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Contributed Paper

Screening of Antagonistic Bacterial Isolates from Hives of *Apis cerana* in Vietnam Against the Causal Agent of American Foulbrood of Honey Bees, *Paenibacillus larvae*

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ABSTRACT

American foulbrood (AFB) is a virulent disease of honey bee brood caused by the Gram-positive, spore-forming bacterium; *Paenibacillus larvae*. In this study, we determined the potential of bacteria isolated from hives of Asian honey bees (*Apis cerana*) to act antagonistically against *P. larvae*. Isolates were sampled from different locations on the fronts of *A. cerana* hives in Vietnam. A total of 69 isolates were obtained through a culture-dependent method and 16S rRNA gene sequencing showed affiliation to the phyla Firmicutes and Actinobacteria. Out of 69 isolates, 15 showed strong inhibitory activity against *P. larvae*; *Bacillus pocheonensis* (VN101) showed the largest zone of inhibition (26 ± 1 mm). In this study, the diversity and richness of antagonistic isolates indicated that *Bacillus* spp. are the most promising as inhibitors of *P. larvae*. These finding suggested that certain bacterial isolates can act as antagonist to control *P. larvae* and may have other biotechnological applications.

Keywords: honey bee, 16S rRNA, microbial ecology, *Paenibacillus larvae*, antagonistic bacteria

1. INTRODUCTION

The Asian honey bee, *Apis cerana*, along with the European honey bee, *A. mellifera*, serve as critical insect pollinators of multiple economically important crops in Southeast Asia [1]. In recent years, the health of *A. mellifera* colonies has been a major concern because extensive colony losses threaten agriculture's reliance on pollination services from honey bees [2]. Various factors are most likely acting in concert to fuel this pollinator

crisis, including pests and pathogens such as viruses [3], microsporidians [4], ectoparasitic mites [5], fungi and bacteria [6]. In response to increased *A. mellifera* colony losses, *A. cerana* has received increased interest from researchers as a "healthier" honey bee alternative for beekeeping, with their demonstrably faster hygienic response that has the potential to better control the spread of pathogens within colonies [7]. Coupled with the drive to identify worker traits that could improve honey bee health is an increased interest in understanding how nest microenvironments [8] and the colony microbiome (both in the nest and from the bees themselves) contributes to the health and productivity of colonies [9-11]

There is a growing understanding of the ways in which bacterial communities interact with their hosts as well the wider colony environment [11]. Past studies have examined the relationship amongst colony microbes, fitness, nutrition and disease resistance in honey bees [10-12]. Recently, much focus has centered on bacterial honey bee communities and their presence in various in bee body parts [13], their contribution to bee bread [14] and their influence on honey [15]. The majority of research has focused on bacterial communities in *A. mellifera* colonies, but studies are broadening to investigations of *A. cerana* colonies [13]. Of great relevance to honey bee health are microbes that might act against the Gram-positive spore-forming bacterium *Paenibacillus larvae*; a causative agent of American foulbrood (AFB). This disease is among the most virulent of honey bee brood [16]. Recent studies have demonstrated the potential of honey bee bacterial symbionts that have with clear antagonistic effects against *P. larva*e; these bacteria have been isolated from *A. mellifera* larvae [17] and the gut of *A. cerana japonica* [18].

AFB is frequently reported in *A. mellifera*, and often results in death of an infected colony. AFB is known in parts of Asia, but has not been documented in some regions, such as Thailand, India and Vietnam [1]. Despite a lack of information regarding its prevalence in *A. cerana* and other Asiatic honey bee species [1], it is important to gain a deeper understanding of the microbial symbionts found in microenvironments of the Asian honey bee, and how these bacteria may interact with pathogenic bacteria such as *P. larvae*. Therefore, the objectives of this study were to improve our understanding of microbial community of *A. cerana* hives and to investigate the potential use of these microbes as natural antagonistic agents against *P. larvae*. We achieved this using 16S rRNA gene sequence analysis to identify cultivable bacterial species that were present in samples collected from the fronts of *A. cerana* hives in Vietnam. These microbes were then cultured so that they could be evaluated *in vitro* for potential antagonist activity against *P. larvae*.

2. MATERIALS AND METHODS

2.1 Sampling and Isolation of Hive Associated Bacteria

Microbial samples were collected from three apiaries of *A. cerana* colonies that were housed in wooden hives and maintained by a commercial beekeeper in Da Chong, Ba Vi District, Hanoi Province, Vietnam in year 2013. A total of 9 field samples were collected using sterile cotton swabs that were rubbed on three different locations of the hives: (a) at top of the hive front, (b) 5 cm inside the entrance on the hive bottom, and (c) 10 cm above the entrance. The swabs were aseptically packed and sent to the Bee Research Laboratory, USDA-ARS in Beltsville, MD, USA, where they were processed following Good Laboratory Practices (GLP), with all

materials autoclaved and properly disposed of following use.

Prior to cultivation, swabs were moistened with 100 µl of sterile distilled water. All samples were cultivated on nutrient agar (NA) plates at 37 °C for 24 h. Bacterial morphology was primarily used to distinguish isolates; quantity was measured as cfu/plate. All bacterial isolates were re-cultured and kept in 50% glycerol solution and stored at -20 °C for further study.

2.2 DNA Extraction and Sequencing

Bacteria were taken directly from NA plates for DNA extraction. One full loop of bacteria was collected to be re-suspended in 1 ml of sterile water in a microcentrifuge tube. This tube was then centrifuged at 12,000 rpm for 1 min so that the supernatant could be removed. Following the manufacturer's instructions, 200 ul of InstaGene™ Matrix (Bio-Rad, Hercules, CA) was added to the pellet. 16S rRNA genes were amplified by PCR using universal eubacterial primers eu27.F (5′ GAGAGTTTGATCCTGGCTCAG 3′) and eu1495.R (5′ CTACGGCTACCTTGTT ACGA 3′)[17]. PCR was performed in a final volume of 30 µl, which consisted of 1 µl bacterial extract (approximately 200 ng/µl of total genomic DNA), 2 U Taq DNA polymerase (Qiagen, Germantown, MD) with 1× reaction buffer, 1 mM dNTPs mix, and 0.1 µM of each primer. PCR was performed using a thermal cycler set at 30 cycles of 93 °C 1 min, 54 °C 1 min, and 72 °C 1 min. Bands of an appropriate size were confirmed using 1.5 % agarose gel electrophoresis. PCR products were then purified directly with QIAquick® PCR purification kit (Qiagen, Germantown, MD). Sequencing was performed using Big Dye 2.0 (Applied Biosystems, Foster City, CA) end-terminal cycle sequencing, followed by separation and analysis on an Applied Biosystems 3130 DNA Genetic Analyzer.

2.3 Phylogenetic Analysis

DNA sequence data from this study were submitted to GenBank (accession numbers KU060150-KU060220), and compared to closely related 16S rRNA sequences retrieved from GenBank database by BLAST-N searches (http:// blast.ncbi.nlm. nih.gov/Blast.cgi). Multiple sequence alignments were performed with CLUSTALW multiple alignment software embedded in BioEdit [19]. A phylogenetic relationship was constructed using MEGA6 software that employed maximum likelihood algorithms, based on paired alignments of nucleotide sequences of the 16S rRNA genes, to compare our isolates with other corresponding organisms. The Kimura-2 parameter was applied for generating phylogenetic tree [20], which were bootstrapped with 1,000 bootstrap replications; only values greater than 70% were considered above the branches.

2.4 Antagonist inhibition assays

P. larvae strain ATCC9545 was used as a type reference strain. It was obtained from the American Type Culture Collection (ATCC, USA) and cultivated in brain heart infusion (BHI) agar (Difco, Sparks, MD) at 37 °C for 3 days. Inhibitory activities of each isolate against *P. larvae* were assayed using a modified well diffusion technique, as described previously [18]. Wells (6 mm in diameter) were cut on an agar plate using a cork borer. Bacterial suspensions from *P. larvae* cultures, grown overnight, were suspended in sterile distilled water and measured comparing McFarland standard no. 1. The suspension then spread evenly over the surface of each BHI agar plate, as previously described by Evans and Armstrong (2006) [17]. At the same time,

each cultivable bacterial colony on NA agar plate was suspended as described above. Then, 100 µl of each bacterium suspension was applied to one well of each plate. Tests were performed in triplicate. Positive and negative controls were prepared using reference commercial antibiotic discs, lincomycin (2 µg/disc) (BD BBL™, Sparks, MD), and 100 µl of sterile distilled water, respectively. The modified well systems were incubated at 37 °C for 48 h, and then inhibition zone diameters were measured.

3. RESULTS AND DISCUSSION

3.1 Characterization of Isolates by 16S rRNA Gene Sequence Analysis

The microbial communities were identified using a culture-dependent method. All were clearly identified based on colony size, color, and morphology. All the partial bacterial 16S rRNA gene sequences (n = 69) were subjected to an NCBI BLAST-N search (https://www.ncbi.nlm.nih.gov/BLAST/) with the highest sequence similarities and designated name id; VN (accession numbers KU060150-KU060220) (Table 1). A phylogenetic tree of the sequenced isolates was constructed by maximum likelihood analysis (Figure 1). The isolates could be grouped into two major bacterial phyla: Firmicutes and Actinobacteria (Table 1), and represented 10 known genera: *Bacillus, Staphylococcus, Enterococcus, Kucuria, Rhodococcus, Brevibacillus, Arthobacter, Kuthia, Paenibacillus* and *Lysinibacillus* (Figure 2). Dominant isolates showed the best match to *Bacillus* spp. Three bacterial isolates showed <96 % sequence similarity. VN 59 and VN 95 showed the best match to *Bacillus odysseyi*, whereas VN109 was closest to *Paenibacillus ginsengarvi*. Moreover isolates VN104, VN111, VN113, VN114 and VN115 matched uncultured bacterium in the 16S RNA gene database. Therefore, they were assigned *Bacterium* spp. accession numbers on Genbank; however, the phylogenetic tree revealed they were closest to the phylum Actinobacteria.

Honey bees are generalist pollinators that fill their hives with nutritionally rich resources. The core bacterial microbiota of the honey bee contains lactic acid bacteria (LAB), generally affiliated with the honey bee gut [21]. Whereas the hive microenvironment is highly diverse, little is known about sharing of bacterial taxonomic groups worldwide [14]. Previous studies suggest that differences in bacterial diversity among colonies reflect environmental conditions. [17]. Moreover, our finding showed some cultivable bacteria as *Staphylococcus* spp. and *Enterococcus* spp., which are frequently found for vertebrate animal like livestock [22-23]. We hypothesize that the microorganisms associated in or near the hive entrance provide a symbiotic relationship that may be critical to bee health and can provide defense against enteric pathogens. Conversely, the top of hive we did not observed any culturable bacteria and thus it is very likely that bees culture and or maintain the bacteria in the hive entrance as an active means of defense against microorganisms.

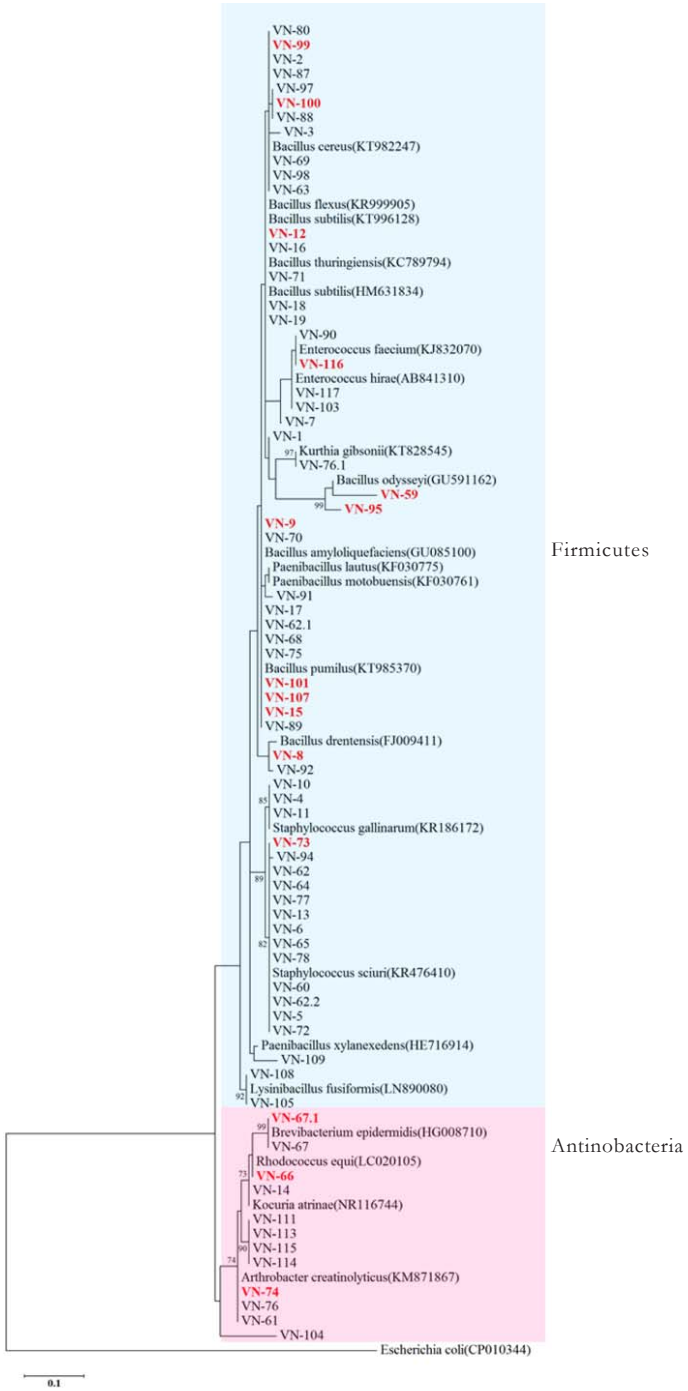

Figure 1. **16S rRNA phylogenetic relationships.** Maximum likelihood tree showing relationships among bacteria isolates from this study. The resulting trees were generated by conducting 1,000 bootstrap replicates. The bar indicates the genetic distance scale (number of nucleotide differences per site). Bootstrap values >70 are shown in the corresponding nodes. *Escherichia coli* (CP010344) is presented as outgroup sequence. Antagonistic bacterial isolates against *P. larvae* are highlighted in bold red type.

Table 1. Identification of cultivation bacteria isolates and their closest affiliations based on 16 rRNA gene sequences.

Isolation no.	Accession number	Closest species	Bacteria division	Similarity (%)
VN-1	KU060150	*Bacillus subtilis*	Firmicutes	100
VN-2	KU060151	*Bacillus thuringiensis*	Firmicutes	100
VN-3	KU060152	*Bacillus thuringiensis*	Firmicutes	100
VN-4	KU060153	*Staphylococcus gallinarum*	Firmicutes	100
VN-5	KU060154	*Staphylococcus sciuri*	Firmicutes	99
VN-6	KU060155	*Staphylococcus sciuri*	Firmicutes	100
VN-7	KU060156	*Enterococcus casseliflavus*	Firmicutes	100
VN-8	KU060157	*Bacillus mycoides*	Firmicutes	100
VN-9	KU060158	*Bacillus pumilus*	Firmicutes	100
VN-10	KU060159	*Staphylococcus gallinarum*	Firmicutes	100
VN-11	KU060160	*Staphylococcus gallinarum*	Firmicutes	99
VN-12	KU060161	*Bacillus subtilis*	Firmicutes	100
VN-13	KU060162	*Staphylococcus sciuri*	Firmicutes	99
VN-14	KU060163	*Kocuria atrinae*	Actinobacteria	100
VN-15	KU060164	*Bacillus aerophilus*	Firmicutes	100
VN-16	KU060165	*Bacillus subtilis*	Firmicutes	100
VN-17	KU060166	*Bacillus aerophilus*	Firmicutes	100
VN-18	KU060167	*Bacillus subtilis*	Firmicutes	100
VN-19	KU060168	*Bacillus subtilis*	Firmicutes	100
VN-59	KU060169	*Bacillus odysseyi*	Firmicutes	90
VN-60	KU060170	*Staphylococcus sciuri*	Firmicutes	100
VN-61	KU060171	*Arthrobacter creatinolyticus*	Actinobacteria	99
VN-62	KU060172	*Staphylococcus sciuri*	Firmicutes	99
VN-62.1	KU060173	*Bacillus amyloliquefaciens*	Firmicutes	100
VN-62.2	KU060174	*Staphylococcus sciuri*	Firmicutes	99
VN-63	KU060175	*Bacillus cereus*	Firmicutes	100
VN-64	KU060176	*Staphylococcus sciuri*	Firmicutes	100
VN-65	KU060177	*Staphylococcus sciuri*	Firmicutes	99
VN-66	KU060178	*Rhodococcus equi*	Actinobacteria	100
VN-67	KU060179	*Brevibacterium epidermidis*	Actinobacteria	100
VN-67.1	KU060180	*Brevibacterium epidermidis*	Actinobacteria	100
VN-68	KU060181	*Bacillus amyloliquefaciens*	Firmicutes	99
VN-69	KU060182	*Bacillus thuringiensis*	Firmicutes	100
VN-70	KU060183	*Bacillus methylotrophicus*	Firmicutes	100
VN-71	KU060184	*Bacillus subtilis*	Firmicutes	100
VN-72	KU060185	*Staphylococcus sciuri*	Firmicutes	99
VN-73	KU060186	*Staphylococcus sciuri*	Firmicutes	99
VN-74	KU060187	*Arthrobacter creatinolyticus*	Actinobacteria	99
VN-75	KU060188	*Bacillus methylotrophicus*	Firmicutes	100

Table 1. Continued.

Isolation no.	Accession number	Closest species	Bacteria division	Similarity (%)
VN-76	KU060189	*Arthrobacter creatinolyticus*	Actinobacteria	100
VN-76.1	KU060190	*Kurthia gibsonii*	Firmicutes	99
VN-77	KU060191	*Staphylococcus sciuri*	Firmicutes	100
VN-78	KU060192	*Staphylococcus sciuri*	Firmicutes	100
VN-80	KU060193	*Bacillus cereus*	Firmicutes	100
VN-87	KU060194	*Bacillus thuringiensis*	Firmicutes	100
VN-88	KU060195	*Bacillus aryabhattai*	Firmicutes	100
VN-89	KU060196	*Bacillus pumilus*	Firmicutes	100
VN-90	KU060197	*Enterococcus faecium*	Firmicutes	100
VN-91	KU060198	*Paenibacillus xylanilyticus*	Firmicutes	100
VN-92	KU060199	*N/A*	Unidentified	100
VN-94	KU060200	*Staphylococcus lentus*	Firmicutes	100
VN-95	KU060201	*Bacillus odysseyi*	Firmicutes	96
VN-97	KU060202	*Bacillus aryabhattai*	Firmicutes	100
VN-98	KU060203	*Bacillus thuringiensis*	Firmicutes	99
VN-99	KU060204	*Bacillus cereus*	Firmicutes	100
VN-100	KU060205	*Bacillus aryabhattai*	Firmicutes	100
VN-101	KU060206	*Bacillus pocheonensis*	Firmicutes	99
VN-103	KU060207	*Enterococcus hirae*	Firmicutes	100
VN-104	KU060208	*N/A*	Unidentified	100
VN-105	KU060209	*Lysinibacillus fusiformis*	Firmicutes	100
VN-107	KU060212	*Bacillus pumilus*	Firmicutes	100
VN-108	KU060213	*Lysinibacillus contaminans*	Firmicutes	99
VN-109	KU060214	*Paenibacillus ginsengarvi*	Firmicutes	97
VN-111	KU060215	*N/A*	Unidentified	100
VN-113	KU060216	*N/A*	Unidentified	100
VN-114	KU060217	*N/A*	Unidentified	100
VN-115	KU060218	*N/A*	Unidentified	100
VN-116	KU060219	*Enterococcus faecium*	Firmicutes	99
VN-117	KU060220	*Enterococcus hirae*	Firmicutes	100

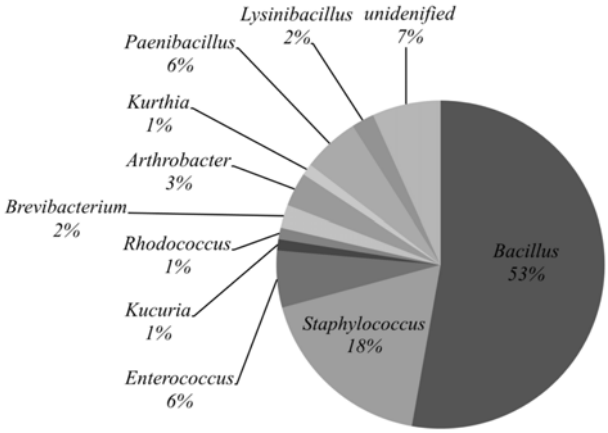

Figure 2. Diversity of total species and genera found within different honey bee (*A. cerana*) apiary positions.

3.2 Species Distribution

The cultivable isolates (n = 69) represent two phyla: Firmicutes and Actinobacteria, and ten known genera: *Bacillus, Staphylococcus, Enterococcus, Kucuria, Rhodococcus, Brevibacillus, Arthobacter, Kuthia, Paenibacillus* and *Lysinibacillus* (Figure 2). A total of 69 16S haplotypes with similarity to a minimum of 26 bacterial taxa were identified from the survey; 6 isolates were presented to unidentified cultivable bacteria (Table 1).

Three genera were extensively observed in this experiment. Fifty-three percent of all isolates fell within the genus *Bacillus* (n = 30). Of these, many isolates (n = 6) belonged to the *B. subtilis* group, with 100% similarity. Five isolates were best matched to *B. thuringiensis* and three were closest to *B. pumilus*. Eighteen percent of all isolates fell within the genus *Staphylococcus* (n = 16); most of these isolates (n = 12) belonged to *S. sciuri*. Finally, six percent of all isolates (n = 5) belonged to the genus *Enterococcus* (Figure 2).

This study revealed that microbial contributions from different locations on the hive fronts are diverse. On the top of the hive, no obvious bacteria were observed. In the entrance of the hive, 48% of isolated bacteria (n = 12 isolates) belonged to genera *Bacillus*, 32% were *Staphylococcus* (n = 8), whereas others fell into the genera *Kucuria, Rhodococcus* and *Brevibacterium*. In the site 10 centimeters above the entrance, a similar trend of two main genera of cultivable bacteria was observed, *Bacillus* and *Staphylococcus* (n = 26 isolates) and lower abundances of a broad array of other bacteria were found (Table 2). Our results show that there is potential for a broad diversity of cultivable bacteria to interact with honey bees as they move in and out of their hive. It is possible that some of these bacteria might be obtained from horizontal transmission from sources, such as flower nectar [14], bee bread [24], and other sources such as materials collected via the environment surrounding [25].

3.3 Antagonistic Activity Against *P. larvae*

All bacterial isolates from this study were assessed for possible antagonistic effect against *P. larvae* by using *in vitro* inhibition assays. Of the 69 isolates tested, 15 (21.74%) demonstrated inhibition zones (Figure 3 and Figure 4); VN101 showed the largest inhibitory zone (26±1 mm) (Figure 3). In phylogenetic tree, 11 isolates that inhibited *P. larvae* were distributed among *Bacillus* spp. (Figure 1): *B. mycoides* (VN8), *B. pumilus* (VN9,

VN107), *B. subtilis* (VN12), *B. aerophilus* (VN15), *B. odyssey* (VN59 and VN95), *B. epidermidis* (VN67.1), *B. cereus* (VN99), *B. aryabhattai* (VN100), and *B. pocheonensis* (VN101). Our results showed some isolates corresponded to previous report by Yoshiyama and Kimura (2009) [18], who found various bacteria genera in the guts of *A. cerana* worker bees. However, that study found that *Bacillus* spp. showed a strong inhibitory effect against *P. larvae*. Furthermore, aerobic spore-forming bacteria isolated from *A. mellifera* hives and honey, such as *B. subtilis, B. pumilus, B. licheniformis, B. cereus, B. megaterium* and *B. laterosporus* also revealed antagonistic activity against the AFB pathogen [26] that some bacterial groups are corresponding with our finding.

It is well known that members of the genus *Bacillus* produce a wide spectrum of antimicrobial substances, including peptide and lipopeptide antibiotics, as well as bacteriocins [27]. Several studies have shown that *Bacillus* can kill other bacteria. For example, *B. cereus* produce antimicrobial products like a bacteriocin-like inhibitory substance (BLIS) to restrict the growth of several closely related bacteria and other clinically important Gram-positive bacteria such as *Staphylococcus aureus* and *Micrococcus luteus* [28]. Similarly, *B. subtilis* produce powerful antibiotics: surfactin acts against the growth of *P. larvae* and an antifungal substance acts against a causative pathogen of chalkbrood disease, *Ascosphaera apis* [29]. Certainly the substances produced by *Bacillus* species can only be effective against the same or closely related bacterial species [30]. This explains why almost *Bacillus* species show antagonistic activity against *P. larvae*.

Table 2. Sequence abundance of the 10 observed taxonomic keys from each position of honey bee samples that affiliated with distinct phylogenetic groups.

Genus	Phylum	Position of honey bee samples		
		Top hive front	In entrance	10 cm above entrance
Bacillus	Firmicutes	0 % (0)	48.0% (12)	40.9 % (18)
Staphylococcus	Firmicutes	0 % (0)	32.0 % (8)	18.2% (8)
Enterococcus	Firmicutes	0 % (0)	4.0 % (1)	9.1 % (4)
Kucuria	Actinobacteria	0 % (0)	4.0 % (1)	0 % (0)
Rhodococcus	Actinobacteria	0 % (0)	4.0 % (1)	0 % (0)
Brevibacterium	Actinobacteria	0 % (0)	8.0 % (2)	0 % (0)
Arthrobacter	Actinobacteria	0 % (0)	0 % (0)	6.8 % (3)
Kurthia	Firmicutes	0 % (0)	0 % (0)	2.3 % (1)
Paenibacillus	Firmicutes	0 % (0)	0 % (0)	4.5 % (2)
Lysinibacillus	Firmicutes	0 % (0)	0 % (0)	4.5 % (2)
Unidenified bacteria	-	0 % (0)	0 % (0)	13.6 % (6)

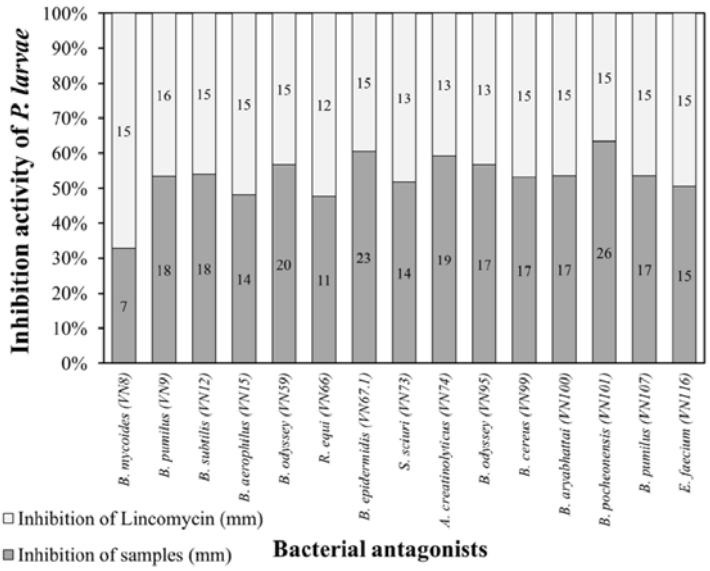

Figure 3. *In vitro* inhibitory activity of bacterial isolates against *P. larvae*. Data were calculated from experiments performed in triplicate. Lincomycin (2 µg/disc) was used as a positive control.

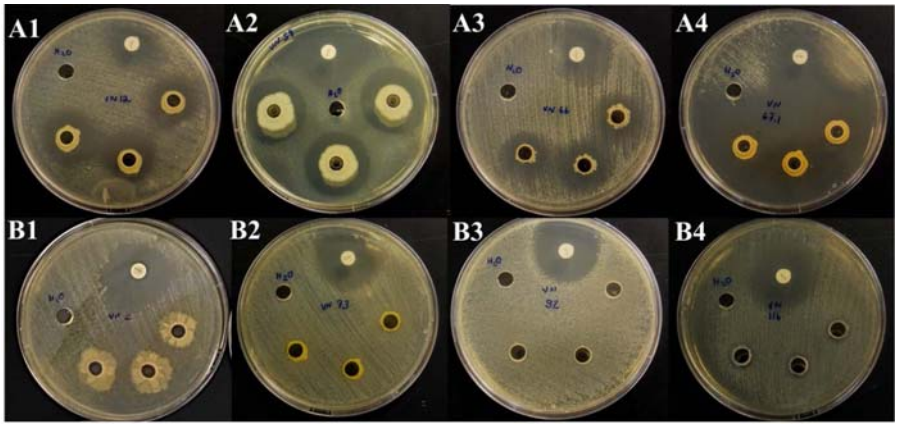

Figure 4. *In vitro* antagonistic activity of some bacterial strains. Agar well-diffusion assay of some bacterial isolates against *P.larvae* strain ATCC9545. **(A)** inhibition of *P. larvae* by cell suspensions of isolate VN12 (*B. subtilis*)(A1), VN59 (*B. odyssey*)(A2), VN66 (*R. equi*)(A3) and VN67.1 (*B. epidermidis*)(A4) (Positive results). **(B)** No inhibition of *P. larvae* by cell suspensions of isolates VN2 (*B. thuringiensis*) (B1), VN73 (*S. sciuri*) (B2), VN92 (unidentified bacterium) (B3), and VN116 (*E. faecium*) (B4) (Negative results). Lincomycin (2 µg/disc) and sterile distilled water (H_2O) were used as positive and negative control for all experiments.

4. CONCLUSIONS

This study was the first to study bacteria associated with the *A. cerana* hive microenvironment, and the first to investigate their potential inhibitory activity against *P. larvae*. We have presented results from both the traditional bacterial culture method and 16S rRNA gene sequencing to explore the bacterial communities. Based on data derived from our study, the majority of the cultivated bacteria fell into phyla Firmicutes and Actinobacteria. Out of 69 isolates, 15 exhibited strong antagonistic activity toward *P. larvae*. Most these isolates belong to *Bacillus* spp. According to the preliminary results presented here, different locations on the hives (i.e. top of the hive, center of the hive front, and inside the hive entrance) differed strikingly in diversity of cultivable and bacterial species. Lastly, we find that bacteria diversity presumably reflects transmission mechanisms of honey bees, microbes and their environment. Bacteria may play important role to honey bee health, social levels and protecting against pathogens, as well as being detrimental to colony health. Importantly, this study highlights the complexity of hive microenvironments. Further study is needed to better understand the role of these bacteria in the hive.

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