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ISOLATION AND CHARACTERIZATION OF BENEFICIAL BACTERIA FROM *APIS CERANA* HONEYBEES FROM HANOI, VIETNAM

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ABSTRACT

Beneficial bacteria are vital for maintaining honeybee health by outcompeting pathogenic microorganisms, boosting immunity, and enhancing resilience to diseases. Identifying the specific bacterial strains associated with honeybees enables the development of targeted probiotics that can improve the health of bees and humans. The present study describes the isolation and identification of bacterial strains from *Apis cerana* honeybees in Hanoi, Vietnam, utilizing a culture-based method, Matrix-Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF) analysis, and 16S rRNA sequencing. MALDI-TOF analysis revealed several beneficial bacterial species, including *Lactobacillus kunkeei, Lactobacillus plantarum, Pediococcus pentosaceus, Leuconostoc mesenteroides, Leuconostoc citreum, Bacillus subtilis*, and *Bacillus megaterium*. Antimicrobial spectrum analysis showed that 16 out of the 23 identified isolates exhibited inhibitory effects against tested bacteria. Selected isolates with broad antimicrobial spectra, including *L. kunkeei, L. plantarum, P. pentosaceus, L. mesenteroides, L. citreum*, and *B. subtilis*, were further validated through 16S rRNA gene sequencing. The results confirmed the identity of these strains, emphasizing the probiotic potential of *L. kunkeei, L. plantarum, L. mesenteroides, L. citreum, P. pentosaceus*, and *B. subtilis* for honeybee health. Our findings provide valuable insights into the bacterial diversity and antimicrobial properties associated with honeybees, suggesting their use as probiotics in beekeeping and beyond.

Keywords: 16S rRNA gene, *Apis cerana*, *Bacillus*, honeybees, *Lactobacillus*, MALDI-TOF, probiotics.

INTRODUCTION

Honeybees are crucial for pollination and maintaining ecosystem stability, yet they are facing stress from various biotic and abiotic factors. These stressors include pathogens, parasites, agro-chemicals, climate change, and human activities, all of which can adversely affect their health and hive productivity (Daisley *et al.*, 2020).

Additionally, the overuse or misuse of antibiotics in apiculture has raised concerns about antibiotic resistance and the accumulation of antibiotic residues in honeybee products (Alberoni *et al.*, 2018; Daisley *et al.*, 2020). These concerns have led researchers to explore natural alternatives to conventional treatments to enhance honeybee health (Alberoni *et al.*, 2018).

A previous study has emphasized the importance of a balanced gut microbiota in maintaining honeybee health (Alberoni *et al.*, 2016). Honeybees, like other animals, possess a distinct core microbiota that influences their health (Alberoni *et al.*, 2016; Daisley *et al.*, 2020). A healthy gut microbiome supports bees through various metabolic, trophic, and protective functions (Alberoni *et al.*, 2016; Baffoni *et al.*, 2016; Motta *et al.*, 2022; Wu *et al.*, 2020). Factors such as temperature fluctuations (Zheng *et al.*, 2017), diet (Huang *et al.*, 2018), seasonal changes, pathogens (Baffoni *et al.*, 2021; Motta *et al.*, 2022; Raymann & Moran, 2018; Wu *et al.*, 2020), and chemical exposures can disrupt the gut microbiota, leading to dysbiosis. This imbalance can negatively affect honeybee health and their role in ecosystems and agriculture (Anderson & Ricigliano, 2017; Baffoni *et al.*, 2021; Kwong & Moran, 2016; Motta *et al.*, 2022).

Maintaining a healthy gut microbiota is essential for protecting honeybees from various stressors (Daisley *et al.*, 2020). Probiotic treatments using beneficial microorganisms offer a promising approach for enhancing bee health. Beneficial microbes, including *Lactobacillus*, *Bifidobacterium*, and *Bacillus* strains, have shown potential for improving honeybee health and productivity (Alberoni *et al.*, 2018; Alberoni *et al.*, 2016; Baffoni *et al.*, 2016; Sabaté *et al.*, 2012). These beneficial bacteria support immune function, nutrient absorption, and resistance to pathogens and environmental stressors. Moreover, several studies have indicated that these microbes can mitigate the negative impacts of antibiotics and pesticides, promote detoxification, and help maintain a balanced gut microbiota (Alberoni *et al.*, 2018; Motta *et al.*, 2022). However, commercial probiotics often use non-native strains that do not establish well in bee guts (Motta *et al.*, 2022). Therefore, developing natural probiotics specific to the honeybee microbiota is crucial. Exploring microorganisms from honey and bee guts, which have shown antagonistic effects against pathogens, offers new opportunities for probiotic development (Schell *et al.*, 2022; Silva *et al.*, 2017).

This study aims to isolate lactic acid bacteria and *Bacillus* strains from *Apis cerana* honeybees and evaluate their potential for combating pathogenic bacteria. The results will provide valuable data, genetic resources, and candidates for developing biological products to improve the well-being of bees and humans.

MATERIALS AND METHODS

Sample collection

Six samples (comprising adults, pupae, and larvae) were collected from six healthy *Apis cerana* colonies at a honeybee farm in Gia Lam, Hanoi, Vietnam by the Research Center for Tropical Bees and Beekeeping, Vietnam National University of Agriculture. The samples were kept on ice and promptly transported to the Laboratory of Molecular Microbiology, IBT, VAST. The health status

of the honeybee colonies was initially confirmed through the absence of disease symptoms and further verified by Reverse Transcription Polymerase Chain Reaction (RT-PCR) using specific primers for *Nosema* spp. and seven significant honeybee viruses: Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Cloudy wing virus (CWV), Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), and Sacbrood virus (SBV) as reported in previously (Lanh *et al.*, 2024).

To prepare the adult samples, we humanely euthanized them by removing their heads. Using alcohol-sterilized forceps, we carefully collected the guts by clamping the tip of the last abdominal segment and gradually removing the entire gut, including the hindgut, midgut, and honey stomach. The extracted samples were then stored at 4ºC for subsequent analysis.

Isolation of bacteria

Larvae, pupae, and the entire gut of adult bees were separately homogenized in a saline solution (0.85% NaCl). Then, 100 microliters of each appropriate dilution of the homogenized bee gut solution were spread onto De Man-Rogosa-Sharp (MRS; Oxoid, Basingstoke, UK) agar plates and Luria-Bertani (LB) broth agar plates [1.5% (w/v) agar]. The MRS plates were incubated at 37°C for 48-72 hours, and the LB plates were incubated at 37°C for 24 hours, both under microaerophilic conditions. The colonies were randomly selected and streaked on a new MRS or LB plate for pure culture isolation and incubated as mentioned above. The isolates were kept in 25% (v/v) glycerol at -80ºC for long-term storage.

Identification of isolates using MALDI-TOF

All isolates were identified using the MALDI-TOF Biotyper (Bruker Daltonics, Bremen, Germany) and then automatically evaluated by a Biotyper Compass Explorer software (version 4.1.100) (Bruker, Germany). The identification probability was expressed as a score ranging from 0 to 3.0. A score above 2.0 indicated a reliable genus identification and a likely species identification. Gram staining was employed to observe the morphological characteristics of the isolates.

Antimicrobial spectrum

The antimicrobial activity of the selected isolates against a variety of indicator bacterial species, including *Klebsiella* spp., *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, was determined using an agar well-diffusion method with some modifications (Ewnetu *et al.*, 2013). These strains were recovered from the guts of *A. cerana* honeybees and provided by the Laboratory of Molecular Microbiology, IBT, VAST. The turbidity of bacterial suspensions, adjusted to comply with the standard McFarland 0.5 (~10^8 colony forming units, CFU/mL), was spread onto the plate (Ewnetu *et al.*, 2013). A 7-mm diameter well was punched aseptically onto the Mueller–Hinton agar (Oxoid, Basingstoke, UK) with the reverse end of a sterile 1-mL pipette tip. A total of 100 µL of test agent (bacterial inoculum, 10^9 CFU/mL) was seeded into each well. Two negative controls, MRS medium and MRS at pH 4.0 were used to account for media components and low pH effects to specifically address inhibition caused by the low pH in the

inoculum produced by lactic acid bacteria (LAB). The measurement of clear zones was determined after 16-24 hours of incubation at 37°C. All assays were done in triplicate.

16S rRNA gene amplifying and sequencing

The total DNA of chosen strains were extracted using a DNA extraction kit (Thermofisher, USA) following the instructions provided by the manufacturer. One hundred ng of the extracted DNA was used as a template for amplifying the 16S rRNA genes by PCR using a specific pair of primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR thermal cycle was 94ºC for 5 min and 35 cycles of 94ºC for 1 min followed by 56ºC for 45s and 72ºC for 1 min, and a final cycle at 72º for 10 min. The reactions were carried out in a C1000 Thermal Cycler (Bio-Rad, USA). The PCR products were electrophoresed on a 1 % (w/v) agarose gel, stained with ethidium bromide, and visualized using UV light. The purified PCR products were sequenced by the capillary sequencing system, ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

The obtained sequences were assembled, subsequently aligned using Bioedit version 7.0.5.3 (Hall, 1999), and compared in the NCBI GenBank to determine the closest relatives. MEGA X was used to generate a neighbor-joining tree of LAB and *Bacillus* based on the 16S rRNA sequences (Kumar *et al.*, 2018; Saitou & Nei, 1987; Tamura *et al.*, 2004).

RESULTS

Isolation and identification of bacterial strains

A total of 48 isolates were obtained from the investigated samples. Figure 1 shows representative results of bacterial isolation. These strains were subsequently analyzed using MALDI-TOF. The MALDI-TOF analysis identified 23 isolates with the highest probability scores as follows: four *L. kunkeei* strains, one *L. plantarum* strain, one *Pediococcus pentosaceus* strain, four *Leuconostoc mesenteroides* strains, three *Leuconostoc citreum* strains, six *B. subtilis* strains, and four *B. megaterium* strains (Table 1). The remaining isolates included *E. coli*, *Enterobacter* spp., *Staphylococcus* spp., *Klebsiella* spp., *Enterococcus* spp., and several yeast species (*Hanseniaspora opuntiae*, *Pichia kluyveri*, and *Candida glabrata)*. The morphology of some isolates is shown in Figure 2.

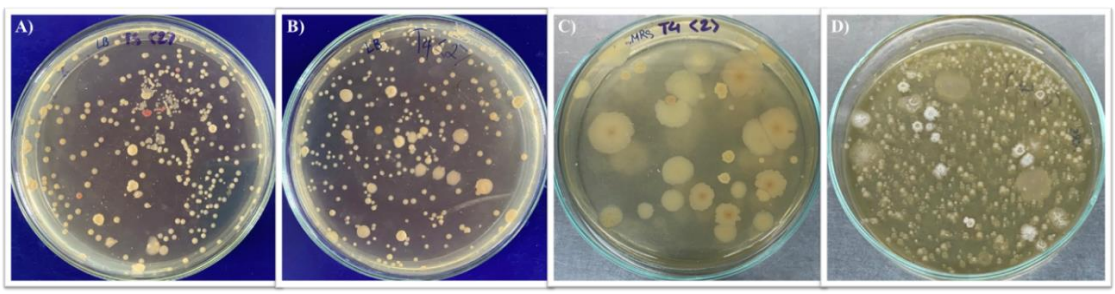

Figure 1. Isolation of bacteria from the guts of *A. cerana* adults on (A and B) LB agar plates and (C and D) MRS agar plates.

Table 1. MADI-TOF results of isolated strains from *Apis cerana* honeybees.

No	Identified strains	Source	Score value	Reference	Note
1	*Lactobacillus kunkeei*	Pupae	1.73	*Lactobacillus kunkeei* DSM 12361T DSM	LK_VN01
2	*Lactobacillus kunkeei*	Adults	1.78	*Lactobacillus kunkeei* DSM 12361T DSM	LK_VN02
3	*Lactobacillus kunkeei*	Adults	1.80	*Lactobacillus kunkeei* DSM 12361T DSM	LK_VN03
4	*Lactobacillus kunkeei*	Adults	1.78	*Lactobacillus kunkeei* DSM 12361T DSM	LK_VN04
5	*Lactobacillus plantarum*	Adults	2.11	*Lactobacillus plantarum* DSM 1055 DSM	LP_VN01
6	*Pediococus pentosaceus*	Adults	2.11	*Pediococus pentosaceus* DSM 20206 DSM	PP_VN01
7	*Leuconostoc mesenteroids*	Adults	1.85	*Leuconostoc mesenteroids* spp *mesenteroides* DSM 20343T DSM	LM_VN01
8	*Leuconostoc mesenteroids*	Adults	1.95	*Leuconostoc mesenteroids* spp *mesenteroides* DSM 20241 DSM	LM_VN02
9	*Leuconostoc mesenteroids*	Adults	2.00	*Leuconostoc mesenteroids* spp *mesenteroides* DSM 20241 DSM	LM_VN03
10	*Leuconostoc mesenteroids*	Adults	1.81	*Leuconostoc mesenteroids* spp *mesenteroides* DSM 20241 DSM	LM_VN04
11	*Leuconostoc citreum*	Adults	2.17	*Leuconostoc citreum* DSM 5577T DSM	LC_VN01
12	*Leuconostoc citreum*	Adults	2.25	*Leuconostoc citreum* 1616_B NEQAS	LC_VN02
13	*Leuconostoc citreum*	Adults	2.22	*Leuconostoc citreum* 1616_B NEQAS	LC_VN03
14	*Bacillus subtilis*	Pupae	1.94	*Bacillus subtilis* spp *subtilis* DSM 10T DSM	BS_VN01
15	*Bacillus subtilis*	Pupae	1.72	*Bacillus subtilis* DSM 5611 DSM	BS_VN02
16	*Bacillus subtilis*	Pupae	1.73	*Bacillus subtilis* spp *subtilis* DSM 10T DSM	BS_VN03
17	*Bacillus subtilis*	Pupae	1.72	*Bacillus subtilis* spp *subtilis* DSM 5660 DSM	BS_VN04
18	*Bacillus subtilis*	Pupae	1.82	*Bacillus subtilis* DSM 5611 DSM	BS_VN05
19	*Bacillus subtilis*	Adults	2.06	*Bacillus subtilis* spp *subtilis* DSM 10T DSM	BS_VN06
20	*Bacillus megaterium*	Adults	2.25	*Bacillus megaterium* DSM 32T DSM	BM_VN01
21	*Bacillus megaterium*	Adults	2.19	*Bacillus megaterium* DSM 1668 DSM	BM_VN02
22	*Bacillus megaterium*	Adults	2.21	*Bacillus megaterium* DSM 1668 DSM	BM_VN03
23	*Bacillus megaterium*	Adults	2.11	*Bacillus megaterium* DSM 1668 DSM	BM_VN04

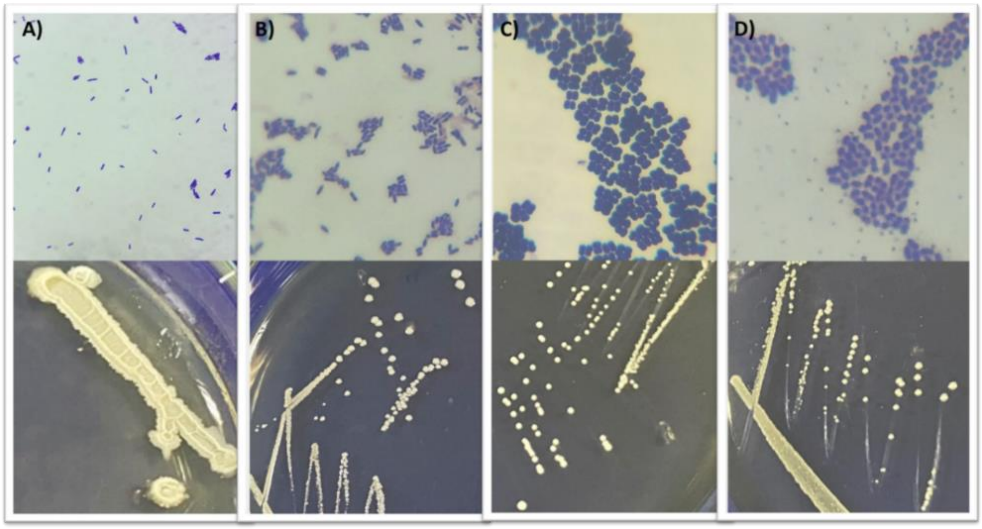

Figure 2. Images of some Gram-positive isolates and their colony morphology, respectively. A) *B. subtilis*, B) *L. kunkeii*, C) *P. pentosaceus*, D) *L. mesenteroids.*

Antimicrobial spectrum

The agar-diffusion experiment was used to rapidly screen the antimicrobial spectra of 23 isolates identified by MALDI-TOF. Results showed that 16 out of these 23 isolates inhibited at least one of the tested bacteria (Table 3 and Figure 3). Among them, 10 isolates exhibited antimicrobial activity against all eight bacteria tested, including *L. kunkeii* strain LK_VN01, four *L. mesenteroids* strains, three *L. citreum* strains, *L. plantarum* strain LP_VN01, and *P. pentosaceus* strain PP_VN01. The other two *L. kunkeei* strains inhibited 6 out of 8 tested strains, two *B. subtilis* strains inhibited 4 out of 8 tested strains, and one *B. subtilis* strain can only inhibit 2 out of 8 tested strains. Three *B. subtilis* strains (BS_VN04 – BS_VN06) and all four *B. megaterium* strains were unable to inhibit all tested strains (Table 2). A negative control (MRS medium) and MRS medium (at pH 4) did not affect all investigated indicator strains.

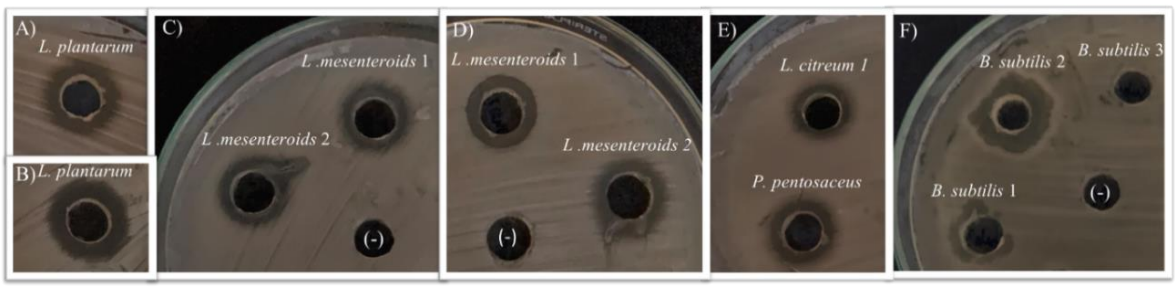

Figure 3. Antimicrobial activity exhibited by *L. plantarum* on (A) *K. oxytoca* and (B) *S. aureus*; 2 *L. mesenteroids* strains on (C) *K. pneumonia* and (D) *S. aureus*; *L. citreum* strain and (E) *P. pentosaceus* on *K. oxytoca* and 3 *B. subtilis* strains on (F) *K. pneumonia*. MRS or LB medium was used as a negative control (-).

Molecular identification of selected isolates

L. kunkeei strain LK-VN01, *L. plantarum* strain LP_VN01, *P. pentosaceus* strain PP_VN01, *L. citreum* strain LC_VN01, *L. mesenteroids* strains LM_VN01 and 02), and 3 *B. subtilis* strains (BS_VN01−03), all exhibiting broad antimicrobial spectra, were further characterized through 16S rRNA gene sequencing and phylogenetic analysis. *E. coli* ATCC 1175 (T) was used as the outgroup. The 16S rRNA gene sequence analysis revealed that isolate LK_VN01 is 100% identical to *L. kunkeei* DSM 12361 (accession number Y11374.1) and *L. kunkeei* strain isolated from Japan honey (accession number AB559820.1). The sequence analysis verified that this strain was *L. kunkeei*. The phylogenetic tree was constructed using the 16S rRNA gene sequence of the *L. kunkeei* isolate as well as that of related species. The NJ tree analysis clearly placed our strains in the same group as other *L. kunkeei* isolates from honeybee guts (*A. melifera*) (Figure 4).

Table 2. Antimicrobial spectrum of the isolated strains on pathogenic bacteria including *E. coli*, *Klebsiella* spp., *E. faecalis, P. aeruginosa,* and *S. aureus.*

Test strains	***L. kunkeei***				***L. mesenteroides***				***L. plantarum***	***P. pentosaceus***	***L. citreum***		
	LK_VN01	LK_VN02	LK_VN03	LK_VN04	LM_VN01	LM_VN02	LM_VN03	LM_VN04	LP_VN01	PP_VN01	LC_VN01	LC_VN02	LC_VN03
E. coli AC1	+++	+	+	+	+++	+++	+	+	+++	++	+++	+	+
E. coli AC2	+++	+	+	+	++	++	+	+	+++	+++	++	+	+
K. pneumonia	+++	-	-	-	+++	+++	+	+	+++	++	+++	+	+
K. varicola	+++	+	+	+	++	++	+	+	++	+++	++	+	+
K. oxytoca	+++	+	+	+	+++	+++	+	+	+++	+++	+++	+	+
P. aeruginosa	+++	-	-	-	+++	+++	+	+	+++	+	+++	+	+
E. facialis	+++	+	+	+	+++	+++	+	+	+++	+++	+++	+	+
S. *aureus*	+++	+	+	+	++	++	+	+	+++	+++	++	+	+

*Note: clear zone around well, +: 1-3 mm, ++: > 3-5 mm, +++: >5 mm, -: no inhibition zone was detected

Table 2. (continued).

Test strains	***B. subtilis***						***B. megaterium***			
	BS_VN01	BS_VN02	BS_VN03	BS_VN04	BS_VN05	BS_VN06	BM_VN01	BM_VN02	BM_VN03	BM_VN04
E. coli AC1	-	+++	-	-	-	-	-	-	-	-
E. coli AC2	+	+++	+	-	-	-	-	-	-	-
K. pneumonia	+	++	-	-	-	-	-	-	-	-
K. varicola	-	-	-	-	-	-	-	-	-	-
K. oxytoca	+	+++	-	-	-	-	-	-	-	-
P. aeruginosa	++	-	+	-	-	-	-	-	-	-
E. facialis	-	-	-	-	-	-	-	-	-	-
S. *aureus*	-	-	-	-	-	-	-	-	-	-

*Note: clear zone around well, +: 1-3 mm, ++: > 3-5 mm, +++: >5 mm, -: no inhibition zone was detected.

Additionally, the 16S rRNA sequences of two *L. mesenteroids* strains were identical to each other and shared 100% sequence identity with *L. mesenteroids* ATCC 8293 (accession number KC429780.1). These two strains were confirmed as *L. mesenteroids*. In addition, the NJ tree analysis convincingly clustered these two strains, along with *L. mesenteroids* ATCC 8293 and other *L. mesenteroids* strain TBE-8 isolated from honeybee guts (*A. melifera*) (accession number MN629244.1) into one group (Figure 4).

Similarly, analysis of the 16S rRNA sequences of remaining lactic acid bacteria, including *L. plantarum* strain LP_VN01 and *P. pentosaceus* strain PP_VN01, *L. citreum* strain LC_VN01 indicated that their 16S rRNA sequences were 100% identical to *L. plantarum* strains (GU290217.1 and OM320641), *P. pentosaceus* strains (MT000126.1 and AJ305321.1) and *L. citreum* strain ATCC 49370 (NR041727.1), respectively. The NJ phylogenetic tree showed that these three LAB species, together with their corresponding strains, were in three distinct clades (Figure 4).

Three isolated *B. subtilis* strains that exhibited broad antimicrobial activity were further characterized using 16S rRNA gene

sequencing and phylogenetic analysis. The results showed that these three strains shared identical 16S rRNA sequences (with a length of 1381 bp) and had 100% sequence identity to *B. subtilis* strain KSU 43 (accession number MN208471.1) confirming that they were *B. subtilis*. The phylogenetic tree was constructed using 16S rRNA gene sequences of *B. subtilis* isolates and related species. As indicated by the NJ tree analysis, all three isolates were clustered in one group along with other *B. subtilis* isolated from honeybee stomachs (*A. melifera*) (Figure 5).

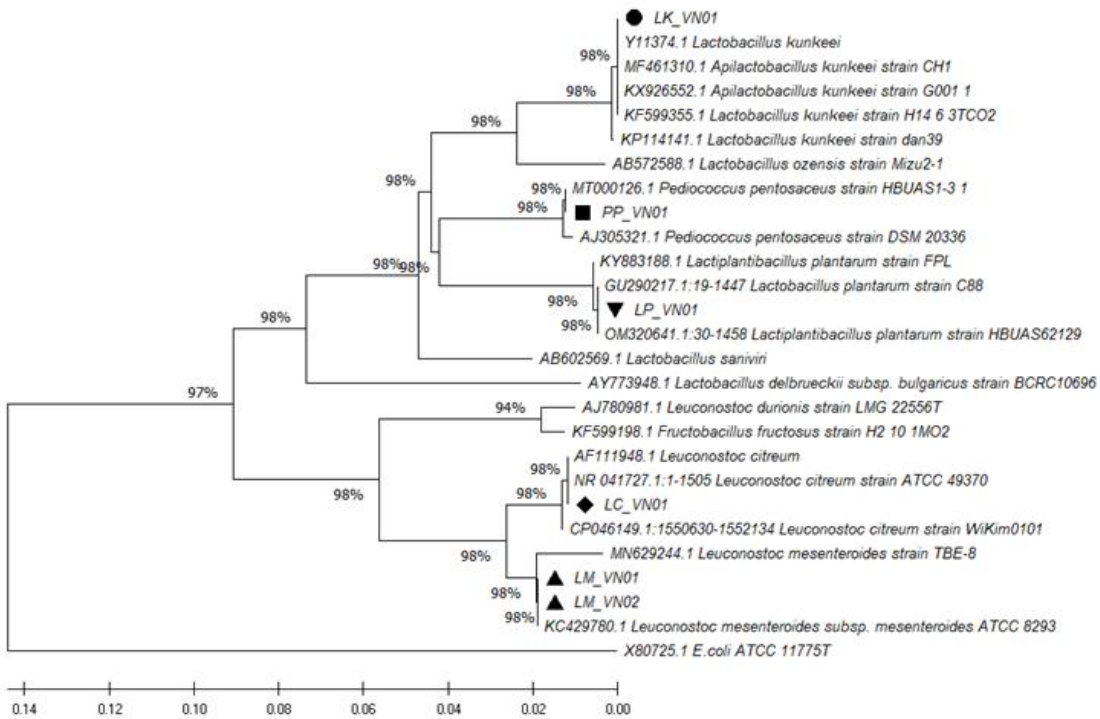

Figure 4. The phylogenetic tree of the isolated lactic acid bacteria was constructed using their sequences of 16S rRNA. *E. coli* ATCC 1175 was used as the outgroup. Isolates obtained from this study are underlined.

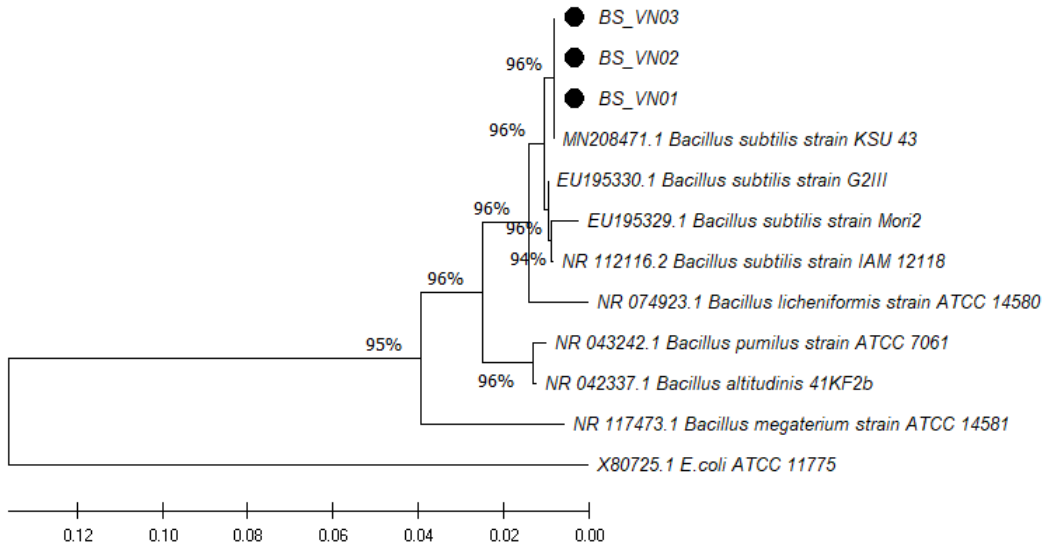

Figure 5. Phylogenetic tree constructed using the neighbour-joining method according to 16S rRNA gene sequence of *B. subtilis* strains (BS_VN01–BS_VN03) and other *Bacillus* species. *E. coli* ATCC was usd as an outgroup. The strains isolated from this study are underlined.

DISCUSSION

Honeybees heavily rely on their microbial communities to resist pests, pathogens, environmental toxins, and nutrient-poor food sources. These factors are major contributors to the high rates of honeybee colony loss (Daisley *et al.*, 2020; Wu *et al.*, 2020). Additionally, the use of veterinary medicines in beekeeping is limited due to concerns about antibiotic resistance, antibiotic residues in hive products, and potential disruption of the bee gut microbiota (Daisley *et al.*, 2020). Thus, managing honeybee health should prioritize responsible antibiotic use and explore alternative disease prevention and treatment methods.

Evidence suggests that probiotics can augment antibiotic effects and mitigate antibiotic-induced dysbiosis in humans and other animals, providing a potential alternative in beekeeping (Butler *et al.*, 2016). In this study, we aimed to isolate bacterial strains with potential probiotic benefits for honeybees and humans, focusing on lactic acid bacteria (LAB) and bacilli. The isolated strains were first screened by MALDI-TOF. As a result, we obtained four *L. kunkeei* strains, one *L. plantarum* strain, one *Pediococus pentosaceus* strain, four *Leuconostoc mesenteroids* strains, four *Leuconostoc citreum* strains, six *B. subtilis* strains, and four *B. megaterium* strains. These bacteria have previously been isolated from honey (Endo *et al.*, 2012), the guts of *A. melifera*, bee bread (Janashia *et al.*, 2016), honeybee hives (Daisley *et al.*, 2020), and other sources.

In all vertebrates and invertebrates, LABs are the most common bacteria in the

digestive system (Alberoni *et al.*, 2016). They produce antibacterial compounds such as organic acids, hydrogen peroxide, diacetyl benzoate, and bacteriocins, which benefit both humans as well as animals. The honeybee digestive system provides suitable pollen for the colonization of lactic acid bacteria (Royan, 2019). Analyses of the symbiotic systems in different bee colonies have revealed the association of *Lactobacillus* and *Leuconostoc* with honeybee health and colony size (Mathialagan *et al.*, 2018). In our study, 13 bacterial strains belonging to 5 LAB species, *L. kunkeei, L. plantarum, P. pentosaceus, L. mesenteroids,* and *L. citreum*, were isolated and characterized.

L. kunkeei has previously been isolated from honey (Endo *et al.*, 2012), the intestines of *A. melifera*, bee bread (Janashia *et al.*, 2016), honeybee hives (Daisley *et al.*, 2020), etc. Studies have indicated that this LAB is effective against a wide range of human and honeybee pathogens (Berríos *et al., 2018;* Butler *et al.*, 2016; Lashani *et al.*, 2020; Olofsson *et al.*, 2016). In this investigation, *L. kunkeei* strain LK_VN01 exhibited antimicrobial activity against all bacterial indicators tested, including pathogenic bacteria originating from the sick *A. cerana* honeybee guts, such as *Klebsiella* spp., *E. coli*, *E. faecalis*, *P. aeruginosa*, and *S. aureus*. This isolate exhibited a 100% identity to *L. kunkeei* DSM 12361 (accession number Y11374.1) and *L. kunkeei* strain isolated in Japan honey (accession number AB559820.1) in terms of the 16S rRNA gene sequence. Our findings align with a previous study that found *L. kunkeei* strains isolated from honeybee guts (*A. melifera*) possess antimicrobial activity against most honeybee pathogens (Lashani *et al.*, 2020), *Paenibacillus larvae, E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 (Pachla *et al.*, 2018). *L. kunkeei*, a member of the LAB group, is a well-known probiotic bacterium (Ramos *et al.*, 2020). The supplement *L. kunkeei*, whether used alone or in combination with other LABs, can reduce honeybee morbidity, significantly improve lifespan, induce immune stimulation, and boost honey yield (Iorizzo *et al.*, 2022; Rangberg *et al.*, 2015). It can also correct antibiotic-associated microbiota imbalances and immunodeficiencies in honeybees (Daisley *et al.*, 2020). Furthermore, recent studies have shown that several proteins produced by these bacteria can act as antibiotics against pathogens associated with human wound infections (Butler *et al.*, 2016; Olofsson *et al.*, 2016; Schell *et al.*, 2022).

L. plantarum is a well-studied lactic acid bacterium known for its probiotic properties in various hosts, including animals and humans. In honeybees, it can positively influence the gut microbiota, enhance the stability and diversity of the microbial community, potentially improve digestion and nutrient absorption, and contribute to overall health and immune system function (Daisley *et al.*, 2020). In this study, the *L. plantarum* strain LP_VN01 was isolated from *A. cerana* adults in Vietnam. This strain exhibited 100% identity in the 16S rRNA sequence with *L. plantarum* strains found in fermented food (GU290217.1) and rice wine (HBUAS62129). Previous studies reported that *L. plantarum* possesses antimicrobial properties that inhibit the growth of certain pathogens in the bee gut (Daisley *et al.*, 2023). Our *L. plantarum* strain LP_VN01 demonstrated strong antimicrobial activity against all tested strains. It has been indicated that *L. plantarum* supplementation in honeybee

diets can enhance resistance to stress factors like pesticides and environmental stressors, contributing to the overall well-being of honeybee colonies (Daisley *et al.*, 2020). In addition to *L. plantarum*, other LABs such as *L. mesenteroides*, *P. pentosaceus*, and *L. citreum* were also isolated. These LABs, found in various ecological niches, are associated with healthy honeybee colonies and are known for their fermentative properties and their ability to mitigate the impact of bee pathogens and enhance colony health (Huang *et al.*, 2021). Moreover, it has been indicated that the combination of *L. plantarum* and *L. mesenteroides* can effectively inhibit human seasonal and avian influenza viruses (Bae *et al.*, 2018).

Furthermore, studies provide compelling evidence for the ability of *Bacillus* spp. to improve honeybee gut health, stimulate the immune response, and enhance nutrient absorption (Mustar & Ibrahim, 2022). In the present study, we isolated and characterized 10 bacterial strains from two *Bacillus* species: four *B. subtilis* strains and six *B. megaterium* strains. Among these, only three *B. subtilis* strains exhibited antimicrobial effects against the tested pathogenic bacterial strains, while the remaining *B. subtilis* strains and all *B. megaterium* strains did not inhibit any tested strains. The 16S rRNA gene sequence analysis revealed that our three isolates (BS_VN01 – BS_VN03) were 100% similar to each other and were identical to *B. subtilis* KSU 43 (accession number MN208471.1), which was isolated from honeybees.

Previous research has demonstrated that *B. subtilis* strains promote honeybee gut health by inhibiting *Paenibacillus* larvae, the pathogen responsible for American Foulbrood (Alippi & Reynaldi, 2006). Sabaté et al. (2012) showed that *B. subtilis* subsp. *Subtilis*, which originated from honey, improved bee performance, including stimulating queen egg laying, increasing honey production, and reducing nosemosis and varroosis, two important bee diseases. This probiotic culture can assist beekeepers in managing their colonies and producing late nuclei and/or bee packages (Sabaté *et al.*, 2012). Taken together, our analysis suggests that these lactic acid bacteria species and *B. subtilis* strains found in honeybee resources hold promise as candidates for developing probiotics for both honeybee and human use.

CONCLUSION

Here, we have successfully isolated beneficial bacteria, including LAB and *Bacillus* species from *A. cerana* samples. Notably, among the isolated strains, *L. kunkeei*, *L. plantarum*, *L. mesenteroids*, *L. citreum*, *P. pentosaceus* and *B. subtilis* showed a notable broad antimicrobial spectrum against various foodborne and honeybee pathogens, indicating their potential for use as probiotics. These bacteria can restore microbial balance, inhibit pathogen colonization, and strengthen the immune system. More research is needed to understand their biological and nutritional significance thoroughly and to develop effective probiotics for both honeybees and humans.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Alberoni, D., Gaggìa, F., Baffoni, L. *et al.* (2016). Beneficial microorganisms for honey bees: problems and progresses. *Appl Microbiol Biotechnol* 100, 9469–9482. https://doi.org/10.1007/s00253-016-7870-4.

Alberoni, D., Baffoni, L., Gaggìa, F., Ryan, P., Murphy, K., Ross, P., Stanton, C., & Di Gioia, D. (2018). Impact of beneficial bacteria supplementation on the gut microbiota, colony development and productivity of Apis mellifera L. *Beneficial Microbes*, 9(2), 269–278. https://doi.org/10.3920/BM2017.0061.

Alberoni, D., Gaggìa, F., Baffoni, L., & Di Gioia, D. (2016). Beneficial microorganisms for honey bees: problems and progresses. *Applied microbiology and biotechnology*, 100(22), 9469-9482. https://doi.org/10.1007/s00253-016-7870-4.

Alippi, A. M., & Reynaldi, F. J. (2006). Inhibition of the growth of Paenibacillus larvae, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore–forming bacteria isolated from apiarian sources. *Journal of invertebrate pathology*, 91(3), 141–146. https://doi.org/10.1016/j.jip.2005.12.002.

Anderson, K. E., & Ricigliano, V. A. (2017). Honey bee gut dysbiosis: a novel context of disease ecology. *Current opinion in insect science*, 22, 125–132. https://doi.org/10.1016/j.cois.2017.05.020.

Bae, J. Y., Kim, J. I., Park, S., Yoo, K., Kim, I. H., Joo, W., Ryu, B. H., Park, M. S., Lee, I., & Park, M. S. (2018). Effects of Lactobacillus plantarum and Leuconostoc mesenteroides Probiotics on Human Seasonal and Avian Influenza Viruses. *Journal of microbiology and biotechnology*, 28(6), 893–901. https://doi.org/10.4014/jmb.1804.04001.

Baffoni, L., Gaggìa, F., Alberoni, D., Cabbri, R., Nanetti, A., Biavati, B., & Di Gioia, D. (2016). Effect of dietary supplementation of Bifidobacterium and Lactobacillus strains in Apis mellifera L. against Nosema ceranae. *Beneficial Microbes*, 7(1), 45–51. https://doi.org/10.3920/BM2015.0085.

Berríos, P., Fuentes, J., Salas, D., Carreño, A., Aldea, P., Fernández, F., & Trombert, A. (2018). Inhibitory effect of biofilm-forming Lactobacillus kunkeei strains against virulent Pseudomonas aeruginosa in vitro and in honeycomb moth (Galleria mellonella) infection model. *Beneficial Microbes*, 9(2), 257-268. https://doi.org/10.3920/BM2017.0048.

Butler, É., Oien, R. F., Lindholm, C., Olofsson, T. C., Nilson, B., & Vásquez, A. (2016). A pilot study investigating lactic acid bacterial symbionts from the honeybee in inhibiting human chronic wound pathogens. *International Wound Journal*, 13(5), 729–737. https://doi.org/10.1111/iwj.12360.

Daisley, B. A., Pitek, A. P., Chmiel, J. A., Gibbons, S., Chernyshova, A. M., Al, K. F., Reid, G. (2020). Lactobacillus spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. *Communications biology*, 3(1), 1-13. https://doi.org/10.1038/s42003-020-01259-8.

Endo, A., Irisawa, T., Futagawa–Endo, Y., Takano, K., du Toit, M., Okada, S., & Dicks, L. M. (2012). Characterization and emended description of Lactobacillus kunkeei as a fructophilic lactic acid bacterium. *International journal of systematic and evolutionary microbiology*, 62(Pt_3), 500–504. https://doi.org/10.1099/ijs.0.031054-0.

Ewnetu, Y., Lemma, W., & Birhane, N. (2013). Antibacterial effects of Apis mellifera and stingless bees honeys on susceptible and resistant strains of Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae in Gondar, Northwest Ethiopia. *BMC complementary and alternative medicine*,

13(1), 1–7. https://doi.org/10.1186/1472-6882-13-269.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series (Vol. 41, No. 41, pp. 95-98).

Huang, S. K., Ye, K. T., Huang, W. F., Ying, B. H., Su, X., Lin, L. H., Li, J. H., Chen, Y. P., Li, J. L., & Bao, X. L. (2018). Influence of feeding type and Nosema ceranae infection on the gut microbiota of Apis cerana workers. *MSystems*, 3(6), e00177–18. https://doi.org/10.1128/msystems.00177-18.

Huang, Y. H., Chen, Y. H., Chen, J. H., Hsu, P. S., Wu, T. H., Lin, C. F., Peng, C. C., & Wu, M.-C. (2021). A potential probiotic Leuconostoc mesenteroides TBE–8 for honey bee. *Scientific reports*, 11(1), 18466. https://doi.org/10.1038/s41598-021-97950-9.

Kwong, W. K., & Moran, N. A. (2016). Gut microbial communities of social bees. *Nature Reviews Microbiology*, *14*(6), 374-384. https://doi.org/10.1038/nrmicro.2016.43.

Iorizzo, M., Letizia, F., Ganassi, S., Testa, B., Petrarca, S., Albanese, G., De Cristofaro, A. (2022). Functional properties and antimicrobial activity from lactic acid bacteria as resources to improve the health and welfare of honey bees. *Insects*, 13(3), 308. https://doi.org/10.3390/insects13030308.

Janashia, I., & Alaux, C. (2016). Specific immune stimulation by endogenous bacteria in honey bees (Hymenoptera: Apidae). *Journal of economic entomology*, 109(3), 1474–1477. https://doi.org/10.1093/jee/tow065.

Janashia, I., Carminati, D., Rossetti, L., Zago, M., Fornasari, M. E., Haertlé, T., Giraffa, G. (2016). Characterization of fructophilic lactic microbiota of Apis mellifera from the Caucasus Mountains. *Annals of Microbiology*, 66(4), 1387-1395. https://doi.org/10.1007/s13213-016-1226-2.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, 35(6), 1547. https://doi.org/10.1093/molbev/msy096.

Lanh PT, Duong BT, Thu HT, Hoa NT, and Van Quyen D. 2024. Comprehensive analysis of the microbiome in Apis cerana honey highlights honey as a potential source for the isolation of beneficial bacterial strains. *PeerJ* 12:e17157. https://doi.org/10.7717/peerj.17157.

Lashani, E., Davoodabadi, A., & Soltan Dallal, M. M. (2020). Some probiotic properties of Lactobacillusspecies isolated from honey and their antimicrobial activity against foodborne pathogens. Veterinary research forum: an international quarterly journal, 11(2), 121–126. https://doi.org/10.30466/vrf.2018.90418.2188.

Mathialagan, M., Edward, Y., David, P., Senthilkumar, M., Srinivasan, M., & Mohankumar, S. (2018). Isolation, characterization and identification of probiotic lactic acid bacteria (LAB) from honey bees. *International J Current Microbiol Applied Sci*, 7, 849–906. https://doi.org/10.20546/ijcmas.2018.704.096.

Motta, E. V., Powell, J. E., Leonard, S. P., & Moran, N. A. (2022). Prospects for probiotics in social bees. *Philosophical Transactions of the Royal Society B*, 377(1853), 20210156. https://doi.org/10.1098/rstb.2021.0156.

Mustar, S., & Ibrahim, N. (2022). A Sweeter Pill to Swallow: A Review of Honey Bees and Honey as a Source of Probiotic and Prebiotic Products. *Foods*, 11(14), 2102. https://doi.org/10.3390/foods11142102.

Olofsson, T. C., Butler, È., Markowicz, P., Lindholm, C., Larsson, L., & Vásquez, A. (2016). Lactic acid bacterial symbionts in honeybees–an unknown key to honey's antimicrobial and therapeutic activities. *International Wound Journal*, 13(5), 668-679. https://doi.org/10.1111/iwj.12345.

Pachla, A., Wicha, M., Ptaszyńska, A. A., Borsuk, G., Łaniewska–Trokenheim, Ł., & Małek, W. (2018). The molecular and phenotypic characterization of fructophilic lactic acid bacteria isolated from the guts of Apis mellifera L. derived from a Polish apiary. *Journal of applied genetics*, 59(4), 503-514. https://doi.org/10.1007/s13353-018-0467-0.

Ramos, O. Y., Basualdo, M., Libonatti, C., & Vega, M. F. (2020). Current status and application of lactic acid bacteria in animal production systems with a focus on bacteria from honey bee colonies. *Journal of Applied Microbiology*, 128(5), 1248-1260. https://doi.org/10.1111/jam.14469.

Rangberg, A., Mathiesen, G., Amdam, G., & Diep, D. (2015). The paratransgenic potential of Lactobacillus kunkeei in the honey bee Apis mellifera. *Beneficial Microbes*, 6(4), 513-523. https://doi.org/10.3920/BM2014.0115.

Raymann, K., & Moran, N. A. (2018). The role of the gut microbiome in health and disease of adult honey bee workers. *Current opinion in insect science*, 26, 97–104. https://doi.org/10.1016/j.cois.2018.02.012.

Royan, M. (2019). Mechanisms of probiotic action in the honeybee. Critical Reviews™ in Eukaryotic Gene Expression, 29(2). Royan M. (2019). Mechanisms of Probiotic Action in the Honeybee. *Critical reviews in eukaryotic gene expression*, 29(2), 95–103. https://doi.org/10.1615/CritRevEukaryotGeneExpr.2019025358.

Sabaté, D., Cruz, M., Benítez–Ahrendts, M., & Audisio, M. (2012). Beneficial effects of Bacillus subtilis subsp. subtilis Mori2, a honey–associated strain, on honeybee colony performance. *Probiotics and Antimicrobial Proteins*, 4(1), 39–46. https://doi.org/10.1007/s12602-011-9089-0.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, 4(4), 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.

Schell, K. R., Fernandes, K. E., Shanahan, E., Wilson, I., Blair, S. E., Carter, D. A., & Cokcetin, N. N. (2022). The Potential of Honey as a Prebiotic Food to Re-engineer the Gut Microbiome Toward a Healthy State. Frontiers in nutrition, 9, 957932. https://doi.org/10.3389/fnut.2022.957932.

Silva, M. S., Rabadzhiev, Y., Eller, M. R., Iliev, I., Ivanova, I., & Santana, W. C. (2017). Microorganisms in honey. *Honey analysis*, 500, 233–257. https://doi.org/10.5772/63259.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, 101(30), 11030-11035. https://doi.org/10.1073/pnas.0404206101.

Wu, Y., Zheng, Y., Chen, Y., Chen, G., Zheng, H., & Hu, F. (2020). Apis cerana gut microbiota contribute to host health though stimulating host immune system and strengthening host resistance to Nosema ceranae. *Royal Society open science*, 7(5), 192100. https://doi.org/10.1098/rsos.192100.

Zheng, H., Powell, J. E., Steele, M. I., Dietrich, C., & Moran, N. A. (2017). Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proceedings of the National Academy of Sciences*, 114(18), 4775–4780. https://doi.org/10.1073/pnas.1701819114.