

ANTAGONISTIC EFFECT OF GUT BACTERIA IN THE HYBRID CARNIOLAN HONEY BEE, *APIS MELLIFERA CARNICA*, AGAINST *ASCOSPHAERA APIS*, THE CAUSAL ORGANISM OF CHALKBROOD DISEASE

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Abstract

The objective of this study was to isolate and characterize bacterial strains associated with the gut of the hybrid Carniolan honey bee, *Apis mellifera carnica*, and to determine their in vitro and in vivo potential against *Ascosphaera apis*, the causal organism of chalkbrood disease, with the purpose of exploring feasible biological control. Six bacterial strains were isolated from healthy worker honey bees by culture-dependent methods. Six fungal strains (A3, A4, A7, A8, A9, and A15) of *A. apis* were isolated from larvae suffering from chalkbrood disease on Yeast-Glucose-Starch agar (YGPSA) medium. All bacteria were identified by a combination of morphology, Gram stain, and 16S rRNA sequence analysis, and fungal strains were identified by morphology and 5.8S rRNA. In vitro and in vivo inhibition assays were carried out to determine the ability of bacterial isolates to inhibit *A. apis*, the causal agent of chalkbrood disease. The analysis of 16S rRNA sequences revealed that four bacterial strains (B2, B4, B10, and B100) belong to *Bacillus subtilis* species, and two strains (P1 and P5) belong to *Pseudomonas fluorescens*. Significant differences in antagonistic activity of all bacterial strains were observed. *B. subtilis* isolate B2 showed the highest antagonistic activity, as measured by the inhibition zone against *A. apis*, followed by the P1 strain of *P. fluorescens*. SEM analysis also supports the antagonistic activity of these bacteria against *A. apis*. This study provides a theoretical basis for biological control of honey bee chalkbrood disease.

Keywords: antifungal activity, *Apis mellifera carnica*, *Ascosphaera apis*, growth inhibition assay, gut bacteria, scanning electron microscopy.

INTRODUCTION

The honey bee (*Apis mellifera*) is an important pollinator of various crops and plant species worldwide. The total annual global economic worth of pollination amounts to an estimated 153 billion euro, representing 10% of the value of the

global agricultural production (Gallai et al., 2009). A mysterious decline in honey bee colonies has gained attention worldwide as they are threatened by various pathogens globally (van Engelsdorp and Meixner, 2010). The most contagious and destructive disease that affects honey bee honey brood is chalkbrood, which is caused by the fungus *As-*

cosphaera apis (Maassen ex Claussen) (Spiltoir, 1955). *A. apis* leads to significant losses in terms of both bee numbers and colony productivity (Zaghloul et al., 2005). This disease is now found throughout the world, and there are indications that chalkbrood incidence may be on the rise (Aronstein and Murray, 2010).

A. apis produces only sexual spores and is heterothallic; thus, spores are produced only when mycelia of the two opposite mating types come together and fruiting bodies are formed (Aronstein et al., 2007). Honey bee larvae primarily are infected by ingesting sexual spores of *A. apis* with their food. The spores germinate in the lumen of the gut, which requires very specific conditions in the larval gut environment (Bailey and Ball, 1991). Infected larvae rapidly reduce food consumption and then stop eating altogether. Theantana and Chantawannakul (2008) recently identified several enzymes produced by *A. apis*, some of which are implicated in assisting the pathogen in penetrating the peritrophic membrane of the bee larval midgut. Fungal spores are present on all surfaces within the beehive (Nelson and Gochnauer, 1982) and remain viable for many years, providing a continual source of infection. In addition to environmental conditions, interaction between biotic factors such as differences in fungal strains and the genetic background of the bees may affect the disease incidence and severity (Flores et al., 2005).

A broad range of chemotherapeutic compounds has been tested for their ability to control chalkbrood (Heath, 1982; Liu, 1991; Gliński and Chmielewski, 1996; Davis and Ward, 2003). Hornitzky (2001) listed chemicals that seemed promising for controlling fungal growth either in culture or in bee colonies, but unfortunately, none of the tested compounds achieved the level of control required to fight the disease. Common problems associated with synthetic pesticide and antimicrobial use include the ineffectiveness of these agents against infectious spores, adverse effects on the vitality of the brood and the longevity of the bees (Gliński and Chmielewski, 1996), and an increased rate of resistance of various *A. apis* strains. Furthermore, pesticide and antifungal chemical residues present in honey represent a major human health hazard (Frazier et al., 2008). The discovery of new antibiotics to control chalkbrood could lead to the emergence of additional resistant *A. apis* strains. Therefore, there is great interest in the investigation of alternative and efficient chalkbrood-controlling substances. Over the years, some alternative strategies have

been developed and implemented to control chalkbrood disease (Heath, 1982; Hornitzky, 2001). Initial clues show that in the honey bee, climax gut microbial communities develop under unstressed conditions and act as a barrier against various pathogens while an unbalanced intestinal microbial community is linked to pathogen invasion (Cox-Foster et al., 2007). According to the pioneering work by Sabaté et al. (2009) and Li et al. (2012), some endosymbiotic bacteria that show antibacterial activity against *A. apis* are found in the gut of *A. mellifera*, and these findings suggest a novel mode of controlling honey bee disease.

The hybrid Carniolan honey bee occurs in various parts in Egypt and is commonly used in beekeeping. However, little is known about the interaction between gut bacteria and pathogens in *A. m. carnica*. In particular, the actions of gut bacteria against chalkbrood remain elusive. To improve understanding of gut microbial communities with a view to the future use of antagonistic bacteria as biological agents, we identified the bacterial species present in the gut of *A. m. carnica* by morphology, biochemistry, and Gram staining and evaluated their activity against *A. apis*. The aim of this study was to isolate and characterize bacterial strains associated with the gut of hybrid Carniolan honey bee and to determine their *in vitro* and *in vivo* potential against *A. apis*, with the purpose of exploring feasible biological control.

MATERIAL AND METHODS

Isolation of *Ascosphaera apis*

Six strains (A3, A4, A7, A8, A9, and A15) of *A. apis* were isolated from samples of hybrid Carniolan honey bee infested brood with chalkbrood symptoms at apiaries located at the Shotop, Dauroot, and Dashloot localities of Assiut Governorate, Egypt. These apiaries have known histories of chalkbrood disease and have not used any chemical treatment for overcoming it. Black and white color mummies, depending on whether or not ascospores were present, were collected in the years 2009 and 2010 from these apiaries and used for isolation of the causal pathogen.

Black (sporulating) and white (mycelia only) mummies were washed with sterile water, cut into small pieces (0.25 - 0.5 cm), surface sterilized by dipping into 0.5% sodium hypochlorite solution for 3 min, and then rinsed several times in sterile water. We scraped the outer and inner surfaces of disinfested brood samples with a sterile applicator stick

and plated the scrapings onto sterile Petri dishes containing YGPSA medium (1% yeast extract, 0.2% glucose, 0.1 M KH_2PO_4 , 1% soluble starch, 2% agar) containing 100 $\mu\text{g}/\text{mL}$ of streptomycin to exclude growth of both Gram-positive and Gram-negative bacteria, and incubated the plates at 35°C under 6% CO_2 . The fungi were subcultured on the same medium until a pure culture was established.

Morphological and molecular identification of fungal isolates

Fungi were morphologically identified at 40× magnification (Axio Lab A1, Carl Zeiss, Germany) using reference images from Anderson and Gibson (1998). Molecular identification was carried out by PCR and sequencing of the ITS1–5.8S–ITS2 genomic region. The primers used in PCR were derived from conserved regions of the DNA sequences reported by James and Skinner (2005) for the 5.8S ribosomal DNA. The fungal isolates were examined morphologically using scanning electron microscopy (SEM) (Leo 435, Cambridge, USA) and compared with the model description of *A. apis* as described by Chorbiński (2003).

Isolation of bacteria

To isolate gut bacteria, honey bee workers were collected from healthy and chalkbrood-free hives at the apiary of the Faculty of Agriculture, University of Assiut, Egypt. The adults were surface-sterilized with 70% ethanol for 60 s, followed by 5% NaOCl for 60 s, and washed with sterile distilled water. These samples were placed in separate sterile tubes, each containing 8 mL of normal saline. A gut dissected from the adults was homogenized with a pestle in 100 μL of phosphate-buffered saline. The gut homogenates were incubated on different selective media micro-aerobically at 35°C for 2 days (Jeyaprakash et al., 2003). The colonies grown on the plates were selected according to the colony morphology, Gram staining, catalase reactions, and their appearance based on SEM. The isolated colonies were propagated on solid agar plates.

Morphological and molecular identification of bacterial isolates

Identification of bacterial isolates was carried out according to Bergey's manual of determinative bacteriology (Breed et al., 1957) using the following tests: shape of cells, motility, Gram staining, aerobiosis, starch hydrolysis, gelatin liquefaction, nitrate reduction, acetyl methyl carbinol production (Voges-Proskauer test), fermentation reaction with

mannitol, glucose, sucrose, arabinose, xylose, and lactose, and pigment production. Analysis of morphological characters and 16S rDNA gene sequencing was conducted to identify the producer strain. Genomic DNA was isolated from each bacterial isolate and used as a template for PCR, with primers designed from the conserved sequence in the 16S rRNA genes of bacteria. A set of primers, 16S-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-R (5'-AAGGAGGTGATCCAGCCGCA-3'), was used to amplify the region of interest.

In vitro antagonistic effect of isolated bacteria against *A. apis*

Inhibitory activities of each isolated bacterial colony were performed using the well diffusion assay (Yoshiyama and Kimura, 2009). Wells 7 mm in diameter were made on the nutrient agar plate with the reverse end of a 1 mL pipette tip. Each *A. apis* strain was inoculated into nutrient broth (Oxoid Ltd; Basingstoke, UK), which was incubated overnight at 35°C with shaking. The overnight cultures were diluted with fresh broth to give a final inoculum of approximately 10^5 colony forming units per mL. Nutrient agar plates (Oxoid) were inoculated with each fungal isolate to yield semi-confluent growth. At the same time, each isolated bacterial colony was suspended in distilled water and adjusted to a concentration of 10^6 cells/mL. Then, 100 μL of suspension of each isolated bacterial strain was placed into one well of each plate. As a control, filter paper discs without inoculum were impregnated with 20 μL of sterile water. The agar plates were inverted and incubated at 35°C for 18 h, zones of growth inhibition around the well were measured, and the mean and SD were calculated from triplicate experiments.

In vivo interaction and SEM

An in vivo inhibitory study was performed by treating the chalkbrood-infected honey bee colonies with isolated *B. subtilis* and *P. fluorescence* isolates. Bacterial inoculum from the P1 isolate of *P. fluorescence* and B2 isolate of *B. subtilis* were prepared separately with sterilized normal saline and the number of bacteria adjusted (8×10^8 cell/mL). Then, each solution (for both bacterial isolates) was individually sprayed onto the honey bee colonies using a hand sprayer. Out of 14 infected honey bee colonies selected, 6 colonies were sprayed by inoculum solution directly, 6 colonies were fed with inoculum solution added to 50% sucrose in water, and 2 colonies were used as control without any

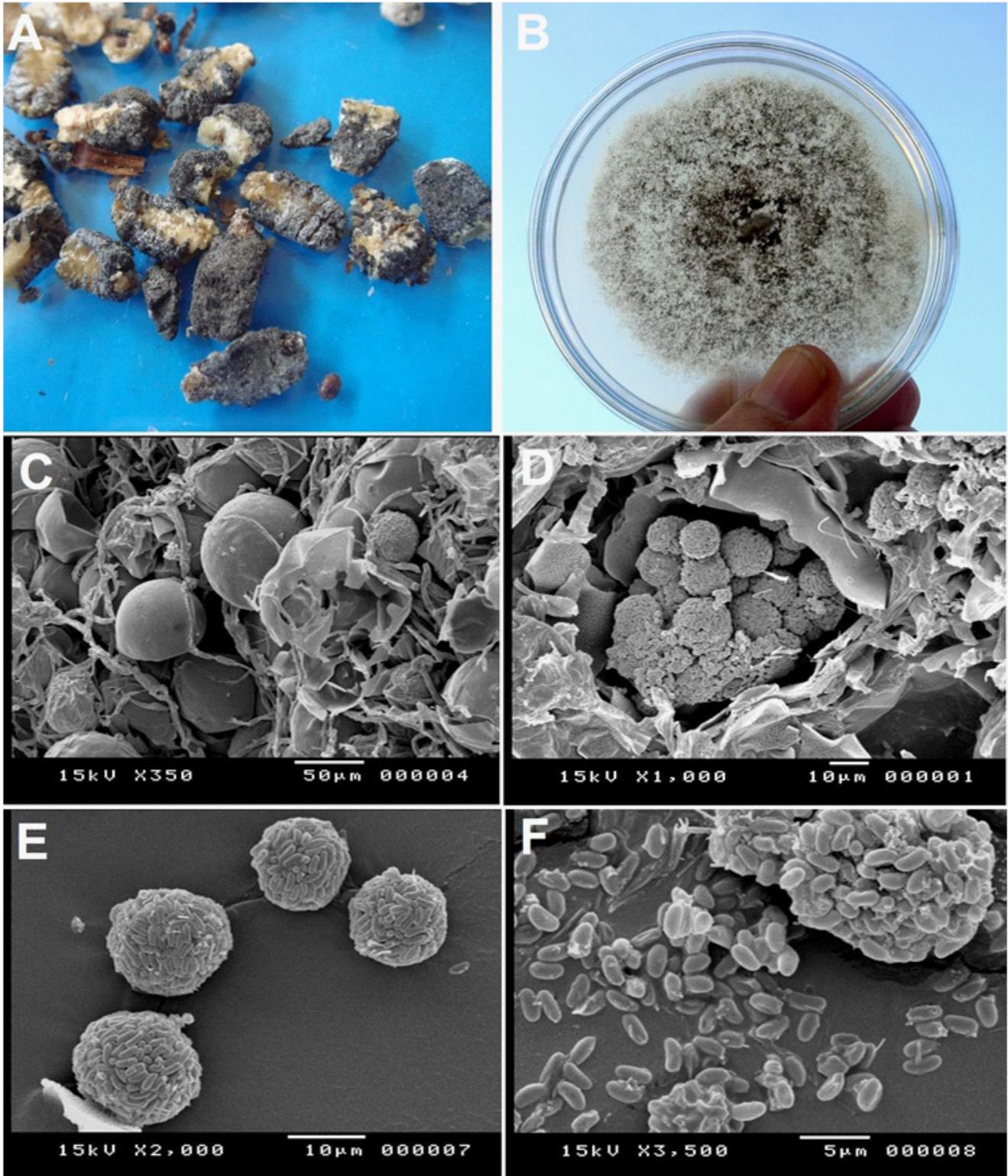


Fig. 1. Various developmental stages of *A. apis*. (A) Chalkbrood mummies. Chalkbrood mummies are white, brown, or black. (B) *A. apis* idiomorphs cultured on solid YGSA culture medium at 35°C under 6% CO₂. (C) SEM micrographs of numerous developing ascoma. (D) SEM micrographs of ruptured mature ascoma releasing a number of small spherical shaped asci. (E) SEM micrographs of asci containing fungal ascospores. (F) SEM micrographs of ascospores produced in a mixed culture of two mating type idiomorphs.

Table 1.

Different strains of *Ascosphaera apis* isolated from infected colonies

Number of isolates	Locality	Isolate type	Sporocysts*
A3	Shotopp	White	-
A4	Dirout	White	-
A7	Shotop	Pale buff	+
A8	Kom Omboha	White	-
A9	Dashloot	White	-
A15	Assiut	Black buff	+

* (-) non-forming sporocysts, (+) forming sporocysts

Table 2.

Morphological and physiological features of *Bacillus subtilis* isolates (B2, B4, B10, and B100) and *Pseudomonas fluorescense* bacterial isolates (P1 and P5)

Tests	Results for <i>Bacillus subtilis</i> isolates (B2, B4, B10, and B100)*	Tests	Results for <i>Pseudomonas fluorescense</i> bacterial isolates (P1 and P5)*
Bacterial cell shape	Rod	Bacterial cell shape	Rod
Motility	Motile	Motility	Motile
Gram staining reaction	+	Gram staining reaction	-
Anaerobic growth	-	Aerobiosis	Aerobic
Catalase activity	+	Voges-Proskauer	-
Acid production from:	+	Catalase activity	+
Glucose	+	H ₂ S production	-
Xylose	+	Acid production from:	+
Mannitol	+	Glucose	+
Gas production from glucose	-	Sucrose	+
Voges-Proskauer test		D-Fructose	+
Hydrolysis of:		Glycerol	+
Casein	+	Aesculin	+
Starch	+	Galactose	+
Esculin	+	Mannitol	-
Urea	-	D-Ribose	+
Gelatin liquefaction	+	Maltose	+
Tyrosinase activity	-	Gelatin liquefaction	-
Nitrate reduction	+	Starch hydrolysis	-
Indole formation	-	Nitrate reduction	+
Growth at pH:		Indole formation	-
5.7	+	Growth at 40° C	-
6.8	+	Aesculin hydrolysis	-
Growth in NaCl:		Oxidation of gluconate	-
2%	+	Citrate use	+
5%	+	Pigment production :	Yellow pigment with weak fluorescence light under ultraviolet light
7%	+		
10%	Weak growth		
Pigment production on:			
Nutrient agar medium	Cream to light brown		
PDA	Cream to brown to black		

* (+) positive reaction, (-) negative reaction

Table 3.

In vitro antagonistic effect of gut bacterial isolates against different strains of *Ascosphaera apis*, the causal agent of chalkbrood disease of honey bee

Bacterial isolates	Mean (\pm SD) diameter of inhibition zone against <i>A. apis</i> strains (in mm)					
	A3	A4	A7	A8	A9	A15
B2	20.25 \pm 1.2a	21.50 \pm 1.4a	21.25 \pm 1.1a	14.75 \pm 0.9b	23.00 \pm 1.2a	18.75 \pm 0.7a
P1	12.25 \pm 0.7b	19.25 \pm 1.0a	12.00 \pm 0.6c	21.25 \pm 1.3a	12.50 \pm 0.7d	19.25 \pm 1.1a
B10	11.75 \pm 0.5b	12.50 \pm 0.4bc	13.25 \pm 0.4bc	12.00 \pm 0.8c	16.00 \pm 1.0c	13.25 \pm 0.5b
B4	10.75 \pm 0.6bc	12.25 \pm 0.4c	14.50 \pm 0.5b	09.25 \pm 0.7d	19.50 \pm 1.2b	12.00 \pm 0.5bc
B100	10.75 \pm 0.7bc	10.75 \pm 0.3c	04.50 \pm 0.4e	09.00 \pm 0.9d	15.50 \pm 1.1c	04.50 \pm 0.3d
P5	08.25 \pm 0.3c	15.00 \pm 0.9b	08.25 \pm 0.7d	14.75 \pm 0.9b	12.00 \pm 0.7d	11.00 \pm 0.7c

Different letters in a column denote means with significant differences, $P < 0.05$.

treatment. Four replicates for each experimental group were run.

After 7 days, brood samples were collected from brood combs of beehives with clinical symptoms of chalkbrood disease and externally sterilized with 70% alcohol and dissected under sterile conditions. The samples were transferred into vials containing 4% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4). After being washed three times with the same buffer, all of the samples were post-fixed with 1% OsO₄ in cocodylate buffer for 20 min, rinsed in the same buffer, and dehydrated using a graded series of ethanol. Dehydrated specimens were dried in a critical point drier, mounted on a specimen stub, and repeatedly pierced with a fine needle to break the ascoma and expose the interior structure. Specimens were coated with gold and examined by SEM (Jeol JSM-5400) at the Electron Microscopy Unit, University of Assiut, Egypt.

Statistical analysis

All statistical calculations were expressed as mean \pm SD of three replicates. ANOVA and t tests were applied to identify significant differences in antimicrobial activity. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Physico-morphological characteristics of *A. apis* isolates and ITS1-5.8S-ITS2 genomic region sequence analysis

A total of 6 fungal biotypes (A3, A4, A7, A8, A9, and A15) belonging to *A. apis* were isolated from infested honey bee broods of *A. m. carnica*, collected from different localities of Assiut Governorate, Egypt. All fungal isolates identified as *A. apis*, as described by Heath (1982), Bailey and Ball (1991),

and Bissett et al. (1996). These fungal isolates were further identified by ITS1-5.8S-ITS2 genomic region sequence analysis and morphological analysis (Fig. 1). *A. apis* is a heterothallic organism that sporulates only when mycelia of opposite sexes (designated + and -) come together. Out of 6 biotypes, 2 (A7 and A15) could form ascoma on culture medium; however, 4 isolates (A3, A4, A8, and A9) did not form ascoma on culture medium. When A8 and A9 grew together on the previous culture medium, they showed compatibility and formed ascoma in the line of isolates mating. Meanwhile, the white isolates (A3 and A4) showed no compatibility with each other or with A8 and A9 together (Tab. 1).

Physico-morphological characteristics of bacterial isolates and the 16S rRNA genomic region sequence analysis

The gut microbial community of individual healthy workers of *A. m. carnica* was analyzed using a culture-dependent method. A total of 11 colonies were isolated. After eliminating the identical colonies on the basis of size, color, and morphology, we selected 6 bacterial representatives. A total of 4 isolates were endospore-forming bacteria (B2, B4, B10, and B100) and identified as the *B. subtilis* strain on the basis of their physico-morphological characters and 16S rRNA sequence. Two isolates were non-endospore-forming bacteria (P1 and P5) and identified as *P. fluorescence* on the basis of their physico-morphological characters and 16S rRNA gene sequence (Tab. 2).

In vitro antagonistic effect of isolated bacteria against *A. apis*

To investigate whether the bacterial isolates had antagonistic activity against *A. apis*, the causal agent of chalkbrood disease of honey bees, all 6 bacterial

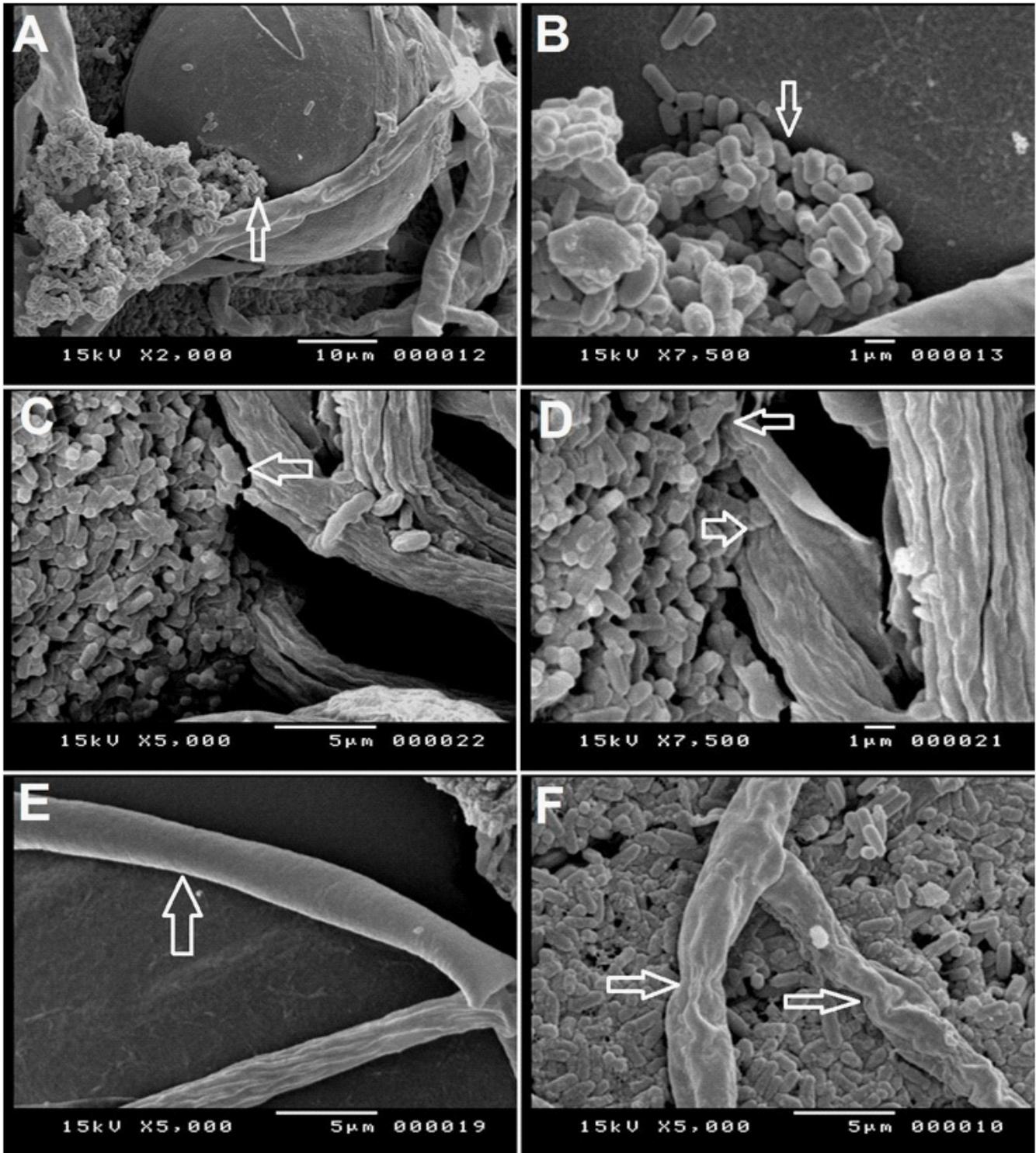


Fig. 2. SEM micrographs of the antagonistic interaction between bacterial isolates and *A. apicola* after treatment of the infected colony with bacterial isolates.

(A, B) B2 strain of *B. subtilis* showing invasion of cells into ascomata. The white arrow indicates the depression in the cell wall of ascoma due to cell wall lysis. (C, D) P1 strain of *P. fluorescence* showing disintegration of *A. apicola* hyphae. The white arrow indicates the lysis of the hyphae cell wall *A. apicola*. (E) No shrinkage was observed in the *A. apicola* hyphae without any antagonistic bacteria, as indicated by the white arrow. (F) The white arrow indicates the rough cell wall and shrinkage of fungal hyphae in the presence of the B2 strain of *B. subtilis*.

isolates were further investigated using an in vitro inhibition assay. All isolates tested showed inhibitory activity as measured by inhibitory zones. Different bacterial isolates showed different sizes of inhibition zones against different isolates of *A. apis*. This result suggested that a range of different mechanisms is involved in *A. apis* isolate inhibition by these bacteria. There was a significant difference between antimicrobial activities among the different bacterial isolates, but none of the *A. apis* isolates showed resistance to any bacterial isolates. Isolate B2, belonging to *B. subtilis* species, showed the most significant effect with the largest inhibitory zone against the A9 isolate (23.00 ± 1.2 mm) and A4 isolate (21.50 ± 1.4 mm) of *A. apis*, while the B100 bacterial isolate showed a less pronounced effect against the A15 fungal isolate with a minimal inhibitory zone (04.50 ± 0.3 mm). The mean \pm SD values of the inhibitory zone size of all the bacterial isolates against the *A. apis* isolates were B2 (23.00 ± 1.2 mm) against A9, P1 (*P. aeruginosa*) (21.25 ± 1.3 mm) against A8, B10 (16.00 ± 1.0 mm) against A9, B4 (19.50 ± 1.2 mm) against A9, B100 (15.50 ± 1.1 mm) against A9, and P5 (15.00 ± 0.9 mm) against *A. apis* isolates ($P < 0.05$). The most susceptible isolates among *A. apis* were A9 isolates, which showed the highest sensitivity against all 4 *B. subtilis* isolates (Tab. 3).

SEM study of in vivo interaction between bacteria and *A. apis*

SEM study of mature ascoma of *A. apis* showed a large number of globules (ascoma), which varied in size and were scattered on the surface of diseased brood. Inside some ascoma, immature spore formation had begun, which aggregated to form asci. After one week of bacterial solution spray application on an infected colony, numerous bacterial cells of antagonistic bacteria, the P1 isolate of *P. fluorescence*, had invaded into the *A. apis* hyphae. These bacteria caused the disintegration and cell wall lysis of the fungal hyphae (Fig. 2C, D). On the other hand, mature ascoma of *A. apis* were also invaded by numerous bacterial cells of the B2 isolate of *B. subtilis*. These bacteria lysed the cell wall of the mature ascoma, forming a cavity to enter the ascoma (Fig. 2A, B). *A. apis* hyphae showed a shrunken appearance when grown together with an antagonistic B2 strain of bacteria *B. subtilis* (Fig. 2E, F). The antifungal agent gluconic acid (Kaur et al., 2006) was produced by a number of honey bee gut bacteria. In this study, bio control of *A. apis* isolates could be the result of

gluconic acid inhibition of *A. apis* hyphae, leading to fungal cell shrinkage, cytoplasmic contraction, and hyphal fragmentation.

DISCUSSION

A. mellifera, the most commonly managed honey bee, is a highly valued resource worldwide and is of great relevance for humans and the whole ecosystem, not only as honey and wax producers but also as pollinators of agricultural and horticultural crops and wild flora (van Engelsdorp and Meixner, 2010). Apiculture has been facing huge economic losses worldwide for a few decades, and a definitive cause of the losses has not yet been identified. Much attention has been paid to some bacterial and fungal diseases. Microbes play an essential role in the health of nearly every organism, and the bacterial community of the honey bee gut is an important factor for honey bee health at the individual and colony levels. To date, most studies of honey bee bacterial communities have focused on different pathogens while little is known about non-pathogenic organisms and their probable benefits for individual bees or entire colonies. There is, however, increasing awareness of the significance of the composition of the gut microbial community for honey bee health, and the genetics of the bees might affect colony microbial diversity. For example, African and European honey bee colonies might have slightly different microbial communities. The European honey bee, *A. mellifera*, harbors various bacterial species in the gut (Gilliam, 1997), some of which have been isolated, including those antagonistic to several pathogens (Evans and Armstrong, 2006). Some of these gut microorganisms produce antibiotics that are antagonistic to fungal pathogens of bees, such as the chalkbrood pathogen (Gilliam, 1997). Microorganisms associated with honey bees are novel sources of bioactive compounds (e.g., antifungal agents) and may have uses beyond the field of apiculture. Antagonistic molds and *Bacillus* spp. that inhibit the chalkbrood pathogen have been found in beebread and the guts of worker honey bees. Bees or larvae have not been found to produce antimycotic substances active against the chalkbrood fungus (Gilliam, 1997). In the present study, we found few bacteria in the gut of the hybrid Carniolan honey bee, a result that is in contrast to that with the European honey bee, which has a large range of bacterial species in the gut. Many factors likely influence insect gut communities. Diet, pH, host specificity (e.g., co-evolutionary

effects), life stage, and host environment can all do so (Mohr and Tebbe, 2006). The cultivable isolates represent *B. subtilis* and *P. fluorescence* species. *Pseudomonas* species have been reported to have antagonistic activity against entomopathogenic fungi in the diamondback moth (Indiragandhi et al., 2007), and *P. aureginosa* has been shown to prevent parasite establishment in the midgut of mosquitoes (Azambuja et al., 2005). It is likely from our findings that the *P. fluorescence* bacteria isolated in this study also play important roles in protecting honey bees against pathogens, including *A. apis*, the causal organism of chalkbrood disease.

Several species of *Bacillus* bacteria have been reported from *A. m. capensis* and *A. m. scutellata* (Jeyprakash et al., 2003; Mohr and Tebbe, 2006). Recently, a novel probiotic bacterium *Lactobacillus* spp. in the workers of the Indian honey bee, *A. cerana indica*, has been isolated from the gut (Pattabhiramaiah et al., 2012). In contrast, Evans and Armstrong (2006) failed to find *Lactobacillus* species in *A. mellifera*, suggesting that the gut microbiota is not constant. The gut microbial diversity of *A. mellifera* is likely to be related to the species and races, variations in the food sources, and seasonal and geographical variations. Furthermore, different honey bee species are likely to visit flowers of different species. Thus, variations in the food source are expected to be correlated with the diversity of the gut microbial community in *Apis* species and races.

We next investigated the bacterial isolates obtained in this study to evaluate their antagonistic effect against *A. apis*. All 6 isolates showed significant antagonistic activity against *A. apis* in the in vitro inhibition assay. Isolate B2 of *B. subtilis* showed the highest antagonistic activity, as measured by the inhibition zone against *A. apis*, followed by the P1 strain of *P. fluorescence*, which is in agreement with results reported for the Australian honey bee (Nayadu and Khan, 2009). Therefore, the findings of the present study suggest that distinct honey bee species possess common representatives of the antagonistic bacteria that may be potential candidates for the controlling of chalkbrood disease of the honey bee. Some broad spectrum antibiotics have been reported to be produced by *Bacillus* species (Foldes et al., 2000). For example, macrolactin produced by *B. subtilis* shows an antagonistic effect against methicillin-resistant *S. aureus* (Romero-Tabarez et al., 2006), which might explain the antagonistic activity of *Bacillus* species against *A. apis*. Evans and Lopez (2004) reported that some non-pathogenic bacteria

of the genera *Lactobacillus* and *Bifidobacterium* can stimulate the innate immune response of honey bee. It might be helpful to evaluate whether the bacterial isolates from the hybrid Carniolan honey bee *A. m. carnica* contribute to host health and fitness by inhibiting the growth of pathogenic microbes.

In addition to chalkbrood disease, honey bee populations are also affected by various pathogenic microorganisms. Most of these pathogens penetrate through the digestive tract and cause serious damage to the honey bee population. For example, the bacteria *Paenibacillus larvae* and *Melissococcus plutonius* and the microsporidian *Nosema ceranae* cause American foulbrood, European foulbrood, and Nosema disease, respectively (Bailey and Ball, 1991; Chen et al., 2008; Yoshiyama and Kimura, 2009). Recently, it has been shown that a *B. subtilis* strain (G2III) isolated from the gut of the honey bee inhibits the growth of *A. apis* by secreting a fungicide and also affects the growth of *P. larvae* by surfactin synthesis (Sabaté et al., 2009). It will be interesting to determine whether 4 strains of *B. subtilis* (B2, B4, B10, and B 100) in this study are also inhibitory against *P. larvae*.

CONCLUSION

The results presented in this paper demonstrate that most of the honey bee gut bacteria have potential antifungal activities and that bacteria associated with the hybrid Carniolan honey bee *A. m. carnica* could be used to develop various pathogen management strategies. The use of symbiotic gut bacteria could represent a natural alternative to synthetic antibiotics in the control of chalkbrood disease, which would reduce antibiotic resistance and antibiotic residue levels. Further research must be conducted on these beneficial bacteria to isolate the active antagonistic compound that kills *A. apis*.

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