by gauging out from the pot using a small knife, and then stored inside a small plastic jar in the refrigerator prior to the research. Bee pollen was characterized using Scanning Electron Microscope (SEM) to determine the morphology of its constituent pollen. The samples were tapped on a copper disk using a double-sided conductive carbon adhesive tape. The samples were then coated with gold using HITACHI MC1000 Ion Sputter, set at 20 mA for 60 s. After coating, the samples were observed using HITACHI SU3500 SEM, with Vacc set at 3 kV,

SI (Spectrum Imaging) 30%, and magnification of 100× and 400×.

Microbials Isolations and Purification

For microbial isolation, the following media were used: Chloramphenicol Yeast Glucose Agar (CYGA), Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Sabouraud Dextrose Agar (SDA). Five gram of Bee bread samples were diluted in 45 mL of NaCl 0.86% solution and serially diluted up to 10,000× (Damayanti et al., 2025). One hundred microliters of samples from the last two dilutions were plated onto the surface of agar medium using the spread plate method and then incubated for 3–5×24 h at a temperature of 30°C. A single colony that grew on the surface of the medium was purified using the streak plate method with the same medium, temperature, and incubation time. The resulting pure isolate was then sampled for Gram staining and catalase testing. The pure colony was then stored in 50% glycerol in a -80°C freezer for stock cultures for subsequent tests.

Antimicrobial Assay

Antibacterial assays were performed using the well diffusion method. Antibacterial tests were performed using pathogenic microorganism: *Candida albicans* ATCC 10231, *Escherichia coli* FNCC 0091, *Staphylococcus aureus* FNCC 0047, and *Pseudomonas aeruginosa* FNCC 0070. The test bacteria at 10^7 CFU/mL of cell density were inoculated onto the surface of Mueller Hinton Agar (MHA). Wells with an 8 mm diameter were made on the surface of the agar. A 48-hour-old microbial isolate previously cultured in liquid medium was added to the wells in a volume of 100 µL. The medium was then incubated at 37°C for 2 × 24 h. The experiment was conducted with duplicates. The inhibition zone formed around the wells was observed, and its diameter was measured using a caliper (Damayanti et al., 2025).

Statistical Analysis

Antimicrobial test data are expressed as the mean and standard deviation (SD) of the inhibition zones. Statistical analysis to determine the significance of the tests against the four test microorganisms was performed using single-factor ANOVA in Excel; if significant differences were found at an $\alpha = 0.05$, further analysis was conducted using Duncan's test.

DNA Isolation and PCR Analysis

The 16S rRNA sequence analysis was conducted following the procedures based on previous studies (Damayanti et al., 2012; Kislichkina et al., 2024). Isolates demonstrating the highest antibacterial properties were cultivated in Tryptic Soy Broth (TSB) at 37 °C for 24 h. DNA bacterial extraction was performed using the FavorPrep Stool DNA Isolation Mini Kit, following manufacturer's instructions. Post-isolation, DNA concentration was measured using a NanoDrop spectrophotometer, and its quality was assessed using 1.8% (w/v) agarose gel electrophoresis. DNA amplification was achieved through PCR, employing the following protocol: an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 75 s, annealing at 55°C for 45 s, extension at 72°C for 1.5 min, and a final elongation at 72°C for 10 min. The primers used for this process were 1429f (5`- GGTTAC CTT GTT ACG ACT T-3`) and 27r (5`- AGAGTT TGA TCC TGG CTC AG-3`). Electrophoresis was used to analyze the PCR products, followed by 16S rDNA sequencing. Sanger sequencing was performed at the Integrated Laboratory for Research and Testing (LPPT) UGM, Yogyakarta, Indonesia.

16S rRNA Sequence and Phylogenetic Tree Analysis

The sequence data were initially edited and compiled using BioEdit software (Hall, 2013) before being transformed into FASTA format. To determine homology, the DNA sequencing data in FASTA format were analyzed using the Basic Local Alignment Search Tool (BLASTN) within the National Center for Biotechnology Information (NCBI) database. For phylogenetic analysis, the neighbor-joining (NJ) tree method was employed using Clustal X2 (Thompson et al., 1997) and MEGA X software (Kumar et al., 2018). The stability of the groupings was assessed using 1000 bootstrap values.

RESULTS

Bee Bread Analysis

Bee bread was harvested directly from the pot of the beehives, marked by a red circle (Figure 1.A). The color of the bee bread was dark yellow (Figure 1.B) with a prominent honey scent and sour smell as product of spontaneous fermentation. Figure 1.C and 1.D shows pollen morphology as observed by SEM at 100x and 400x magnification. Two types of pollen were present in the bee bread sample: smooth, round pollen with a larger size and jagged, round pollen with a smaller size.

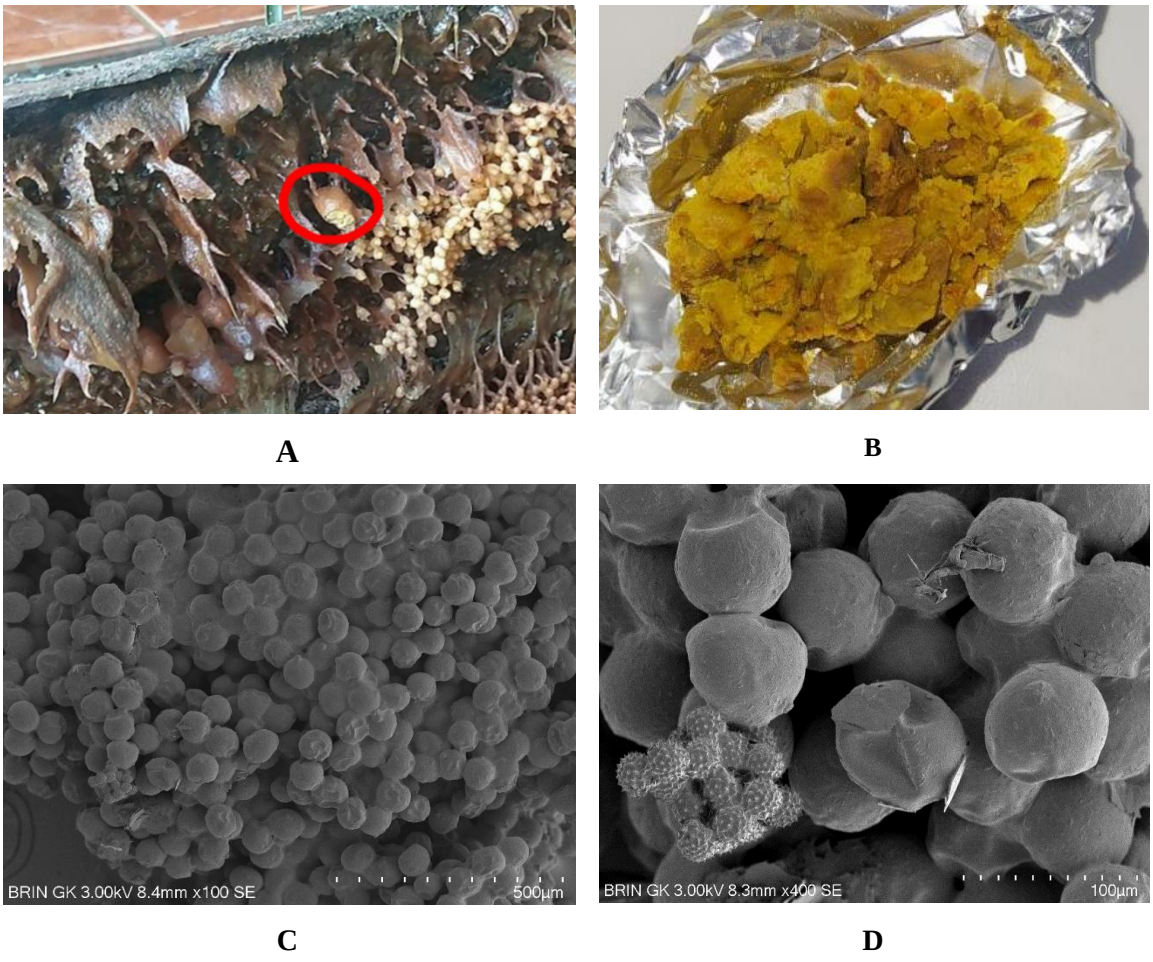


Figure 1. A: Bee bread from the beehives, shown by the red circle. B: Bee bread sample after being taken and collected from the bee pollen pot. C: SEM image of bee pollen at 100× magnification and D: at 400× magnification.

Characterization and Antibacterial Activities of Selected Isolated

Table 1 shows that 33 isolates were successfully isolated from bee bread on various media. Based on the results, all isolates were catalase-positive, and nine isolates were Gram positive while the others were negative. In terms of morphology, although the media used were intended for yeast growth, the microbes that grew included not only yeasts but also bacteria in both rod and cocci shape. Regarding antimicrobial activity, only 1 isolate demonstrated antibacterial activoty against all four tested pathogenic microorganism, namely isolate-19.

Table 1. Catalase, Gram-staining result, morphological analysis and antimicrobial assay of stingless bee bread associated microbials

No.	Isolates Name	Catalase	Gram staining	Shape	Antimicrobial Activity (mm)			
					***Candida albicans* ATCC 10231**	***E. coli* FNCC 0091**	***S. aureus* FNCC 0047**	***P. aeruginosa* FNCC 0070**
1	SDA 1 10-1A	+	-	Bacil		-	-	-
2	SDA 2 10-1A	+	-	Bacil		-	-	-
3	SDA 5 10-2A	+	-	Bacil		-	-	-
4	SDA 6 10-2A	+	-	Bacil		-	-	-
5	SDA 8 10-2A	+	+	Coccus		-	-	-
6	SDA 3 N-3	+	-	Coccus		-	-	-
7	SDA 1 N-4	+	-	Bacil		-	-	-
8	SDA 1 N-5	+	-	Bacil		-	-	-
9	SDA 2 N-4	+	-	Bacil		-	-	-
10	SDA 4 N-5	+	-	Bacil		-	-	-
11	MEA 1 10-1B	+	+	Yeast		-	-	-
12	MEA 2 10-1B	+	+	Yeast		-	-	-
13	MEA 2 10-1A	+	+	Yeast		-	-	-
14	MEA 1 10-1A	+	+	Yeast		-	-	-
15	MEA 1 N-3	+	-	Bacil		-	-	-
16	MEA 1 N-4	+	-	Bacil		-	-	-
17	MEA 2 N-4	+	+	Bacil		-	-	-
18	MEA 1 N-5	+	-	Bacil		-	-	-
19	PDA 1 10-1A	+	+	Bacil	3.65 ± 0.01^{c}	0.92 ± 0.01^{a}	4.48 ± 0.30^{d}	1.69 ± 0.05^{b}
20	PDA 2 10-1A	+	-	Coccus		-	-	-

No.	Isolates Name	Catalase	Gram staining	Shape	Antimicrobial Activity (mm)			
					***Candida albicans* ATCC 10231**	***E. coli* FNCC 0091**	***S. aureus* FNCC 0047**	***P. aeruginosa* FNCC 0070**
21	PDA 1 10-3B	+	+	Coccus		-	-	-
22	PDA 1 N-3	+	-	Bacil		-	-	-
23	PDA 1 N-4	+	+	Coccus		-	-	-
24	PDA 2 N-4	+	-	Bacil		-	-	-
25	PDA 3 N-4	+	-	Bacil		-	-	-
26	PDA 1 N-5	+	-	Coccus		-	-	-
27	YGC 2 N-3	+	-	Coccus		-	-	-
28	YGC 3 N-3	+	-	Coccus		-	-	-
29	YGC 1 N-4	+	-	Coccus		-	-	-
30	YGC 2 N-4	+	-	Bacil		-	-	-
31	YGC 3 N-4	+	-	Bacil		-	-	-
32	YGC 1 N-5	+	-	Bacil		-	-	-
33	YGC 3 N-5	+	-	Bacil		-	-	-

Notes: Antibacterial tests were performed in duplicate. Values are presented as mean ± standard deviation. Statistical analysis was conducted using single factor ANOVA and Duncan; different notation letters indicate that the four microorganisms exhibit significant differences at the 95% confidence level.

Molecular Identification of Selected Isolate

Table 2. show that based on molecular identification of isolate-19, the highest degree of similarity was found in *Bacillus subtilis* strain BCRC 10255, with a Percent Identity value of 94.91% (Accession Number: NR_116017.1). Other reference strains, including *Bacillus velezensis* strain FZB42, *Bacillus subtilis* subsp. *subtilis* strain 168, *Bacillus nakamurai*, and *Bacillus stercoris*, all display a uniform similarity level of 94.74%. This strongly confirms that the isolate is taxonomically classified within the *Bacillus subtilis* species complex.

The phylogenetic tree used *Lacticaseibacillus rhamnosus* strain NBRC 3425 at the bottom as an outgroup. This choice taxonomically serves as an evolutionary anchor from the same phylum (Bacillota) to validate the separation of the core *Bacillus* genus relationships. Isolate 19 was found in the closest cluster (sister clade) to *Bacillus subtilis* strain SBMP4, supported by a strong bootstrap value of 71. This conclusively demonstrated at the molecular level that isolate 19 taxonomically identified as a *Bacillus subtilis* species. The *Bacillus subtilis* cluster (Isolate 19 + strain SBMP4) formed a monophyletic group closely related to *Bacillus stercoris* strain D7XPN1 and *Calidifontibacillus erzurumensis* strain P2 (NR 178988.1) with bootstrap values of 28 and 60, respectively. This tree also successfully separated Isolate 19 from other classical *B. subtilis* reference strains, such as *Bacillus subtilis* subsp. subtilis strain 168 and *Bacillus subtilis* strain BCRC 10255. The separation of this group indicates certain local genetic variations or uniqueness in Isolate 19 compared to other type strains.

Morphological Characterization of Selected Isolate

The bacterial cells displayed a short cylindrical rod morphology (rod-shaped/bacilli), which is typical of the genus Bacillus. The cell ends are characteristically rounded (rounded ends) and resemble an elongated capsule or oval. The size was consistent with the dimensions of vegetative cells or the early endospore formation phase in standardized *Bacillus subtilis*. The cells were clustered, forming dense and overlapping colony networks. Most cells are arranged singly (single arrangement) or in pairs without showing rigid long-chain formations (streptobacilli). Under scanning electron microscopy at 3.00 kV, the bacterial cell wall surface showed a relatively smooth texture (smooth surface) but was closely undulating, indicating healthy, compact peptidoglycan integrity in Gram-positive cell walls with no signs of cell lysis.

Table 2. BLAST analysis results of isolate-19 from bee bread *Tetragonula laeviceps* based on 16S rRNA gene sequences

Species homolog	Per. Identity (%)	Accession number of NCBI database
Bacillus subtilis strain BCRC 10255	94.91	NR_116017.1
Bacillus velezensis strain FZB42	94.74	NR_075005.2
Bacillus subtilis subsp. subtilis strain 168	94.74	NR_102783.2
Bacillus nakamurai strain NRRL B-41091	94.74	NR_151897.1
Bacillus stercoris strain D7XPN1 16S	94.74	NR_181952.1

DISCUSSION

Pollen identification relies on morphological traits such as ornamentation, shape, and size. The pollen morphology of *T. laeviceps* bee bread samples was compared with the primary pollen database using PalDat (https://www.paldat.org/). Bee bread from *T. laeviceps* includes two distinct pollen types: one is circular, in a monad unit, inaperturate, large, and has psilate ornamentation, similar to *Hedychium gardnerianum* of the Zingiberaceae family (Halbritter & Heigl, 2021). The other type is also circular, in a monad unit, but is colporate, small, and features echinate ornamentation, resembling *Bidens pilosa* of the Asteraceae family (Halbritter et al., 2021).

Bacillus subtilis strains are occasionally found in bee pollen and mature bee bread, functioning alongside lactic acid bacteria to contribute to the hive's microbial ecosystem, enzymatic breakdown, and pathogen defense (Didaras et al., 2024; Hwang et al., 2026; Pełka et al., 2021). Bees store raw pollen in a pot along with honey and secretions, forming an environment that ranges from anaerobic to microaerophilic conditions. This setting supports a variety of microbes, such as *Bacillus* species, which assist in the decomposition of complex nutrients. *B. subtilis* strains extracted from bee bread exhibit significant activity in breaking down proteins, starches, and cellulose (Pełka et al., 2021, 2025)

Some *Bacillus* strains residing in the hive contribute to its defence by generating metabolites and antagonistic substances that suppress harmful pathogens, such as *Staphylococcus aureus* or foulbrood bacteria specific to honeybees (Hwang et al., 2025). Studies on the antibacterial activity of *Bacilli* obtained from bee bread was reported in previous studies. *Bacillus amyloliquefaciens* SGMT3 had antibacterial activity against *S. aureus* (Mohammad et al., 2020). Several *Bacillus* species had antibacterial activity against pathogenic bacteria, with interest in *Bacillus amyloliquefaciens* PD9 having antibacterial activity against *E. coli, S. aureus,* and *P. aeruginosa* (Ngalimat et al., 2019). In another study, several *Bacillus* species, dominated by *B. subtilis,* and followed by *B. licheniformis* demonstrated strong antibacterial activity, especially against *E. coli, P.*

aeruginosa, and *S. aureus*, with the exception of *B. licheniformis* (ZB03), which only demonstrated antibacterial activity against *E. coli* (Toutiaee et al., 2022).

B. subtilis has demonstrated potential as a safe probiotic agent and tolerance to stress in several previous studies. Genetic testing of specific *B. subtilis* strains isolated from bee bread confirmed that they lack harmful virulence genes and are safe for potential probiotic use, particularly in producing bacteriocins and other secondary metabolites (Pełka et al., 2025). Another advantage of *B. subtilis* is its ability to tolerate environmental stresses. These strains demonstrate a strong ability to survive high acidity and bile salt conditions, matching the key traits required for functional probiotics (Hwang et al., 2026).

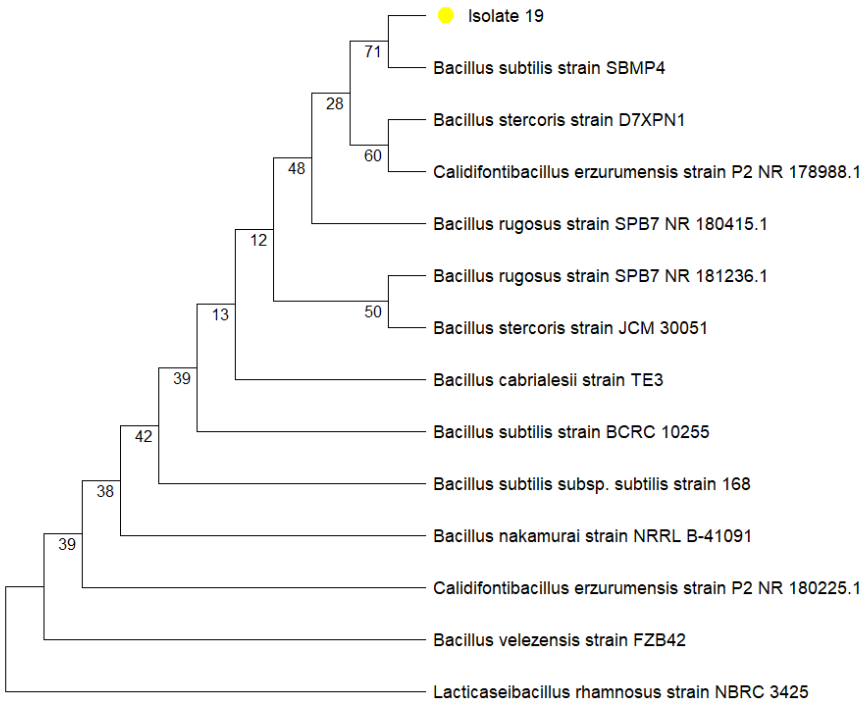
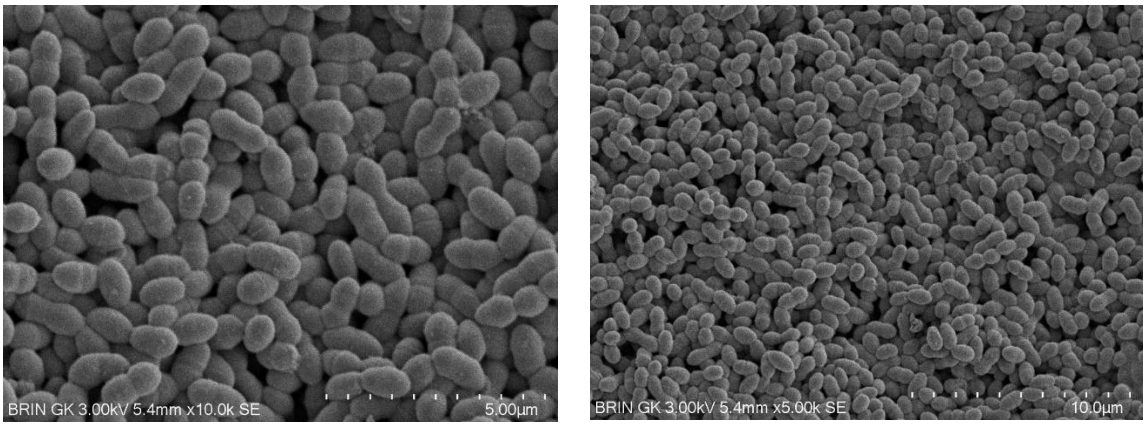


Figure 2. Phylogenetic tree analysis of isolate 19 isolated from *Tetragonula laeviceps* bee bread. The numbers at each node indicate the bootstrap values (e.g., 71, 60, and 50). These values reflect the level of confidence or statistical reliability of the branching topology after repeated computational resampling (1.000x).



A **B**

Figure 3. Morphology of *Bacillus subtilis* isolate 19 isolated from the bee bread of *Tetragonula laeviceps* as observed with an electron microscope at 10,000x and 5,000x magnification

This study effectively investigated *Bacillus subtilis* derived from *Tetragonula laeviceps* bee bread, highlighting its potential as an antibacterial agent. Nonetheless, this study is constrained by limitations, particularly in the use of 16S rRNA gene sequence analysis for species identification. Conventional microbiological taxonomy, based on partial 16S rRNA sequences, typically employs a similarity threshold of ≥ 97% to ascertain that two isolates belong to the same species. The highest similarity value for isolate 19 was 94.91% when compared to the nearest reference strain, suggesting two possibilities: the presence of unique genetic variability (indicating a novel strain or species) and limitations in sequence length. Isolate-19 exhibits ma exhibit highly diverse or unique nucleotide sequence variations relative to the type-strain database. This suggests that isolate 19 may represent a novel strain or even a new species within the genus *Bacillus*, that has not been previously documented. Additionally, the limitation of the 16S rRNA sequence length necessitates whole genome analysis in future research. Whole-genome sequencing (WGS) can also be employed to further investigate the genetic potential of isolate-19.

This study is currently at the stage of screening microorganisms associated with bee bread. It has limitations, as the antimicrobial assay did not include a reference antimicrobial agent, such as an antibiotic. Furthermore, additional testing has not yet been conducted to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Consequently, further testing is required to elucidate the biological potential of isolate-19, which was isolated from the bee bread of *T. laeviceps* bees from Sumbawa, Indonesia.

CONCLUSIONS

Thirty-three isolates were recovered from *Tetragonula laeviceps* bee bread samples. All isolates were catalase-positive, with 9 Gram-positive and mixed bacilli, cocci, and yeasts. Well diffusion assays against *Candida albicans*, *E. coli*, *S. aureus*, and *P. aeruginosa* showed only isolate-19 inhibited all pathogens. 16S rRNA analysis identified isolate 19 within the *Bacillus subtilis* species, showing 94.91% identity to BCRC 10255. NJ phylogeny and SEM confirmed short rod *Bacillus* morphology. This study showed 16S rRNA identity suggests a unique strain requiring WGS analysis.

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