Isolation and Characterization of European Foulbrood Antagonistic Bacteria from the Gastrointestine of the Japanese Honeybee *Apis cerana japonica*

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Summary

Bacteria were isolated from the digestive tract of the Japanese honeybee using a culture-dependent method to investigate antagonistic effects of honeybee intestinal bacteria. Forty-five bacterial strains belonging to nine genera, *Bifidobacterium*, *Lactobacillus, Bacillus, Streptomyces, Pantoea, Stenotrophomonas, Paenibacillus, Lysinibacillus and Staphylococcus* were obtained in this study. Among these, 11 strains were closely related to bifidobacteria previously isolated from the European honeybee *A. mellifera*, which are distinct from bumblebee gastrointestinal bifidobacteria. On the other hand, 17 strains were identified as lactobacilli, another important lactic acid bacteria. According to the results of 16S rRNA gene sequence similarity and phylogenetic analysis, some lactobacilli strains are likely novel species. In addition, lactobacilli obtained in this study were similar to lactobacilli associated with *Apis* species but distant from those of bumblebees, implying that honeybee species share some *Apis* species-specific bacteria in their gut bacterial communities. Eight strains of the genus *Bacillus,* one genus broadly used as probiotics, clustered closely with 3 different *Bacillus* species. Diversity at strain levels within bacterial species was also confirmed by biochemical property analyses, suggesting that these isolates have diverse functions in host honeybees.

For confirmation of the virulence of recently isolated and artificially cultured *M. plutonius* bacterium to larvae under experimental conditions, bioassays were performed by feeding of *in vitro* reared larvae. Atypical strains of *M. plutonius*, which are phenotypically and genetically different from typical strains, succeeded to establish EFB symptoms and led to larval mortality in the bioassays. Thereby, all 45 isolates were investigated for potential effects on the pathogenic bacterium *M. plutonius* atypical strains, causal agent of EFB, by *in vitro* and *in vivo* assays. Evaluations revealed that one isolate of *Bacillus* together with all 11

bifidobacteria and 17 lactobacilli inhibited the growth of *M. plutonius in vitro* and significantly reduced the mortality of *M. plutonius* infected larvae *in vivo* implying antagonism of these isolates to this pathogen. Although more intensive studies are required to clarify the mechanisms of *in vitro* and *in vivo* antagonistic activity of these gut isolates against *M. plutonius*, I predict that antibacterial compound(s) produced by gut isolates mainly function in *in vitro* growth inhibition activities and multiple mechanisms, such as growth inhibition or competitive exclusion of pathogens and stimulation of the immune responses may also be involved in *in vivo* antagonisms and enhanced EFB resistance of larvae.

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Chapter 1. General Introduction

Honeybees, one well-known bee species, are classified into the family Apidae of Hymenoptera, which is a grand order of insects related to ants, wasps and sawflies. Honeybees are included in one genus, *Apis,* comprised of one Western honeybee species, *Apis mellifera*, and eight Asian honeybee species: *Apis cerana, Apis koschevnikovi, Apis nuluensis, Apis nigrocincta, Apis dorsata, Apis laboriosa, Apis florea, and Apis andreniformis* (Oldroyd and Wongsiri, 2006). Honeybees are very distinct eusocial insects and a fascinating insect with a long history of mutualistic relationships between honeybees and mankind. Humans harvest honey, pollen, wax, royal jelly, propolis and venom from honeybees for nutrition, crafts, manufacturing and medical applications. More importantly, honeybees also benefit mankind by providing an essential pollination service to both natural and agricultural ecosystems (Southwick and Southwick, 1992). Pollination is a vital ecosystem service contributing to human health and wellbeing. One mouthful in three of the foods human consumption depends directly or indirectly on insect mediated pollination. As a most important pollinator, honeybees play a key role in modern agricultural development all over the world. According to recent estimations, 52 of 115 leading global food commodities benefit from honeybees and have 10-90% yield reduction without honeybees (Klein et al., 2007). The value of honeybee pollination to United States agriculture is more than 14 billion dollars annually (Morse and Calderone, 2000), and the worldwide total economic value of honeybee pollination amounted to €153 billion in 2005 (Gallai et al., 2009). The value of bee pollination to natural plant biodiversity is not simply estimable (Moritz et al., 2010).

Recently, however, honeybees are facing a serious health threat and widespread population declines in both the United States (Ellis et al., 2010) and Europe (Potts et al.,

2010), well known as the phenomenon colony collapse disorder (CCD), especially in commercial beekeeping industries. Such population declines in honeybees and other wild bees in the United States, Europe and elsewhere has led to worldwide concerns about the impacts on yield of agriculture and biodiversity of natural plants (Biesmeijer et al., 2006; Breeze et al., 2011; Garibaldi et al., 2011; Gallai et al., 2009). Many researchers have studied and revealed risk factors that threaten honeybee colonies, including pathogens, malnutrition, pesticides, beekeeping practices, climate change and genetic diversity (Stokstad, 2007; vanEngelsdorp et al., 2008). Although multiple drivers and interactive effects may be responsible for the widespread declines in pollinator bees (Neumann and Carreck, 2010; Oldryod, 2007), diseases caused by various pathogens are recognized as one considerable factor afflicting honeybee health and causing huge colony losses (Cox-Foster et al., 2007). Although there has been an almost 50% decrease in world honeybee stocks over the last century, human demands for pollinator-dependent crops to maintain health are simultaneously increasing by >300% (Aizen and Harder, 2009). Because of such importance of honeybee to human welfare, there is great concern about honeybee health and consequently disease control becomes a most challenging task for preventing decline of honeybee populations.

Honeybees are vulnerable to various pathogens such as bacteria, viruses, protozoa, fungi, as well as parasitic mites and subjected to heavy damages every year. These diseases cause large amounts of economic losses in apiculture and agriculture of the world by affecting survival of managed and wild honeybees. There are many types of diseases that threaten honeybee colonies such as bacterial diseases (i.e. American Foulbrood, caused by *Paenibacillus larvae*; European Foulbrood, caused by *Melissococcus plutonius*), fungal diseases (i.e. Chalkbrood, caused by *Ascosphaera apis;* Stonebrood, caused by *Aspergillus fumigatus*, *Aspergillus flavus and Aspergillus niger*), viral diseases (i.e. caused by Sacbrood

Virus, Chronic Paralysis Virus, Black Queen Cell Virus and Kashmir Bee Virus) and protozoan diseases (i.e. Nosemosis, caused by *Nosema apis* and *Nosema ceranae*) (Allen and Ball, 1996; vanEngelsdorp and Meixner, 2010). Besides these diseases, honeybees are also afflicted with diseases mediated by pests and parasites such as Varroa mites and Acarine mites (Sammataro et al., 2000). Among the above diseases, European Foulbrodd (EFB) is one contagious honeybee larval disease caused by the pathogenic bacterium *M. plutonius*. EFB kills larvae when they are 4–6 days old and the tissues of infected larvae are decomposed by the bacteria and cause them to turn brown in color (Forsgren, 2010). EFB is currently one of the most serious threats to beekeeping worldwide, particularly in Great Britain (Wilkins et al., 2007) and Switzerland where the incidence of EFB has increased markedly in recent years (Roetschi et al., 2008).

For preventing lose of honeybees, EFB management becomes important task in the apicultural industry. Similar to the control of other diseases that typically rely on chemicals such as antibiotics and acetic acid (Arbia and Babbay, 2011; Wolfgang and Pongthep, 2006), present control measures for EFB consist primarily of antibiotics, such as oxytetracycline hydrochloride (OTC) that inhibits the multiplication of the causal agent *M. plutonius* (Thompson and Brown 2001). However, the application of chemicals in apiculture is of great concern or legally banned in many countries of the European Union (Mutinelli, 2003), because of chemical residues in honeybee products for human consumption (Martel et al., 2006), toxicity to honeybee broods and honeybee beneficial intestinal micro-flora (Pettis et al., 2004; Thompson et al., 2005) as well as emergence of resistant pathogenic bacterial strains (Miyagi et al., 2000). Due to the above problems in disease control with chemicals, novel and sustainable disease management strategies are becoming an urgent need for controlling diseases including EFB and consequently improving honeybee health. Although the bacterium *M. plutonius,* one fastidious anaerobic Gram-positive bacterium, has

been well proven as a causal agent of EFB, the epizootiology of EFB has not been fully understood until recently because of difficulties in culturing the bacteria *M. plutonius* under experimental conditions without second invaders (Forsgren, 2010). Several recent isolated and characterized strains of *M. plutonius* were artificially cultured and provide the possibility for more intensive studies on the relationships between EFB and *M. plutonius*, as well as for development of novel and sustainable EFB control technology.

Honeybees are eusocial insects and a high level of cohesion in colonies may particularly increase the risk of disease outbreaks. In response, honeybees have established and evolved multiple disease defense strategies to combat and limit the impact of the increased disease risk caused by various pathogens. These strategies involve resisting pathogens by building barriers to infection and mounting defense responses, or tolerating pathogens by compensating for the energetic costs or tissue damage (Evans and Spivak, 2010). In addition, as a eusocial insect, group living and multi mating habits of honeybees facilitate disease defense capabilities with behavioral defense including grooming, hygienic behavior, behavioral fever and social organization (Evans and Spivak, 2010), and with increasing genetic diversity in colonies that are composed of two components: within-individuals and between individuals (Ugelvig et al., 2010). Besides eusocial insect unique group level defense systems, honeybees, like any other animal and insect, also have successfully evolved multiple individual level defense systems. Primary lines of individual honeybee disease defense are mechanical barriers constructed by cuticle and epithelial layers that prevent microbes from adhering to or entering the body (Schiffrin and Blum, 2002). Such primary layers not only function as physical barriers, but also function as particularly important biochemical barriers by secreting various antimicrobial compounds that inhibit invasion by pathogens (Stow and Beattie, 2008). This type of antimicrobial activity strength increases with group size (Turnbull et al., 2011). If pathogens invaded through physical and

biochemical barriers, honeybees, like other insects, mount a set of physiological immune responses, including cellular or humoral immune processes, to defend themselves against infection with pathogens. In addition, another important method used to resist pathogens is a symbiont mediated defense mechanism (Evans and Spivak, 2010). Honeybees harbor a diverse assemblage of microbes including bacteria in their gut, other body regions and hives (Gilliam, 1997). Interactions of hosts and symbiotic bacteria play vital roles in disease defense, particularly the beneficial symbiotic bacteria inhabiting the gastrointestinal tract, where many pathogens invade and colonize, enhance the nutritional and immune defensive functions of hosts and consequently increase disease resistance and tolerant levels by diverse mechanisms (Cebra, 1999).

Gastrointestinal tracts of animals are a complex ecosystem that protects hosts from attack by pathogens with physical and chemical barriers created by the gastrointestinal epithelium and mucosa (Bevins et al., 1999). In addition, microbiota colonizing the gastrointestinal tract environment together with epithelial cells play an important role in normal gut function and maintaining host health (Berg, 1996). Many pathogenic infections start with microbial invasion of mucosal surfaces, which are typically colonized by a complex and dynamic community of microorganisms. Most protection against infection by pathogens associated with the intestine involve immune system mechanisms of innate and adaptive immunity as well as the secondary lymphoid organ, and gut-associated lymphoid tissues (GALT) (Bauer et al., 2006). Microorganisms colonizing the intestine have a major role in development of the intestinal immune system, both in terms of GALT development and mucosal immunity. Besides the stimulation of the intestinal immune system, the gastrointestinal microbiota confer many other benefits to intestinal physiology including functioning in development of intestinal integrity by control of epithelial cell proliferation, and differentiation, development of vasculature and GALT, production of essential mucosal

nutrients, and prevention from pathogenic organisms by colonizing resistance mechanisms (Tappenden and Deutsch, 2007). The gastrointestine of animals, together with their diverse microbes, are major factors that influence host disease defense capabilities.

As in all animals, the gastrointestinal tract of adult honeybees is a complex ecosystem that harbors diverse microbial communities including bacteria and plays a key role in maintaining host honeybee health (Gilliam, 1997; Servin, 2004). Recent culture-independent 16S rRNA sequencing and metagenomic surveys suggest that a set of consistent and distinctive microbial communities. This is contrary to results of previous culture-dependent studies that argue variation in species composition of gastrointestinal microbes with honeybee age, season and geographical differences (Gilliam, 1997), dominated in the honeybee digestive tract despite differences in honeybee species, colonies and geographic location (Mohr and Tebbe, 2006; Babendreier et al., 2007; Cox-Foster et al., 2007). Exception of qualitative constancy in phylotypes of dominant bacteria, the relative frequency of bacterial phylotypes and bacterial community structure in the honeybee digestive tract is affected to some degree by life stage, geographic location and species (Ahn et al., 2012; Disayathanoowat et al. 2012). Colonization of honeybee characteristic bacteria in the digestive tract favor different organs of the gut (Martinson et al. 2012) and may serve particular functions for their hosts. Compared to other insects, honeybees worldwide harbor a set of simple and distinctive gastrointestinal microbial communities, whereas the genetic diversity within the bacterial species dominated in honeybee guts lead to functional diversity in host interaction, biofilm formation and nutritional digestion (Engel et al., 2012). Functional diversity in bacterial strain levels and differences in colonization patterns of gut niches may diversify and enhance the beneficial roles of simple sets of gastrointestinal bacteria in maintaining honeybee health.

Early studies on interactions between honeybee and gastrointestinal microbes have mainly focused on the nutritional activities of bacterial communities such as functions in food fermentation and food preservation in the honeybee gut (Human and Nicolson, 2006). The advent of modern microbiology and methodologies have led to the improvement of understanding other many roles of gastrointestinal microbiota to maintain honeybee health by combating disease pathogens with specific mechanisms (Evans and Spivak, 2010; Parker et al., 2011). To provide further insight into the disease preventive functions of gastrointestinal microbiota, many mechanisms by which gastrointestinal bacteria can defend against infection by pathogens have been postulated by intensive studies. These results suggest that in addition to their competitive inhibition of the epithelial and mucosal adherence of pathogens and inhibition of epithelial invasion by pathogens (Gopal et al., 2001; Bibiloni et al., 2001), these bacteria can also enhance the immune system of the host (Evans and Lopez, 2004). Moreover, gut bacteria may also show antagonistic activity against pathogens by producing various antimicrobial substances such as fatty acids and H_2O_2 (Klaenhammer, 1993; Servin, 2004). Although little is known about how members of the intestinal microbiota interact in honeybees to establish mutually beneficial relationships and what mechanisms function in their defense systems, gastrointestinal microbiota attract much attention because of the potential roles in protecting honeybee health and developing honeybee disease management strategies. Therefore, in this dissertation, to further investigate the disease defense roles of honeybee gastrointestinal bacteria and develop sustainable EFB management strategies with such functional bacteria, the isolation and characterization of Japanese honeybee gastrointestinal bacteria were performed and their antagonistic activities to pathogenic bacteria *M. plutonius,* the causal agent of EFB, were assayed *in vitro* and *in vivo*.

Chapter 2. Isolation and Characterization of Gastrointestinal Bacteria from Japanese Honeybee

2.1. Introduction

During their life cycle, honeybee eggs, larvae, pupae and newly emerged adults are usually free of internal microorganisms. However, because of pollen or beebread consumption and food exchange with older honeybees in the colony, mature honeybees become inoculated with various microbes in their digestive tract after emergence (Gilliam and Prest, 1987). Pollen and nectar collection habits and group life style of honeybees increases their exposure to diverse microbes and microorganisms are intensively acquired from the environment. Adult honeybee gastrointestinal tracts harbor a diverse assemblage of microbes including bacteria (Gilliam et al., 1988). Early studies on identification of bacteria associated with honeybee gastrointestines are restricted to only species that can be cultured and revealed that the gastrointestinal microbiota consist of Gram-positive bacteria such as *Bacillus, Lactobacillus, Bifidobacterium, Corynebacterium, Streptococcus*, *Clostridium*, and Gram-negative or Gram-variable bacteria such as *Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus, and Pseudomonas* (Gilliam, 1997). Recently, well-developed molecular techniques have advented the understanding of relationships between honeybee and their gastrointestinal bacteria. One culture-independent study revealed a consistence in bacterial community components dominating the honeybee gastrointestine between different honeybee species and distant honeybee colonies (Martinson et al., 2011). However, other observations indicate that bacterial communities in honeybee digestive tracts are affected to some degree by age, geographic location, colony, species (Ahn et al., 2012; Disayathanoowat et al. 2012) and small amounts of distant bacterial phylotypes exist in some individuals (Moran et al., 2012). This variation in

composition of gut bacteria may be a result of the methodology, culture-dependent and culture-independent differences and different sampling strategies of independent studies.

Previous studies clearly document the roles of beneficial gastrointestinal bacteria as processing of food fermentation, preserving food stores (Gilliam, 1997) and inhibiting the growth of pathogenic bacteria (Evans and Armstrong, 2006). Other studies have also confirmed that bacterial probiotics identified from honeybee gastrointestine can induce immune responses (Evans and Lopez, 2004) and contribute to maintaining a healthy bee colony (Pătruică and Mot, 2012). There is increasing evidence that some species of honeybee endogenous intestinal bacteria or their metabolic products show antagonistic activities to pathogens threatening honeybee health. For example, the honeybee pathogen *A. apis*, causative agent of Chalkbrood disease (Sabaté et al., 2009), *Paenibacillus larvae*, the causative agent of American Foulbrood (Sabaté et al., 2009; Evans and Armstrong, 2005; Forsgren et al., 2010; Yoshiyama and Kimura, 2009) and *Nosema ceranae*, the causal microsporidian of Nosemosis (Porrini et al., 2010) were inhibited by intestinal bacteria or its metabolic products. Such beneficial gastrointestinal bacteria receive great attention due to their potentiality as an alternative sustainable disease control method for honeybees.

The Japanese honeybee *A. cerana japonica*, a subspecies of the Asian honeybee *A. cerana*, is native to Japan. Compared to the European honeybee *A. mellifera*, the Asian honeybee is considered to be resistant to several pathogens, including AFB and *Varroa* mites (Peng et al. 1987; Chen et al. 2000). To date, no reported cases of AFB or *Varroa* mites are known in Japanese honeybee colonies (Yoshida 2000). Recently, bacteria isolated from the gut of the Japanese honeybee were shown to exhibit inhibitory effects *in vitro* on *P. larvae*, the causative agent of AFB (Yoshiyama and Kimura 2009). It is thus possible that the Japanese honeybee may harbor some different gastrointestinal bacteria that may contribute to the tolerance of this honeybee subspecies to be resistant to various pathogens.

Therefore, to better understand the potential of the Japanese honeybee gastrointestinal bacteria in the development of novel and sustainable disease control strategies, the bacteria in the Japanese honeybee gastrointestine were isolated and characterized for further evaluation.

2.2. Isolation of gastrointestinal bacteria from Japanese honeybee

2.2.1. Materials and methods

2.2.1.1. Collection of *A. cerana japonica* and isolation of gut bacteria

For isolation of gastrointestinal bacteria, foragers of *A. cerana japonica* were collected from three different bee colonies in Tsukuba, Japan in June, July and September 2011. Ten incoming workers were caught at the entrance of hives using forceps and transferred to 1.5 ml tubes each time. The external surface of the forager bees was sterilized with 70% ethanol and washed with distilled water. The digestive tracts were then dissected aseptically before being homogenized in tubes containing cultural medium. Wilkins-Chalgren Medium, Lactobacilli Man Rogosa Sharpe (MRS) Broth and Brain Heart Infusion (BHI) Medium were used as selective media for isolating bifidobacteria, lactobacilli and other bacteria respectively. Digestive tracts were transferred to tubes containing selective liquid medium, homogenized with pestles, and the extracts were then spread on agar plates of the same media. The Wilkins-Chalgren and MRS agar plates were then incubated at 35°C for two days under anaerobic conditions (AnaeroPack system, Mitsubishi Gas Chemical Co., Inc., Japan) and the BHI agar plates were incubated at 35°C for 2 days under aerobic conditions. Bacterial colonies growing on plates were selected according to size, color and morphological appearance. Total of 11 colonies on Wilkins-Chalgre plates, 17 colonies on

MRS plates and 17 colonies on BHI plates were selected and sub-cultured to new plates to obtain pure colonies.

2.2.1.2. Identification of gut bacteria with 16S rRNA gene sequences and phylogenetic relationship analysis

To identify gastrointestinal isolates in this study, the pure isolated colonies were used as templates for polymerase chain reaction (PCR) amplification of 16S rRNA genes. Each colony was taken directly from media plates using sterile toothpicks. The tips of these toothpicks were then immersed in 50 μl of the PCR reaction solution. The 16S rRNA genes were amplified using primers Im26 (5'- GAT TCT GGC TCA GGA TGA ACG -3') and Im3 (5'- CGG GTG CTI CCC ACT TTC ATG -3') for bifidobacteria (Kaufmann et al., 1997); primers 10F (5'-AGAGTTTGATCCTGGCTCAG-3'), 519F (5'-CAGCGGCCGCGGTAAT-3'), 907F (5'-AAACTTAAACGAATTGACGG-3') and 1500R (5'-GGTTACCTTGTTACGACTT-3') for lactobacilli; and primers 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1406R (5'-ACG GGC GGT GTG TAC-3') for other bacteria (Weisburg et al. 1991). The thermocycler program consisted of initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min, and annealing at 60°C for 1 min, and extension at 68°C for 1 min. The PCR reactions (50 μl) contained 1x PCR buffer (with 1.5 mM $MgCl_2$), 0.5 μM primers, 0.2 mM of each dNTP, 1 U KOD *Taq* DNA polymerase (TOYOBO, Japan). PCR products were purified on an agarose gel and then extracted using a Qiaex II Gel Extraction Kit (Qiagen, Germany) before ligation into a pGEM-T vector and cloned into *E. coli* using a pGEM-T Cloning Kit (Promega, USA) according to the manufacturer's instructions. The cloned fragments were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit using a 3130xl Genetic Analyzer (Applied Biosystems, Japan), and the BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST) were

used to analyze the sequences. Sequence similarities were calculated using GENETYX-MAC (ver. 9.0, Software Development Co. Ltd., Japan). The 16S rRNA gene fragments sequenced in this study were deposited in the DDBJ/EMBL/GenBank database under the accession numbers listed in tables of results.

For further understanding the phylogenetic relationships of isolates obtained, a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) based on paired alignments of nucleotide sequences of the 16S rRNA genes obtained in this study and sequences of 16S rRNA genes in the GenBank database (NCBI) from previous studies. The CLUSTAL X program (http://bips.u-strasbg.fr/fr/Documentation/Clustalx/) was used to analyze phylogenetic relationships and phylogenetic trees were bootstrapped with 1000 replicates.

2.2.2. Results

2.2.2.1. Japanese honeybee gut isolates

A total of 45 colonies were selected as isolations for further identification and characterization based on size, color and morphology. To characterize taxonomic positions of these bacterial colonies, we performed homology searches of the obtained 16S rRNA gene sequences against sequences in the GenBank database using the BLAST-N program on NCBI (http:// www.ncbi.nlm.nih.gov/blast/) and constructed the phylogenetic trees using the partial 16S rRNA gene sequences (Fig. 1). All isolates clustered into 9 different clusters of the genera *Bifidobacterium*, *Streptomyces, Pantoea, Stenotrophomonas, Paenibacillus, Lactobacillus, Lysinibacillus, Staphylococcus* and *Bacillus*.

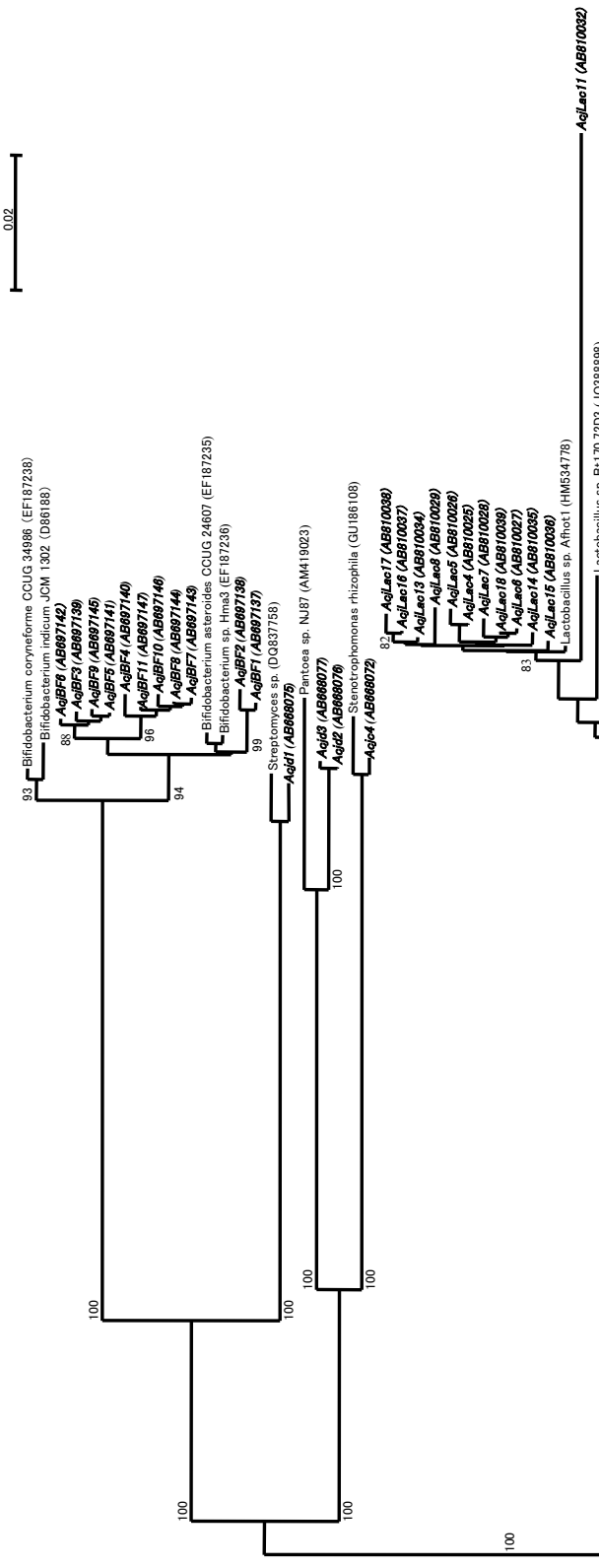

Fig. 1. Phylogenetic tree of gastrointestinal isolates based on the 16S rRNA gene sequences. The phylogenetic tree was constructed using CLUSTAL X program with the neighbor-joining algorithm. Gut isolates obtained in this study were shown in bold italics. Booststrap values >80% are indicated.

2.2.2.2. Phylogenetic relationships of Japanese honeybee gut isolates

A total of 11 colonies obtained from Wilkins-Chalgren agar plates were grouped into one cluster with bifidobacteria isolated in other studies (Fig. 1) and the 16S rRNA gene sequences were designed as AcjBF1-AcjBF11 (Accession numbers as AB697137-AB69714) in DDBJ Database (Table 1). All of the AcjBF isolates obtained in this study showed high similarity with bacteria belonging to the genus *Bifidobacterium* (Table 1). To further elucidate the phylogenetic relationships between the AcjBF isolates from Japanese honeybees and other bifidobacteria isolated in previous studies, we constructed phylogenetic trees only including bifidobacteria (Fig. 2). Phylogenetic analysis indicated that all 11 AcjBF isolates were most closely related to *Bifidobactrium* from honeybees previously deposited, but they formed three different clades that were distinct from the well described species *B. asteroids* and *B. coryneforme*, both from *A. mellifera* and *B. indicum* from *A. cerana*. Five of the AcjBF isolates (AcjBF4, 7, 8, 10, 11) were very closely related to *Bifidobacterium* sp. Aabbto19 isolated from the stomach of *A. mellifera*. AcjBF1 and AcjBF2 formed a distinct cluster with *Bifidobacterium* sp. SHOG615. Isolates AcjBF3, 5, 6, and 9 also formed a well-defined cluster with *Bifidobacterium* sp. SHOG582 from *A. mellifera* (Fig. 2).

Colonies selected from MRS agar plates were grouped into cluster of genus *Lactobacillus* in phylogenetic tree (Fig. 1) and 16S rRNA sequence homology analysis based on the GenBank database revealed that all 17 isolates of this group showed high similarity to bacteria of genus *Lactobacillus* (Table 2). The 17 lactobacilli were designated as AcjLac1-AcjLac18 in the GenBank database with accession numbers as AB10023-AB10039. Among these lactobacilli, thirteen isolates were closely (99%) related to four different species of *Lactobacillus* previously isolated from honeybee gut or honeybee hives, whereas the remaining four isolates (AcjLac9, AcjLac10, Acjlac11, AcjLac12) belonging to

Table 1. Identification of isolates belonging to genus *Bifidobacterium* based on 16S rRNA sequences

Isolate	Database ID	Closest affiliation in GenBank	Similarity (%)
AcjBF1	AB697137	Uncultured *Bifidobacterium sp.* SHOG615 (HM113243)	99%
AcjBF2	AB697138	*Bifidobacterium sp.* Achmro11 (HM534827)	98%
AcjBF3	AB697139	Uncultured *Bifidobacterium sp.* SHOG582 (HM113220)	99%
AcjBF4	AB697140	*Bifidobacterium sp.* Aabbto19 (HM534816)	98%
AcjBF5	AB697141	Uncultured *Bifidobacterium sp.* SHOG582 (HM113220)	99%
AcjBF6	AB697142	Uncultured *Bifidobacterium sp.* SHOG582 (HM113220)	99%
AcjBF7	AB697143	*Bifidobacterium sp.* Aabbto19 (HM534816)	98%
AcjBF8	AB697144	*Bifidobacterium sp.* Aabbto19 (HM534816)	98%
AcjBF9	AB697145	Uncultured *Bifidobacterium sp.* SHOG582 (HM113220)	99%
AcjBF10	AB697146	*Bifidobacterium sp.* Aabbto19 (HM534816)	98%
AcjBF11	AB697147	*Bifidobacterium sp.* Aabbto19 (HM534816)	98%

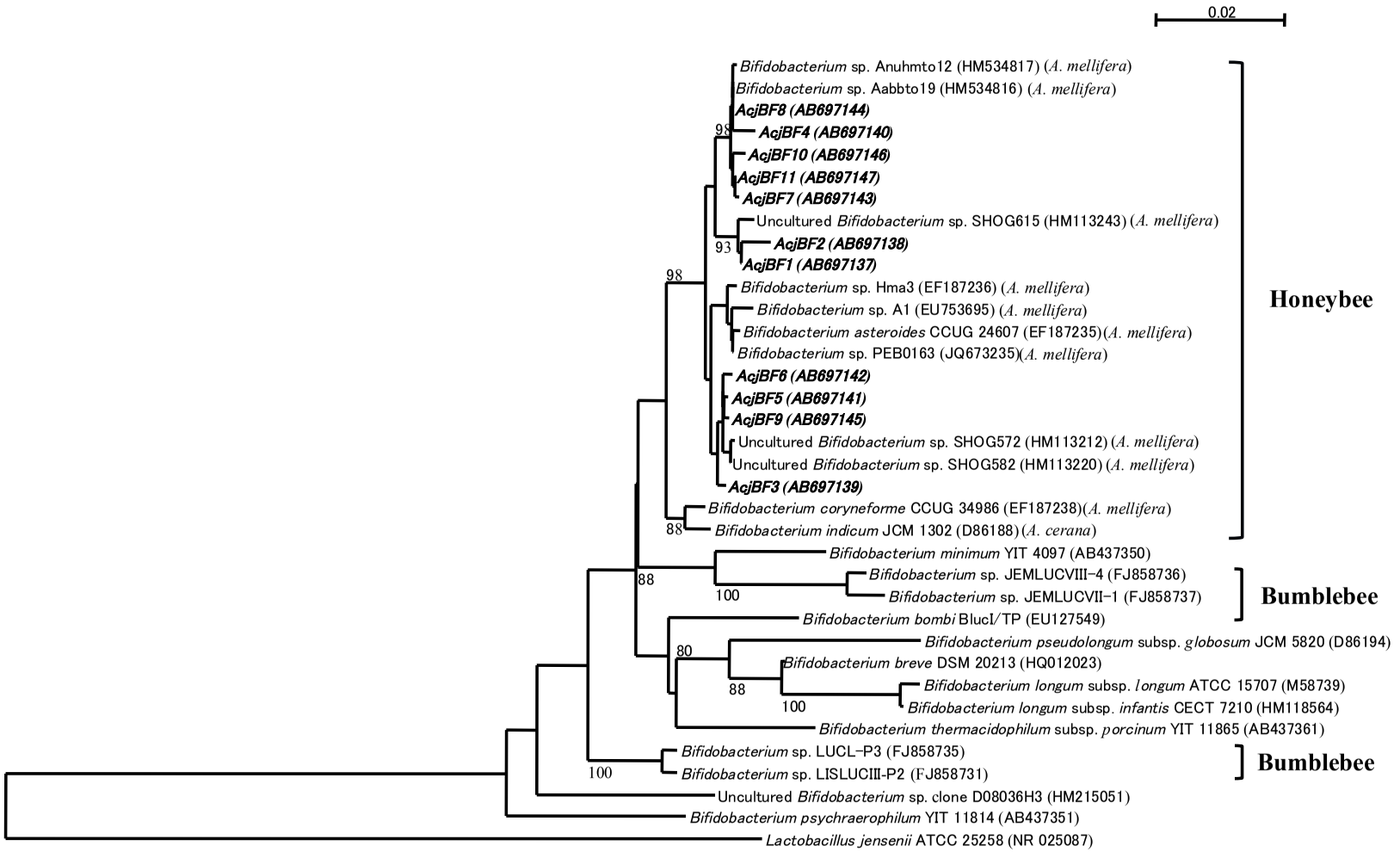

Fig. 2. Phylogenetic tree of genus *Bifidobacterium* based on the 16S rRNA gene sequences. The phylogenetic tree was constructed using CLUSTAL X program with the neighbor-joining algorithm. Bacteria *Lactobacillus jensenii* ATCC25258 was used as the outgroup. Bifidobacteria obtained in this study are shown in bold italics. Booststrap values >80% are indicated.

Table 2. Identification of isolates belonging to genus *Lactobacillus* based on 16S rRNA sequences

Isolate	Database ID	Closest affiliation in GenBank	Similarity(%)
AcjLac1	AB810023	*Lactobacillus sp.* Mbohs2t2 (HM534799)	99%
AcjLac3	AB810024	*Lactobacillus sp.* Afpoto14 (HM534811)	99%
AcjLac4	AB810025	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac5	AB810026	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac6	AB810027	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac7	AB810028	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac8	AB810029	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac9	AB810030	*Lactobacillus kunkeei* 100-1 (JQ009336)	98%
AcjLac10	AB810031	*Lactobacillus kunkeei* Anhst5 (HM534744)	97%
AcjLac11	AB810032	*Lactobacillus sp.* Achmto16 (HM534779)	92%
AcjLac12	AB810033	*Lactobacillus kunkeei* strain 100-1 (JQ009336)	98%
AcjLac13	AB810034	*Lactobacillus sp.* Aabbto16 (HM534779)	99%
AcjLac14	AB810035	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac15	AB810036	*Lactobacillus sp.* Afhot1 (HM534778)	99%
AcjLac16	AB810037	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac17	AB810038	*Lactobacillus sp.* Achmto16 (HM534779)	99%
AcjLac18	AB810039	*Lactobacillus sp.* Afhot1 (HM534778)	99%

distinct species due to their lower 16S rRNA sequence similarity below the species definition threshold level (>98.7%) (Stackebrandf and Ebers, 2006) (Table 2). Further phylogenetic analysis of these isolates demonstrated that lactobacilli obtained from this study clustered as four different groups with *Lactobacillus* isolates related to honeybees from the GenBank database except one isolate named AcjLac11 that showed a different cluster from previously deposited honeybee intestinal bacteria (Fig. 3). Isolates AcjLac9, AcjLac10 and AcjLac12 grouped together with previously described species of *Lactobacillus kunkeei* from the European honeybee *A. mellifera* and isolates AcjLac3 and AcjLac1 were closely related to a phylotype of *Lactobacillus sp*. Afpoto14 and *Lactobacillus sp*. Mbohs2t2 previously isolated from the honeybee *A. florea* and stingless bee *Meliponula bocandeei,* respectively. Other isolates formed a distinct cluster with phylotypes of *Lactobacillus sp*. Achmto16 and Afhot1, which were isolated from the honeybee *A. cerana* in a previous study (Fig. 3).

On the other hand, 16S rRNA gene sequences of the seventeen isolates were selected from BHI agar plates designated as Acja1–Acjd4 (Accession numbers AB668062~AB668078). Based on these 16S rRNA similarities, isolates were classified into seven bacterial genera: *Bacillus*, *Staphylococcus*, *Lysinibacillus*, *Xanthomonas*, *Paenibacillus*, *Streptomyces* and *Pantoea* (Table 3). Three isolates (Acjc4, Acjd2 and Acjd3) were Gram-negative bacteria and the other fourteen isolates were Gram-positive bacteria. Acjc2 and Acjd4 had identical 16S rRNA gene sequences. In addition, based on the 16S rRNA gene sequences of seventeen isolates selected with BHI medium, a phylogenetic tree was constructed using the neighbor-joining method (Fig. 4). The results showed that eight isolates from this study (Acja1, Acja3, Acjb1, Acjb2, Acjc1, Acjc2, Acjc3 and Acjd4) grouped with members of the genus *Bacillus* related to *Bacillus* species isolated in a previous study on Japanese honeybees (Yoshiyama and Kimura 2009), and were subdivided

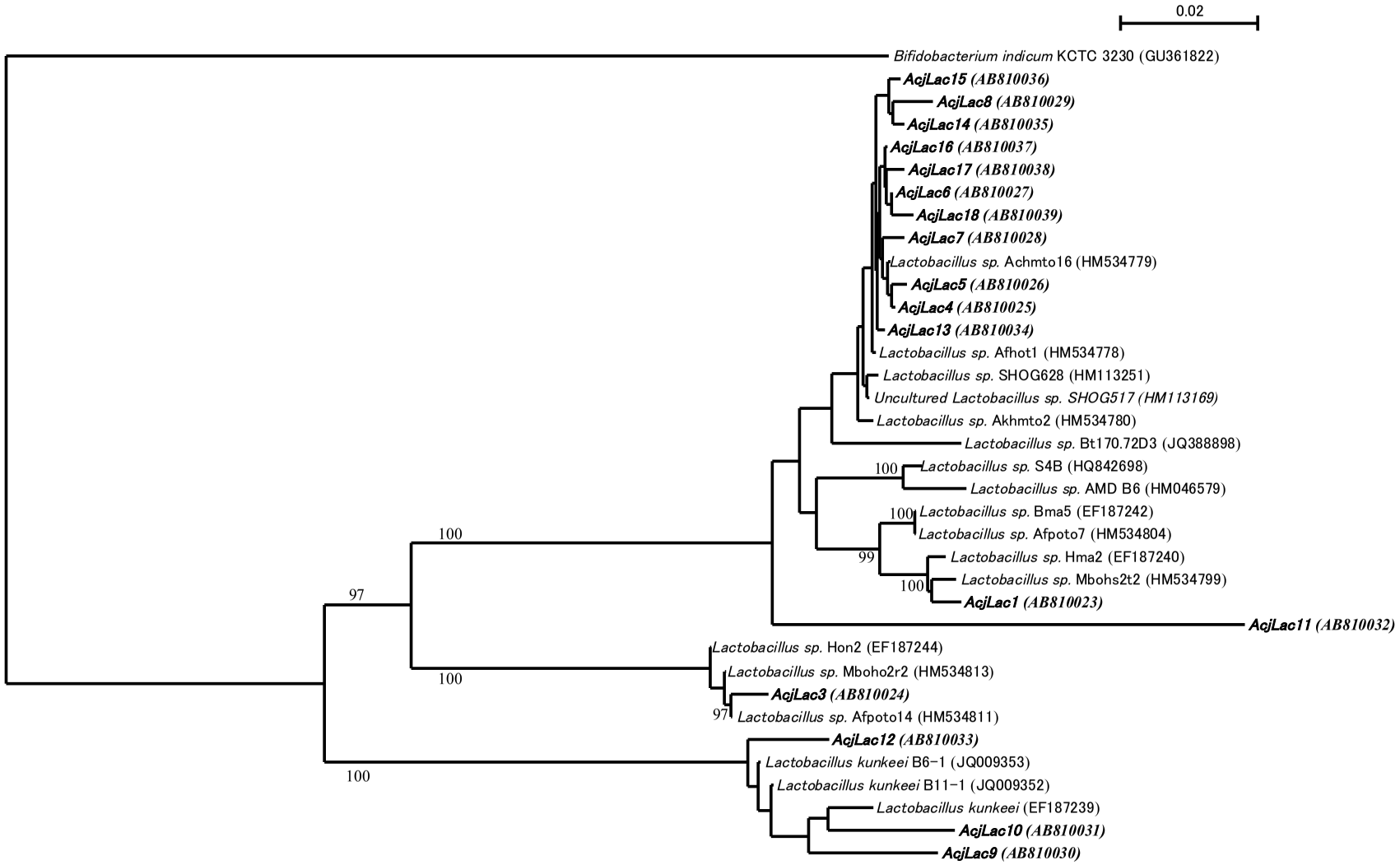

Fig. 3. Phylogenetic tree of genus *Lactobacillus* based on the 16S rRNA gene sequences. The phylogenetic tree was constructed using CLUSTAL X program with the neighbor-joining algorithm. *Bacteria Bifidobacterium indicum* KCTC 3230 was used as the outgroup. Lactobacilli obtained in this study are shown in bold italics. Booststrap values >80% are indicated.

Table 3. Identification of other isolates based on 16S rRNA sequences

Isolate	Database ID	Closest affiliation in GenBank	Bacterial division	Similarity (%)
Acja1	AB668062	*Bacillus cereus* TAUC5 (HQ914780)	Firmicutes; *Bacillus*	99%
Acja2	AB668063	*Staphylococcus sciuri* CTSP9 (EU855191)	Firmicutes; *Staphylococcus*	99%
Acja3	AB668064	*Bacillus subtilis* A97 (AB501113)	Firmicutes; *Bacillus*	99%
Acja4	AB668065	*Staphylococcus sciuri* CTSP9 (EU855191)	Firmicutes; *Staphylococcus*	99%
Acja5	AB668066	*Lysinibacillus sp.* PCSAS2-35 (GQ284494)	Firmicutes; *Lysinibacillus*	100%
Acjb1	AB668067	Bacillaceae bacterium GYPB05 (JF346888)	Firmicutes Unclassified *Bacillaceae*	99%
Acjb2	AB668068	*Bacillus cereus* Aj080319IA-16 (HQ727973)	Firmicutes; *Bacillus*	100%
Acjc1	AB668069	*Bacillus cereus* biovar anthracis str. CI (CP001746)	Firmicutes; *Bacillus*	99%
Acjc2	AB668070	*Bacillus cereus* TAUC5 (HQ914780)	Firmicutes; *Bacillus*	100%
Acjc3	AB668071	*Bacillus cereus* G8639 (AY138271)	Firmicutes; *Bacillus*	99%
Acjc4	AB668072	Uncultured Xanthomonadales bacterium (HM798689)	Proteobacteria; *Gammaproteobacteria*	99%
Acjc5	AB668073	*Paenibacillus sp.* IHB B 2257 (HM233959)	Firmicutes; *Paenibacillus*	99%
Acjc6	AB668074	*Staphylococcus sp.* CTSP32 (EU855210)	Firmicutes; *Staphylococcus*	99%
Acjd1	AB668075	*Streptomyces sp.* ACT-40 (DQ837758)	Actinobacteria;*Streptomyces*	99%
Acjd2	AB668076	*Pantoea sp.* PPE7 (AY501386)	Proteobacteria; *Gammaproteobacteria*	96%
Acjd3	AB668077	*Pantoea sp.* PPE7 (AY501386)	Proteobacteria; *Gammaproteobacteria*	96%
Acjd4	AB668078	*Bacillus cereus* TAUC5 (HQ914780)	Firmicutes; *Bacillus*	100%

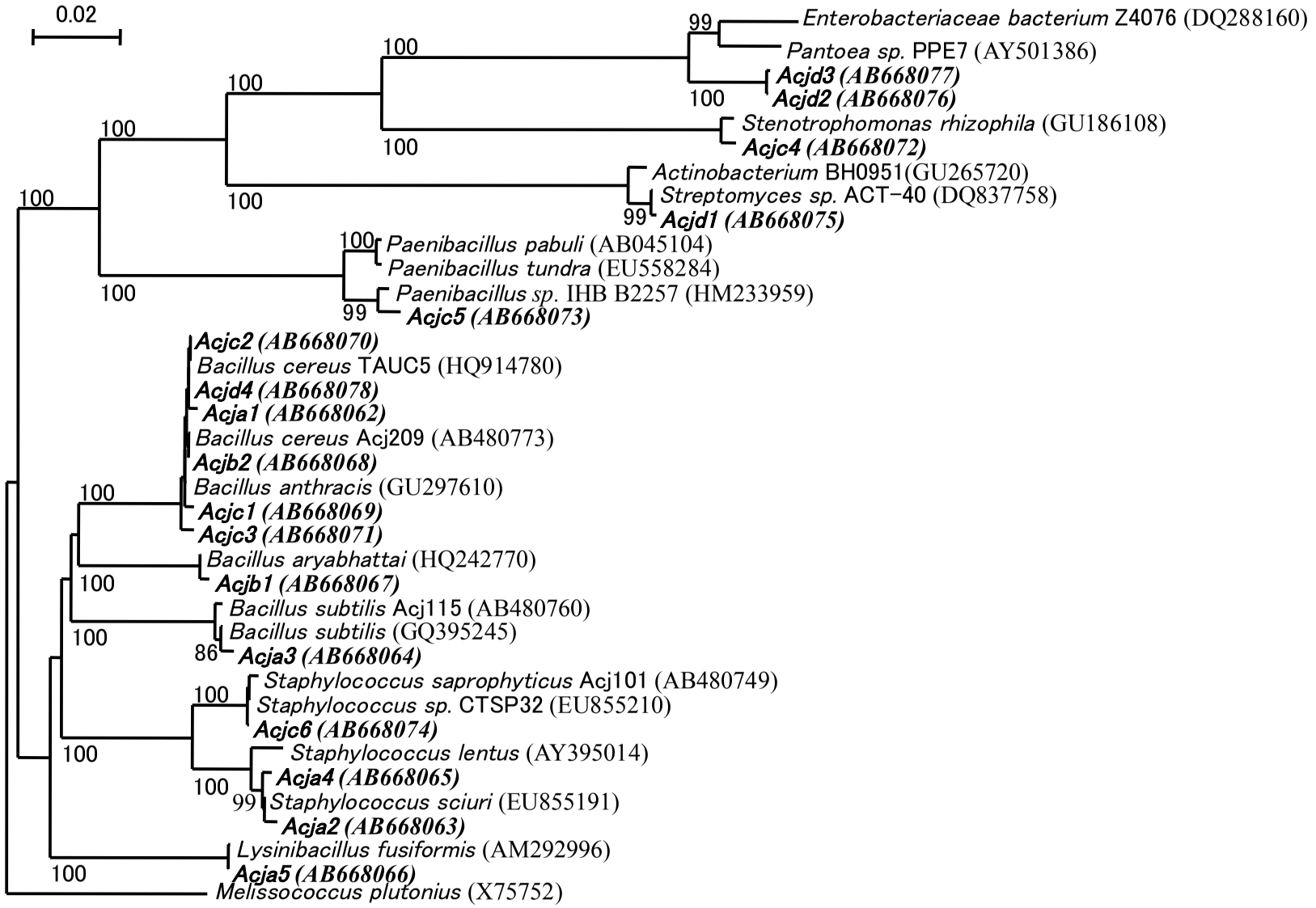

Fig. 4. Phylogenetic tree of bacteria belonging to other bacterial genera based on the 16S rRNA gene sequences. The phylogenetic tree was constructed using CLUSTAL X program with the neighbor-joining algorithm. Gut isolates obtained in this study are shown in bold italics. Booststrap values >80% are indicated.

into three different groups of *Bacillus cereus*, *Bacillus aryabhattai* and *Bacillus subtilis*. Others clustered as six different groups with bacteria of genera *Pantoea, Stenotrophomonas, Streptomyces, Paenibacillus, Staphylococcus and Lysinibacillus* (Fig. 4).

2.3. Characterization of gastrointestinal bacteria from the Japanese honeybee

2.3.1. Materials and methods

2.3.1.1. Characterization of gut bacteria based on biochemical properties

To characterize the biochemical properties of all isolates, carbohydrate substrate utility properties of all pure cultured isolates were performed using API 50CH biochemical kits with the supplied medium (BioMerieux, France). Bacterial suspensions in saline water (100 µl) were mixed with the API 50CH kit supplied liquid medium, API CHL medium for bifidobacteria and lactobacillus bacteria, API CHB/E medium for other gut bacteria, and applied to 50-well strips according to the manufacturer's instructions. The strips were then incubated anaerobically (AnaeroPack System, Mitsubishi Gas Chemical Co., Inc., Japan) at 35°C and changes in color recorded after 48 h or 72 h for bifidobacteria and 24 h or 48 h for other bacteria based on the growth of the bacteria.

2.3.1.2. Characterization of gut bacteria based on observations by SEM

To understand the morphological characteristics of all isolated bifidobacteria and one *Bacillus* isolate Acja3, these isolates were examined under a scanning electron microscope (SEM) (JSM-7600F, JEOL, Japan). Eleven bifidobacteria cultured at 35°C for 48 h under anaerobic conditions in Wilkins-Chalgren liquid medium and one isolate Acja3 cultured at 35°C for 24 h under aerobic conditions in BHI liquid medium were pre-fixed in 2.5%

glutaraldehyde after removing the medium. Droplets of the suspended samples were mounted on the sample stage, Nano-Percolator (JEOL DATUM Ltd., Tokyo), and the liquid aspirated with a syringe for attaching the samples tightly on the membranes. Subsequently, membranes with the samples were post-fixed in 1% osmic tetroxide, dehydrated in absolute ethanol series (50-100%), freeze-dried in t-butyl alcohol, and coated with osmic tetroxide. The prepared samples were then examined with a scanning electron microscope (JSM-7600F, JEOL, Japan).

2.3.2. Results

2.3.2.1. Biochemical properties of Japanese honeybee gut isolates

After phylogenetic analysis to characterize these isolates, the biochemical properties of all isolates obtained in this study were investigated using the API 50CH biochemical system. According to carbohydrate fermentation analysis, all bifidobacteria obtained from this study fermented eleven of forty-nine carbohydrate sources differently, and they did not utilize the remaining thirty-seven carbohydrates in the tests, although some were utilized by well described bifidobacteria from honeybees in a previous study (Killer et al., 2011) (Table 4). In addition, all bifidobacteria isolated in this study utilized esculin ferric citrate, salicin, D-melibiose and D-raffinose as carbohydrate sources, but only one isolate, AcjBF10, was able to utilize methyl-b-xylopyranoside. Despite the high similarity among the 16S rRNA sequences of the AcjBF isolates (>97%), the results of the biochemical analysis differed among all but two of the isolates tested (Table 4). Specifically, AcjBF4 and AcjBF11, which showed high 16S rRNA gene sequence similarity (>99.5%), had identical carbohydrate fermentation patterns.

Biochemical property analysis of lactobacilli revealed that twenty-nine of forty-nine

Table 4. Differential carbohydrate fermentation patterns of bifidobacterial isolates

Substrates	AcjBF 1	AcjBF 2	AcjBF 3	AcjBF 4	AcjBF 5	AcjBF 6	AcjBF 7	AcjBF 8	AcjBF 9	AcjBF 10	AcjBF 11	*B. asteroides*	*B. coryneforme*	*B. indicum*
L- Arabinose	+	++	+	++	++	++	-	++	++	++	++	+	++	++
D- Ribose	-	++	+	++	++	++	+	++	++	++	++	++	++	++
D- Xylose	++	+	-	-	-	-	-	-	-	-	-	++	-	-
D- Galactose	-	++	+	+	+	+	-	++	++	++	+	+	++	++
D- Fructose	-	-	-	-	-	-	-	-	-	-	-	++	++	++
D- Mannnose	-	-	-	-	-	-	-	-	-	-	-	++	-	-
Methyl α-D-glucopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	++	+	++
Arbutin	+	+	-	-	-	-	-	-	-	-	-	++	++	++
Esculin ferric citrate	++	++	++	++	++	++	++	++	++	++	++	ND	ND	ND
Salicin	+	++	+	+	++	++	+	++	++	++	+	++	++	++
D- Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	++	++
D- Maltose	-	-	-	-	-	-	-	-	-	-	-	-	++	++
D- Melibiose	++	++	++	++	++	++	++	++	++	++	++	-	++	+
D- Sucrose	-	-	-	-	-	-	-	-	-	-	-	++	+	+
D- Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D- Melezitose	-	++	+	+	++	+	+	++	++	++	+	++	-	-
D- Raffinose	++	++	++	++	++	++	++	++	++	++	++	-	++	-
5- Ketogluconate	-	+	-	-	-	-	-	+	+	+	-	-	-	-

Data are from this and a previous study (Killer et al., 2011). Results are scored as:++, positive; +, weakly positive; -, negative; ND, no data available

carbohydrates were used as energy sources by these isolates and all isolates showed distinct carbohydrate fermentation patterns except two isolates AcjLac4 and AcjLac7, which showed same carbohydrate fermentation patterns in this analysis (Table 5). Carbohydrates D-arabinose, D-galactose, D-glucose, amygdalin, arbutin and esculin-ferric-citrate were fermented by all seventeen lactobacilli, but some carbohydrates were only used by one isolate. For example, only AcjLac13 fermented substrate erythritol, while carbohydrates D-ribose, D-trehalose and gluconate were used only by strains AcjLac1, AcjLac15 and AcjLac14. Isolates AcjLac15 and AcjLac18 showed higher sequence similarity to *Lactobacillus sp*. Afhot1 (99%) and had significantly different carbohydrate utilization patterns. In addition, isolates (AcjLac9, AcjLac10 and AcjLac12) belonging to the cluster of *Lactobacillus kukeei* species from honeybee also showed distinct properties in carbohydrate utility (Table 5).

As shown in Table 6, all isolates obtained from BHI medium except Acja5 utilized D-fructose, but each isolate had a unique carbon source utilization profile. The differences in these profiles can be used to distinguish between isolates with highly similar 16S rRNA gene sequences. For example, although isolates Acjc2 and Acjd4 had identical 16S rRNA gene sequences, the isolates can be distinguished from each other based on differences in their utilization of four different sugar substrates (Table 6). Isolates Acja2 and Acja4 possessed significantly different biochemical properties although both showed high similarity (99%) with the same species in genus *Staphylococcus*. In addition, two isolates belonging to the genus *Pantoea* also had unique carbohydrate utility patterns (Table 6).

2.3.2.2. Morphological properties of Japanese honeybee gut isolates

To characterize the morphological properties, some bacteria were also observed by SEM. Detailed micrographs of the cell surfaces of all eleven AcjBF isolates are shown in

Table 5. Differential carbohydrate fermentation patterns of *Lactobacillus* isolates

Substrates	Acj Lac1	Acj Lac3	Acj Lac4	Acj Lac5	AcjLa c6	Acj Lac7	Acj Lac8	Acj Lac9	Acj Lac10	Acj Lac11	Acj Lac12	Acj Lac13	Acj Lac14	Acj Lac15	Acj Lac16	Acj Lac17	Acj Lac18
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
D-Arabinose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
L-Arabinose	++	-	-	+	+	-	+	+	+	+	+	+	+	-	-	-	++
D-Ribose	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-D-Xylopyranoside	++	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
D-Galactose	++	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
D-Glucose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
D Fructose	++	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
L-Sorbose	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	+	+
D-Sorbitol	-	-	++	++	++	++	+	++	++	++	+	++	+	-	+	++	++
α-Methyl-D-Mannopyranoside	-	-	++	++	++	++	+	++	+	++	+	++	++	-	++	++	++
α-Methyl-D-Glucopyranoside	++	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
N-Acetyl-glucodamine	+		++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Amygdalin	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Arbutin	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Esculin-ferric-citrate	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	++	++
Salicin	++	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
D-Cellobiose	+	-	++	++	++	++	++	++	++	++	++	++	++	-	++	++	++
D-Maltose	-	-	-	-	-	-	-	++	-	+	+	++	+	-	-	-	++
D-Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Melibiose	-	-	+	+	+	+	++	+	+	+	++	+	++	++	+	+	++
D-Sucrose	++	-	++	++	++	++	++	++	++	++	++	++	++	-	++	++	++
D-Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	+	++	-	-	++
D-Raffinose	-	-	+	+	++	+	++	++	++	+	-	+	-	-	++	++	++
Xylitol	+	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Gentiobiose	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	++	-	++	++	++	++	++	++	++	++	++	++	++	+	++	++	++
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

Results are scored as: ++, positive; +, weakly positive; -, negative

Table 6. Differential carbohydrate fermentation patterns of other isolates

Substrates	Bacterial strains																
	Bacillus							Staphylococcus			Lysinibacillus	Pantoea		Unclassified Bacillaceae	Xanthomonas	Paenibacillus	Streptomyces
	Acja1	Acja3	Acjb2	Acjc1	Acjc2	Acjc3	Acjd4	Acja2	Acja4	Acjc6	Acja5	Acjd2	Acjd3	Acjb1	Acjc4	Acjc5	Acjd1
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	+	+	+	+	+	++	-	++	-	-	-	+	+	++	-
L-Arabinose	-	++	-	-	-	-	-	++	-	++	-	-	-	+	++	++	-
D-Ribose	++	++	++	++	++	++	++	++	-	+	-	-	+	+	+	++	-
D-Xylose	-	+	-	-	-	-	-	-	-	++	-	-	-	+	-	++	-
D-Galactose	-	-	-	++	-	-	-	++	-	+	-	-	-	-	++	++	-
D-Glucose	++	+	++	++	++	++	++	++	-	++	-	++	++	++	++	++	++
D Fructose	++	+	++	++	++	++	++	++	+	++	-	+	+	+	++	++	+
D-Mannose	-	+	-	-	-	-	-	++	-	++	-	-	-	-	++	++	-
Inositol	-	+	-	-	+	-	-	++	-	+	-	-	-	-	-	+	-
D-Mannitol	-	+	-	-	-	-	-	++	-	++	-	++	++	+	+	++	++
D-Sorbitol	-	+	-	-	-	-	-	++	-	++	-	-	-	-	+	+	-
α-Methyl-D-Glucopyranoside	-	++	-	-	-	-	-	++	-	+	-	-	-	-	-	+	-
N-Acetyl-Glucodamine	++	-	++	++	++	++	++	++	-	++	-	++	++	+	++	++	++
Amygdalin	-	+	-	+	-	+	+	++	-	+	-	-	-	-	++	++	-
Arbutin	++	+	+	++	++	++	++	++	-	+	-	-	-	-	++	++	-
Esculin-ferric-citrate	++	++	++	++	++	++	++	++	++	++	+	-	-	++	++	++	-
Salicin	++	+	+	++	++	++	+	++	-	+	-	-	-	-	++	++	-
D-Cellobiose	++	+	+	+	++	+	+	++	-	+	-	-	-	-	++	++	-
D-Maltose	++	+	++	++	++	++	++	++	-	++	-	-	-	++	++	++	-
D-Lactose	-	+	-	-	-	+	-	++	-	++	-	-	-	+	++	++	-
D-Melibiose	-	+	-	-	-	-	-	-	-	+	-	-	-	++	-	++	-
D-Sucrose	++	+	-	-	++	-	++	++	+	++	-	-	-	++	++	++	-
D-Trehalose	++	+	++	++	++	++	++	++	-	++	-	-	-	+	++	++	-
D-Raffinose	-	+	-	-	-	-	-	-	-	+	-	-	-	++	-	++	-
Starch	++	-	++	++	++	++	++	-	-	-	-	-	-	++	+	++	-
Glycogen	++	-	++	++	++	++	++	-	-	-	-	-	-	++	-	++	-
Gentiobiose	-	-	-	-	-	-	-	++	-	++	-	-	-	+	++	++	-
D-Turanose	-	++	-	-	-	-	-	++	-	+	-	-	-	-	+	++	-
D-Fucose	-	-	-	-	-	-	-	++	-	-	-	-	-	-	+	-	-

Results are scored as: ++, positive; +, weakly positive; -, negative

Fig. 5, including the three representative phylotypes (AcjBF1, 3 and 4). The micrographs revealed that cells were irregularly shaped rods without branching and all eleven AcjBF isolates were morphologically similar with bifurcation seldom observed.

2.4. Discussion

To investigate the potential of Japanese honeybee gastrointestinal bacteria for development of novel and sustainable honeybee disease strategies, bacteria in the digestive tract of the Japanese honeybee were isolated using culture-dependent methods and a total of forty-five isolates were obtained. Multiple analyses of 16S rRNA gene sequences, phylogenetic relationships and biochemical characteristics of these isolates revealed that the Japanese honeybee harbors diverse genera of gastrointestinal bacteria and variable phylotypes exist in the same genera or species (Fig. 1). Symbiosis of honeybee and bacteria has been previously studied by culture-dependent methods and diverse assemblages of bacteria demonstrated to inhabit the adult honeybee digestive tract (Gilliam, 1997). A recent culture-independent study suggested that a simple set of gastrointestinal bacteria is associated with honeybees (Martinson et al., 2011). Although some opinions on the components of honeybee gastrointestinal bacteria are yet arguable, honeybees appear to harbor two different types of bacteria in their digestive tract. Some types of gastrointestinal bacteria such as bifidobacteria, lactobacilli and bacilli are resident and inherited between honeybee generations by vertical transmission through social contacts among worker individuals in colonies. Other types of bacteria such as bacteria of the genera *Pantoea* and *Stenotrophomonas* are transient, horizontally acquired from the environment and horizontally transmitted among individuals in colonies under specific conditions but not transmitted to the next generation. Recent findings also support this hypothesis indicating bees acquire lactobacilli by two different means; maternally inherited lactobacilli by vertical

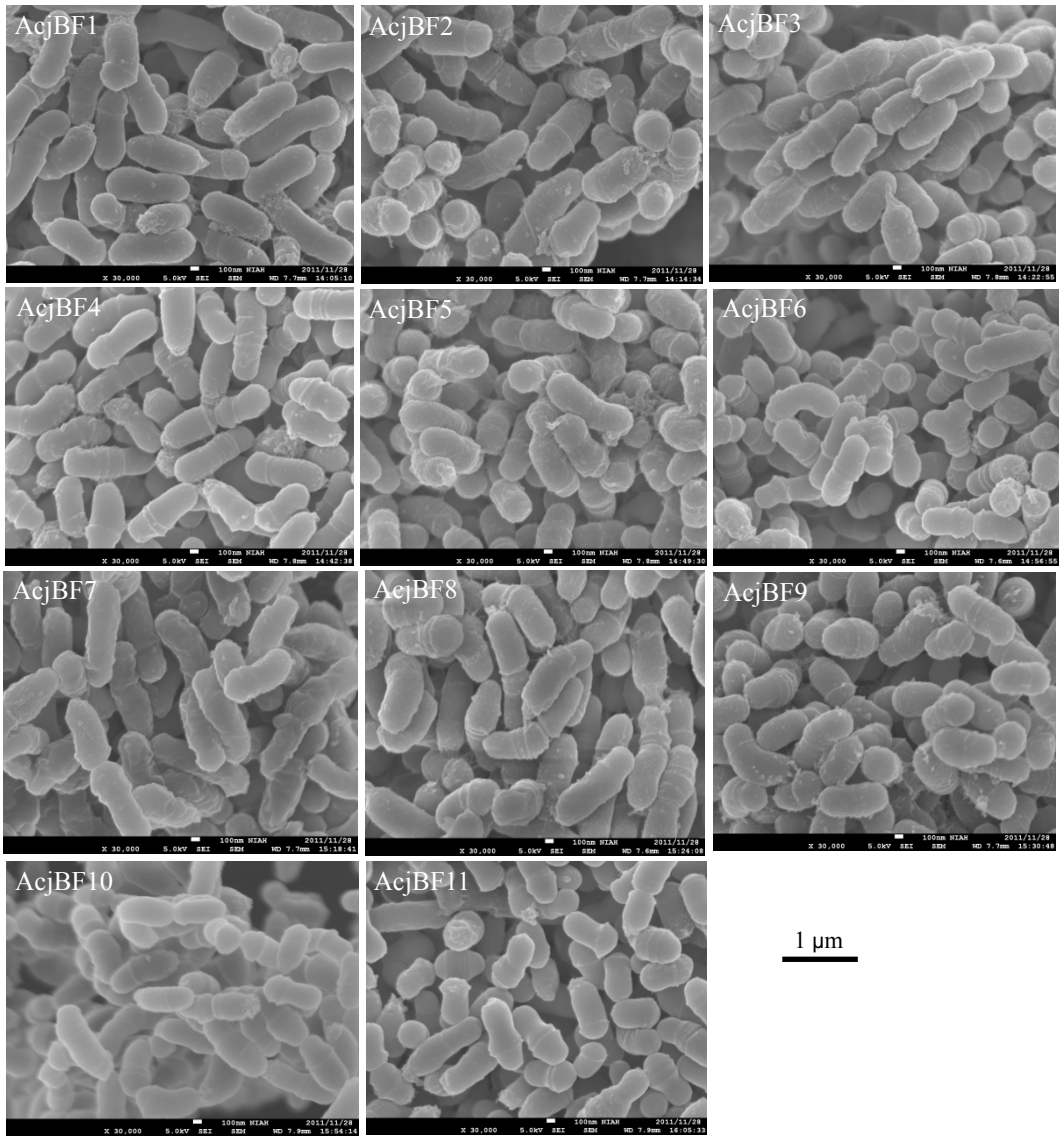

Fig. 5. Scanning electron micrograph of AcjBF isolates (AcjBF1-AcjBF11) obtained in this study. Cells are irregularly shaped rods. The bar represents 1 μm

transmission among bee individuals and others from environmental sources such as flowers by horizontal transmission (McFrederick et al., 2012).

In this study, a total of eleven bifidobacteria were obtained and all these isolates were related to four different bifidobacterial phylotypes deposited in GenBank (Table 1), indicating that bifidobacteria also inhabit the gut bacterial community of the Japanese honeybee. Bifidobacteria have been detected from a variety of insect species, including cockroaches, wasps and hornets (Mrazek et al., 2008). Three *Bifidobacterium* species have also been previously identified from the digestive tracts of honeybees. Of these, *Bifidobacterium asteroides* and *B. coryneforme* were isolated from the intestine of the European honeybee, *A. mellifera*. The third species, *B. indicum*, was isolated from intestines of Asian honeybees, *A. cerana* and *A. dorsata* from the Philippines and Malaysia, respectively (Killer et al., 2010). To the best of our knowledge, this is the first report describing the isolation of bifidobacteria from the Japanese honeybee, *A. cerana japonica*. Results of this study give further evidence to the symbiotic relationships between honeybee species and the genus *Bifidobacterium*.

Seventeen isolates belonging to the genus *Lactobacillus,* one other important genus of lactic acid bacteria was also obtained from the Japanese honeybee gastrointestine in this study and identified as six different phylotypes of *Lactobacillus* (Table 2). This result indicates that Japanese honeybees also harbor *Lactobacillus* bacteria in their digestive tract. Bacteria of the genus *Lactobacillus,* as a main component of lactic acid bacteria, inhabit various niches and play crucial roles in all aspects of ecology (Vandenbergh, 1993). Thus many researchers have devoted great attention to investigating the genus *Lactobacillus* related to humans and animals including invertebrate insects. Some phylotypes of lactobacilli have recently been isolated from the digestive tract of honeybees (Olofsson and

Vásquez, 2008; Tajabadi et al., 2011; Neveling et al., 2012) and bumblebees (Koch and Schmid-Hempel, 2011). First report of gastrointestinal lactobacilli from the Japanese honeybee in this study provides further insights into the mutualistic association between the genus *Lactobacillus* and honeybee species.

In addition to bacteria belonging to the genera *Bifidobacterium and Lactobacillus*, a total of seventeen isolates from the Japanese honeybee gut were identified as seven other different genera; *Bacillus,* Unclassified *Bacillaceae, Staphylococcus, Lysinibacillus, Gammaproteobacteria, Paenibacillus and Streptomyces* (Table 3). Among these isolates, seven isolates belong to the genus *Bacillus* with five different phylotypes closely related to two species (Table 3), indicating the genus *Bacillus* is an important component of the Japanese honeybee gut bacterial community, and the Japanese honeybee and the genus *Bacillus* have stable symbiotic associations. Although the composition of bacterial flora differs between populations in the Japanese honeybee, isolates consist primarily of *Bacillus* species corroborate well with the findings of a previous study on Japanese honeybees (Yoshiyama and Kimura 2009). Other culture-dependent studies have also suggested that *Bacillus* spp. are dominant floral components of the European honeybees (Rada et al., 1997) and some solitary bees (Gilliam et al., 1990a), despite that some recent culture-independent surveys that demonstrated distinctive honeybee specific consistent bacterial flora without *Bacillus* phylotypes (Martinson et al. 2011; Moran et al. 2012). This difference may be due to distinct culture-dependent and culture-independent experimental technology and methodologies. Although more intensive research is needed, it can be confirmed from the above results that lactic acid bacteria and bacilli are resident bacteria in the honeybee gut and can be inherited among generations to establish symbiotic relationships.

On the other hand, bacteria related to the genera *Pantoea*, *Stenotrophomonas*, *Streptomyces, Lysinibacillus* were first isolated and identified by molecular analyses from

the honeybee gut (Table 3), although some studies reported the isolation of bacteria belong to the genus *Streptomyces* from wasps (Poulsen et al., 2011), bacteria of the genera *Pantoea, Stenotrophomonas* and *Streptomyces* from *Lutzomyia* sand flies (Gouveia et al., 2008). These bacterial phylotypes were not detected in the gut of the Japanese honeybee subspecies of the previous study (Yoshiyama and Kimura, 2009). These genera of bacteria are well associated with soil and plants (Delétoile et al., 2009; Hagemann et al., 2008; Song et al., 2004). It is thus possible that Japanese honeybee may acquire these isolates from the environment such as pollen, nectar and water during foraging and these bacteria may be transient bacteria that accidently exist in the honeybee gut because of the horizontal transmission between honeybees and the environment. Research on Western Flower Thrips confirmed that symbionts are acquired from the food source flowers (de Vries et al., 2001). In addition, a recent study have also pointed that insect facultative symbiotic bacteria including extracellular gut bacteria originate from multiple sources such as food and are frequently transmitted horizontally (Kikuchi, 2009). To understand the exact relationships between these genera of bacteria and the Japanese honeybee, further intensive observations with broader sampling of individuals is needed in the future.

Despite the present knowledge of honeybee gastrointestinal bacteria is limited and complex affecting factors and the structure of honeybee gut bacterial community can not be elucidated completely, together with previous and this study, it can be demonstrated that all honeybees harbor some consistent *Apis*-species specific phylotypes of resident bacteria in their gut, but the profiles of gut bacterial communities are variable depends in some degree on the honeybee species, age, season and location. Diversity in the gut bacterial composition may not only due to some distinct phylotypes of resident bacteria, but also due to existence of distinct transient bacteria. For example, Japanese honeybees harbor some *Apis*-species specific phylotypes of bifidobacteria, lactobacilli and bacilli in the digestive tract. While,

other phylotypes of bacteria in the gut are diverse and differ among Japanese honeybee individuals.

Phylogenetic and homology analyses of 16S rRNA gene sequences of eleven *Bifidobacterium* isolates (AcjBF1-AcjBF11) from the Japanese honeybee revealed that all AcjBF isolates were distinct from three previously well described *Bifidobacterium* species *B. asteroids*, *B. coryneforme* and *B. indicum* from honeybee (Fig. 2). However, all AcjBF isolates obtained in this study appear to be composed of three different clusters when compared with other phylotypes of bifidobacteria in GenBank that originated from the European honeybee *A. mellifera,* in phylogenetic tree (Fig. 2). Despite differences in environmental, geographic and phylogenetic histories between two species *A. cerana* and *A. mellifera*, the results of this study demonstrated that some phylotypes of bifidobacteria isolated from the two honeybee species are very similar. Research group of Vásquez also recently identified novel bifidobacteria from all nine recognized honeybee species and suggested that all species of honeybees possess similar phylotypes of bifidobacteria in their honey crop (Vásquez et al., 2012). In addition, a comparative study about lactic acid bacterial flora of *A. mellifera* from USA and Sweden also indicates consistence of related bifidobacterial phylotyps in honeybee digestive tracts regardless of differences in geographical location (Vásquez et al., 2009). Interestingly, however, compared to bumblebee bifidobacteria that were recently isolated from the digestive tract of *Bombus* species (Killer et al., 2009, 2011), the AcjBF isolates obtained in this study were significantly distinct (Fig. 2). It is hypothesized from this result that there are some bumblebee or honeybee species-specialized bifidobacterial phylotypes in the gut. Despite recent observations that honeybees and bumblebees share similar bacterial symbionts including the genus *Bifidobacterium* (Kaltenpoth, 2011, Martinson et al., 2011) and close phylotypes of bifidobacteria have also been detected from honeybees and stingless bees

(Vásquez et al., 2012), unique phylotypes of bifidobacteria have also been isolated from samples of bumblebees but not from samples of honeybees (Killer et al., 2010). These different results may be due to the different sampling locations and methodologies or the results of honeybees and bumblebees sharing gut bacteria in the studies by Kaltenpoth (2011) and Martinson (2011) maybe because of the effects of horizontal transmission between honeybees and bumblebees during foraging. On the other hands, floral foraging behavior and sociality of honeybees and bumblebees may facilitate horizontal transmission of bacteria and such transmission routes may result in establishment of a stable symbiotic relationship between the host and bacteria (Koch and Schmid-Hempel, 2011).

According to phylogenetic analyses, isolates belonging to the *Lactobacillus* in this study were distinct from *Lactobacillus* bacteria from bumblebees (Accession number as JQ388898 in phylogenetic tree) previously deposited in GenBank databases (Fig. 3), this suggests that, in accordance with the results of bifidobacteria in this study, honeybees harbor distinct phylotypes of lactobacilli in their digestive tract. One recent study also reported that bumblebees also possess simpler but distinct phylotypes of *Lactobacillus* in their gut bacterial communities (Koch and Schmid-Hempel, 2011). This observation is in agreement with results of Martinson et al. (2011). However, all but one isolate obtained from the Japanese honeybee clustered with *Lactobacillus* bacteria isolated from other honeybee species such as *A. cerana*, *A. mellifera*, *A. florea* and stingless bees (Fig. 3). Thus it is possible that honeybees have co-evolved symbiotic relationships with bacteria of the genus *Lactobacillus* and *Apis* species-specific phylotypes may be sustained during the long process of evolution. Extensive research emphasizes such views by detecting similar gut *Lactobacillus* phylotypes in the digestive tracts of the honeybees regardless of differences in species (Vásquez et al., 2012), subspecies (Olofsson et al., 2011) and geographical locality (Vásquez et al., 2009). Similar species-specific gut bacterial communities have also been

confirmed in termites (Shinzato et al., 2005). In addition, phylotypes of *Lactobacillus kunkeei* were also detected from the Japanese honeybee, a subspecies of *A. cerana*, and clustered together with phylotypes of *Lactobacillus kunkeei* isolated from the European honeybee (Fig. 3) corroborating the consistent presence of species *Lactobacillus kunkeei* in all species of honeybees. Species *Lactobacillus kunkeei*, well known as wine-spoiling organisms (Edwards et al., 1998), are common in the digestive tract of all nine honeybees species and tested stingless bees (Vásquez et al., 2012).

On the other hand, bifidobacterial phylotype *B. indicum* previously obtained from *A. cerana* (Scardovi and Trovatelli, 1969) were not isolated from *A. cerana japonica*, a subspecies of *A. cerana,* in this study suggesting that different honeybee subspecies may harbor different phylotypes of bifidobacteria in their digestive tract. Similar divergences in bifidobacterial phylotypes between different subspecies of *A. mellifera* were also detected in a previous study by Olofsson et al. (2011). In addition, one isolate AcjLac11 clustered in a different clade from the other isolates (Fig. 3), indicating the Japanese honeybee may harbor some unique species of *Lactobacillus* that show low similarities (92%) to previously identified species. The most likely explanation of such species divergence may be that some *Lactobacillus* bacteria separate to different species or subspecies at some point during evolution and co-evolve with a genetically divergent host to produce specific phylotypes to facilitate symbiosis. Some studies on wasps (Brucker and Bordenstein, 2012) and termites (Hongoh et al., 2005) have highlighted the phenomenon that host genes are more important factors affecting construction of gut bacterial divergence and host-bacteria co-evolutionary relationships are specialized in the composition of gut microbial communities.

Moreover, phylogenetic relationships of seventeen isolates revealed that with the exception of *Bacillus* and *Staphylococcus*, other isolates in this study separated into six distinct clusters with previously identified environmental bacteria in the phylogenetic tree

(Fig. 4). Except for *Bacillus* and *Staphylococcus* bacteria, gut isolate profiles of Japanese honeybees obtained with BHI medium showed significant differences from previous studies in the same Japanese honeybee gut bacteria (Yoshiyama and Kimura, 2009), even when isolated with the same selective medium (BHI) and methods (Table 7). This variance may be attributed to honeybee samples from different colonies, seasons and life stages. The profiles of honeybee gut bacterial communities in various individuals depends on the colonies and life stages (Ahn et al., 2012; Moran et al., 2012) and are also affected by seasonal factors (Sammataro and Yoder, 2012). Differences in gut flora, even among bees from the same colony, have also been reported elsewhere (Mohr and Tebbe 2006). Similarly, the significant influence of colony and season on bumblebee gut bacterial diversity or composition has also been recently observed (Koch et al., 2012). Together with results of previous studies, this study provides a new insight into increasing knowledge about the diversity of honeybee gastrointestinal bacteria among individuals within the same subspecies.

After isolation of gastrointestinal bacteria from the Japanese honeybee, for further understanding of the diversity of isolates obtained in this study, all isolates were characterized with biochemical and morphological observations. The morphological characteristics of all eleven AcjBF isolates were very similar, with all isolates resembling bacilliform cells with non-bifurcations (Fig. 5). Conversely, the substrate utilization profiles obtained with the API 50CH tests produced disparate results, with the eleven AcjBF isolates exhibiting varying abilities to ferment carbohydrates (Table 4). All lactobacilli isolated in this study also exhibited different biochemical properties, showing various patterns of carbohydrate utility by API50CH analysis (Table 5). The biochemical characteristics are markedly various not only between different genera and different *Bacillus* species, but also between different strains within the same species (Table 6). Similar observations of differences in carbohydrate metabolism between highly similar bifidobacterial strains have

Table 7. Diversity of Japanese honeybee gut bacteria in this study and the previous study by Yoshiyama and Kimura (2009)

Bacterial Division		Isolates from this study	Isolates from Yoshiyama and Kimura (2009)
Firmicutes	*Staphylococcus*	Acja2, Acja4, Acjc6	Acj101, Acj114, Acj220
	Bacillus	Acja1, Acja3, Acjb2, Acjc1, Acjc2, Acjc3, Acjd4	Acj115, Acj209, Acj214
	Lysinibacillus	Acja5	-
	Paenibacillus	Acjc5	-
	unclassified Bacillaceae	Acjb1	-
Actinobacteria	*Kocuria*	-	Acj110, Acj112
	Tsukamurella	-	Acj117
	Microbacterium	-	Acj118
	Streptoomyces	Acjd1	-
Alphaproteabacteria	*Sphingomonas*	-	Acj102,Acj111, Acj120, Acj202, Acj210, Acj216
	Mesorhizobium	-	Acj104, Acj124
Betaproteabacteria	*Janthinobacterium*	-	Acj103, Acj119, Acj215
Gammaproteabacteria	*Escherichia*	-	Acj105, Acj212, Acj213
	Pseudomonas	-	Acj106
	Providencia	-	Acj108
	Enterobacteriaceae	-	Acj122, Acj201, Acj204, Acj208
	Erwinia	-	Acj205, Acj211
	Moraxella	-	Acj218
	Xanthomonadales bacturium	Acjc4	-
	Pantoea	Acjd2, Acjd3	-

been reported by Ennahar et al. (2003). In addition, one early report regarding lactobacilli also discussed wide variations in fermentation patterns within the same species (Johansson et al., 1995). This evidence of markedly different carbohydrate fermentation patterns among the same phylotypes (Tables 4, 5, 6), suggests that even the same species based on the phylogenetic analyses may have different strains of bacteria and the bacteria may have diverse activities. Bacteria obtain energy by fermenting many kinds of carbohydrate substrates and produce various metabolic substances that afford antimicrobial properties to bacteria (Russell et al., 2011). Thereby, diversity of Japanese honeybee gut bacteria at the level of genus, species and strain may lead to variability in utilities of different carbohydrate substances as energy sources and result in diverse biochemical properties regardless of the same species contributing to their diverse bio-functions because of distinct metabolic processes. Divergent functional properties within species have also been confirmed by molecular genetic investigations on European honeybee gastrointestinal bacteria (Engel et al., 2012).

The digestive tract of insects including honeybees is the greatest reservoir of bacterial diversity (Dillon and Dillon, 2004) and the symbiotic associations of insects and gastrointestinal bacteria are being gradually elucidated. Beneficial importance of symbionts in sustaining host fitness is established in some insects such as fruit flies (Behar, et al., 2008), mosquitos (Merkling and van Rij, 2012), aphids (Parker et al., 2013) and termites (Hongoh, 2011). Moreover, the crucial roles of the European honeybee gastrointestinal bacteria, which are important symbionts, have also been reported from earlier studies (Gilliam, 1997; Evans and Armstrong, 2006). Symbionts provide nutrients and defense to their host insects and such symbiosis between insects and bacteria are often highly co-evolved (Moran, 2006). Host genotype is considered as one important factor influencing the insect gut bacterial divergence (Moran, 2006; Brucker and Bordenstein, 2012). Some

gastrointestinal isolates obtained in this study may be closely allied with the Japanese honeybee genetics and as such co-evolved unique bacteria may possess important functions for maintaining honeybee health. Therefore, isolation and characterization of Japanese honeybee gastrointestinal bacteria performed in this study are very important for further understanding their functions for health maintenance and investigating their potential as bio-control agents in disease management.

Chapter 3. Evaluation of European Foulbrood Antagonistic Activity of Gastrointestinal Bacteria

3.1. Introduction

Honeybees, like other insects, are associated with various microbes and have co-evolved with diverse gastrointestinal bacteria. As described in Chapter 2, Japanese honeybees also harbor a wide range of bacteria in their digestive tracts. Among these bacteria, lactic acid bacteria (LAB) including bifidobacteria and lactobacilli, as well as bacilli, were detected as the main constituents of gut bacterial communities of Japanese honeybees.

Lactic acid bacteria belonging to the genus *Bifidobacterium* are classified into the class *Actinomycetes* and widely known as commensal microflora, which inhabit the gastrointestinal tracts of humans and animals including invertebrate insects (Turroni et al., 2011). They are non-motile, non-spore forming, and non-pathogenic Gram-positive polymorphic rods bacteria that can occur singly, in chains or in clumps (Reuter, 2001). Species of *Bifidobacterium* have been reported to inhabit seven different ecological niches i.e. human intestine, human vagina, oral cavity, food, sewage and gastrointestinal tracts of animals and insects (Russell et al., 2011). In recent years, bifidobacteria are extensively studied for use as probiotics, because they prevent infection and improve the health of the host through various mechanisms (Reuter, 2001). Probiotics are defined as living organisms that can confer health benefits to the host (de Vrese and Schrezenmeir, 2008). According to a previous review, metabolic activities of bifidobacteria are one major mechanism by which they contribute to host health. Bacteria of the genus *Bifidobacterium* export various proteins such as surface-associated proteins, secreted proteins that play roles in their adhesion to

intestinal surfaces or in bacterial host interactions to enhance the probiotic properties (Russell et al., 2011). The potential health benefits of bifidobacteria to humans have been more extensively clarified by clinical studies that revealed the functions of bifidobacteria in establishment of a healthy microbiota in preterm infants (Mohan et al., 2006), immune-stimulation (Furrie et al., 2005), cholesterol reduction (Kiessling et al., 2002), lactose intolerance (Jiang et al., 1996), prevention of infectious diarrhea (Saavedra et al., 1994) and cancer (Le Leu et al., 2010). Moreover, studies regarding the association of bifidobacteria and insects also suggest that *Bifidobacterium* are predominate in social bee species such as bumblebees and honeybees (Killer et al., 2011). Some distinct species of the genus *Bifidobacterium* have been recently discovered from the honey stomach of the honeybee *A. mellifera* (Olofsson and Vásquez, 2008; Vásquez et al., 2009; Forsgren et al., 2010), and certain isolates of these bacteria exhibited antagonistic activity against *Paenibacillus larvae* (Forsgren et al., 2010).

Bacteria belonging to the genus *Lactobacillus* are classified in the class *Firmicutes* and are Gram-positive, non-spore-forming and non-pathogenic rods or coccobacilli bacteria (Felis and Dellaglio, 2007). Members of the genus *Lactobacillus* are main components of lactic acid bacteria and are predominant inhabitants of the intestinal tract of humans and animals, where they are thought to play crucial roles in prevention of overgrowth of enteric pathogens and in maintenance of resistance to colonization of pathogens (Vandenbergh, 1993). Due to their roles in processing of food fermentation and applications as probiotics, lactobacilli have attracted great attention for scientific research, and beneficial functions are well documented by many *in vitro* and *in vivo* experimental studies. These studies suggest that some strains of *Lactobacillus* show antagonistic activities against certain human pathogenic microorganisms such as *E. coli* (Hirano et al., 2003; Mangell et al., 2002), *Salmonella spp.* (Gill et al., 2001; Jin et al., 1996) and rotavirus (Guerin-Danan et al., 2001).

In addition, these properties were further confirmed by clinical studies. These results have demonstrated considerable potential for promoting new therapeutic strategies by selected probiotics including *Lactobacillus* strains and functions of their products as biotherapeutic agents (Rosenfeldt et al., 2002; Simakachorn et al., 2000). Similar to the probiotic properties of bifidobacteria, lactobacilli also express antimicrobial activities by diverse means. In addition, to exerting beneficial influence on the host by stimulation or modulation of immune responses, bacteria of the genus *Lactobacillus* can also export various functional proteins to exhibit adhesive properties that are responsible for competitive inhibition of the epithelial and mucosal adherence of pathogens (Servin, 2004). Moreover, lactobacilli develop antagonistic activities against microbial pathogens by producing H_2O_2 (Ocana et al., 1999), acids (Alakomi et al., 2000), biosurfactants (Velraeds et al., 1996), and antimicrobial compounds such as bacteriocins, non-bacteriocin peptides (Klaenhammer, 1993; Sablon et al., 2000). Another important optimal niche for lactobacilli is the digestive tract of honeybees (Olofsson and Vásquez, 2008; Tajabadi et al., 2011; Neveling et al., 2012). The lactobacilli associated with honeybees exhibit beneficial effects on honeybee colonies by increasing the number of bees in a population and honey storage (Audisio and Benítez-Ahrendts, 2011). Some previous studies also suggest that bacteria of *Lactobacillus* originating from the European honeybee digestive tract inhibit the pathogens *Paenibacillus larvae* (Forsgren et al., 2010; Audisio et al., 2011) and *M. plutonius* (Vásquez et al., 2012).

The genus *Bacillus* is a spore-forming bacterium classified into the class *Firmicutes.* Their habitat is mainly in soil, plants and gastrointestinal tracts of animals and humans (Mongkolthanaruk, 2012). *Bacillus* species are well known as pathogens, plant biocontrol producers (i.e. fungicides, bactericides and fertilizers) and probiotics. Properties of *Bacillus* bacteria are diverse between species or subspecies (Mongkolthanaruk, 2012). The pathogenic risks of certain *Bacillus* species, some species of *Bacillus* together with

bifidobacteria and lactobacilli are widely used as probiotics by including in various food products and food supplements (Sorokulova et al., 2008). For example, some strains of the species *Bacillus cereus* are known human pathogens due to their production of three enterotoxins and one emetic toxin (Stenfors Arnesen et al., 2008), whereas some species such as *B. clausii*, *B. coagulans, B. subtilis* and *B. licheniformis* have been well studied and used as biocontrol agents or probiotics due to their beneficial functions to plants, humans or animals (Cutting, 2011). According to extensive studies, it is well known that *Bacillus* bacteria interact with their hosts by multiple mechanisms, including stimulation of the immune system, suppression of pathogens and secretion of bioactive compounds (Mongkolthanaruk, 2012). In addition, symbiotic relationships between *Bacillus* species and various insects including honeybees (Gilliam, 1997), solitary bees (Gilliam et al., 1990a), stingless bees (Gilliam et al., 1990b), termites (Margulis et al., 1990), moths (Gilliam, 1985) and cockroaches (Feinberg et al., 1999) are well documented in previous studies and inhibition activity of *Bacillus* bacteria against the honeybee pathogens *Paenibacillus larvae* and *A. apis* are also reported (Sabaté et al., 2009).

European Foulbrood is an infectious disease of honeybee larvae that is caused by the gram-positive bacterium *M. plutonius* (Forsgren et al., 2005). Unlike AFB, in which only one causative agent is involved in the disease process, EFB is considered to associate with a number of secondary invaders (Forsgren, 2010). Several secondary bacteria have been indicated in the EFB disease complex. Bacteria *M. plutonius* extracted directly from naturally infected larvae are capable of causing clinical signs, while artificially cultured *M. plutonius* show virulence when grown in mixed culture with *Achromobacter eurydice* (Forsgren, 2010). Moreover, *in vitro* reared larvae only show EFB symptoms when both *M. plutonious* and *Paenibacillus alvei* are present (Budge et al., 2010). However, other studies showed that *Paenibacillus alvei* has no additive or synergistic effect with *M. plutonius* and

they also suggested that *M. plutonius* collected from different geographic European locations possess different virulence and vary in their ability to cause larval mortality (Charrière et al., 2011). In addition, although the bacteria *M. plutonius* were thought to be homologous and clonal until recently, one study revealed that at least two groups of strains (typical and atypical *M. plutonius*) that are phenotypically and genetically distinguishable exist in this species (Arai et al., 2012). Indeed, EFB has not yet been systematically studied and the pathogenic mechanisms of the disease are poorly understood, primarily due to difficulties in culturing *M. plutonius*, a facultative anaerobe, and establishing EFB under laboratory conditions (McKee et al. 2004). However, recent advances in technology are improving our incomplete understanding about EFB and its causative agent *M. plutonius.*

Similar to probiotic application for humans, members of the genus *Bifidobacterium*, *Lactobacillus* and *Bacillus* have attracted considerable attention for use as probiotic bacteria to protect honeybees from infectious diseases. Although the functions and mechanisms of honeybee gastrointestinal bacteria as probiotics remain obscure, their potentiality in maintaining host health and preventing disease are considerably prospective and increased understanding of the properties of these isolates as a probiotic bacterium is useful for developing honeybee alternative disease control strategies. Because probiotics possess strain specific antimicrobial activities (Servin, 2004), it is necessary to evaluate whether bacterial isolates obtained from the Japanese honeybee gastrointestine do actually display antimicrobial properties. Consequently, isolates of the Japanese honeybee digestive tract were investigated for their potentials in disease management by conducting *in vitro* and *in vivo* antagonistic assays against one important honeybee pathogenic organism *M. plutonius*.

3.2. Experimental reproduction of European Foulbrood with *Melissococcus plutonius*

3.2.1. Materials and methods

3.2.1.1. Virulence of *M. plutonius* strains in *in vitro* reared honeybee larvae

As difficulties in EFB establishment of reared larvae with experimental cultured *M. plutonius* (McKee et al. 2004) and different virulent capabilities of *M. plutonius* isolated in a previous study (Charrière et al., 2011), confirmation of EFB establishment in experimentally reared larvae with cultured *M. plutonius* strains used in this study become an important first step for further *in vivo* antagonistic activity assays of gut bacteria obtained from Japanese honeybees. Thereby, for investigating virulence of the bacterium *M. plutonius* on honeybee larvae under experimental conditions, infectious bioassays were performed by feeding larvae with artificial diet inoculated with cell suspensions of the artificially cultured bacterium *M. plutonius*. Larvae were collected from *A. mellifera* colonies maintained by the National Agriculture and Food Research Organization, National Institute of Livestock and Grassland Science, Honey Bee Research Unit in Tsukuba. The protocol employed for *in vitro* larval rearing followed the methods of Forsgren et al. (2010) with some modifications. A queen was confined in a queen cage for one day and the larvae in the cage were collected from the colony after the fourth day. The first instar larvae (< 24 h) were grafted from the queen cage and transferred to an artificial diet consisting of royal jelly (50%), water (37%), D-glucose (6%), D-fructose (6%) and yeast extract (1%) in a 24-well cell culture plate. The culture plates were kept in a plastic box and incubated at 35°C and a relative humidity of 90%. Thirty-five larvae were used in each group and larvae of the control group were fed with artificial diet only throughout the experimental period. Conversely, experimental groups were fed with artificial diet inoculated with cell suspensions of the bacterium *M. plutonius*

typical strain DAT606 (O.D=0.5) and atypical strain DAT 561 (O.D=0.5) for 24 hours before changing to normal artificial diet only. Larval mortality was then assessed every day under a stereomicroscope over a five-day period and survival rate of larvae were recorded. Dead larvae were distinguished by the absence of respiration, decreased body elasticity, and a change in body color from white to milky yellow. Log-rank tests were performed with software EZR (Easy R) for statistical analysis of survival rate of larvae. Similarly, typical strains DAT583 and DAT585, as well as atypical strains DAT351 and DAT573 were also examined by infectious bioassays. These typical and atypical strains of *M. plutonius* used in this study were isolated from diseased larvae with clinical signs of EFB from the Kanto, Chubu or Chugoku areas of Japan (Arai et al., 2012), and sub-cultured on KSBHI agar plates at anaerobic condition. KSBHI medium was brain heart infusion (BHI; Difco) based media supplemented with 0.15 M KH_2PO_4 plus 1% soluble starch.

3.2.1.2. Optimal lethal concentration of virulent *M. plutonius* strain

For obtaining optimal lethal concentrations of virulent *M. plutonius* strains, relationships between infection amount of the bacterium *M. plutonius* and larvae mortality were further analyzed by infecting larvae with different concentrations of *M. plutonius* atypical strain DAT351 with the methods described above. Experimental groups of larvae were fed with artificial diet inoculated with gradually diluted amounts of *M. plutonius* DAT351 ($1x10^7$ cfu/ml, $1x10^8$ cfu/ml, $1x10^9$ cfu/ml), respectively. After a one-hour infection, all groups of larvae were transferred to fresh artificial diet everyday. Larvae of control group were fed artificial diet only for the full period of experiments. Mortality of different groups was confirmed under a microscopy everyday and survival rate of larvae in all groups were recorded on day five after inoculation.

3.2.1.3. Inter-growth of virulent *M. plutonius* strain in larvae body

To understand the status of the bacterium *M. plutonius* virulent strain in larval guts after infection, inter-growth in the larvae body was detected by plating method that spread the larval intestinal contents on agar plates to count the *M. plutonius* bacteria colony formation units after incubation. Larvae were fed with artificial diet mixed with cell suspensions of *M. plutonius* DAT351 (ca.1x10^7cfu/ml) for one hour similar to the above experiment. Three larvae were collected into 1.5 ml tubes containing the KSBHI liquid medium after one hour infection and homogenized extracts were plated on KSBHI agar for culturing the *M. plutonius* in the larval body when remains of larvae were transferred to fresh diet and rearing continued under the same experimental conditions. This time point was designated as zero hours after infection. Similarly, three larvae from remains were also collected and the homogenized extractions were also cultured on KSBHI agar plates after twenty-four and seventy-two hours. The KSBHI agar plates were spread with larvae intestinal contents from three different time points were incubated forty-eight hours at 35°C on anaerobic conditions for counting colony formation units and the Log CFU/ml were recorded.

3.2.2 Results

3.2.2.1. EFB establishment

To determine whether artificially cultured *M. plutonius* strains isolated recently from diseased larvae in Japan can establish EFB under experimental conditions, infectious feeding assays were conducted and results revealed that *in vitro* reared larvae cause EFB clinical symptoms after infection with the *M. plutonius* atypical strain DAT561 but not with the *M. plutonius* typical strain DAT606 (Fig. 7A). All larvae fed with atypical strains of *M.*

plutonius stopped growing at day two or three and their respiration became slow. Similar to EFB cases in the field, dead larvae lost body elasticity and became yellowish and watery. Larvae in experimental groups infected with the typical strain of *M. plutonius* survived similar to larvae in control groups that were not infected with *M. plutonius* bacterium. Whereas, mortality of larvae in the group infected with the atypical strain of *M. plutonius* were significantly higher than in the control group (Fig. 7B) (Log-rank test, $P<0.05$). Similar results were also observed when other typical (DAT583 and DAT585) and atypical (DAT351 and DAT573) strains were used. Typical strains DAT583 and DAT585 have no virulence to larvae after feeding, conversely, atypical strains DAT351 and DAT573 showed virulence to larvae with high mortality in this experiment (Fig. 8) (Log-rank test, $P<0.05$). Same as strain DAT561, other atypical strains DAT351 and DAT573 also caused normal clinical symptoms of EFB in this assay although the mortalities varied.

3.2.2.2. Lethal concentration

To examine optimal lethal concentrations of *M. plutonius* strain DAT351, bioassays with infected larvae with different concentrations of bacterial cell suspensions were performed. The mortality of larvae in the infected group of atypical strain DAT351 was positively related to the concentrations of *M. plutonius* inoculated in the artificial diets (Fig. 9). The survival rate of larvae in the group infected with $1x10^7$ cfu/ml of *M. plutonius* DAT351 was 40%, whereas the survival rate of larvae in group $1x10^8$ cfu/ml and $1x10^9$ cfu/ml were gradually lower than the group $1x10^7$ cfu/ml with 31% and 18%, respectively. Despite the differences between larval susceptibilities may influence the mortality of larvae to some degree, concentration $1x10^7$ cfu/ml of *M. plutonius* DAT351 was optimal for further antagonistic activity investigations.

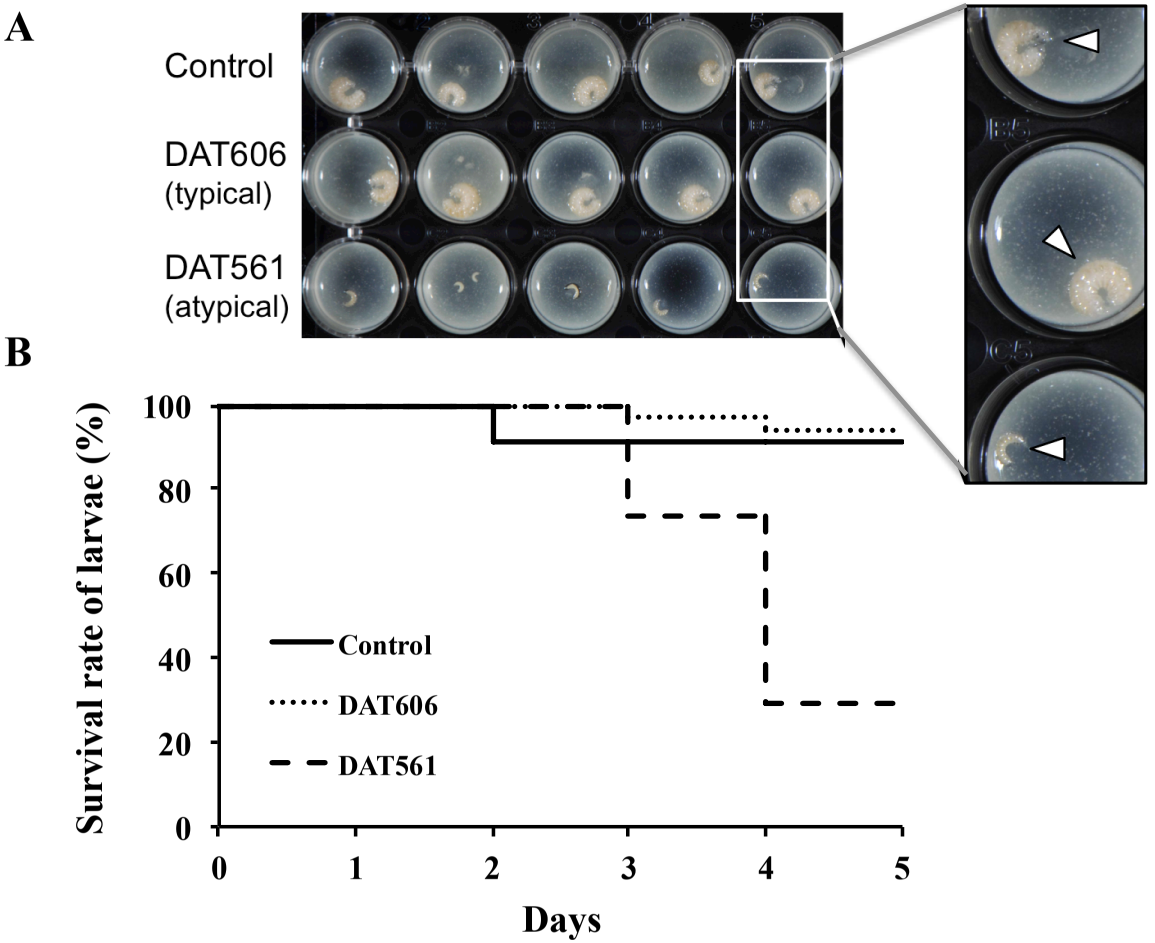

Fig. 7. Experimental reproduction of EFB by feeding honeybee larvae with typical and atypical strains of *M. plutonius*. A) Larvae at day 4. Larvae were transferred onto the surface of an excess amount of artificial diet in 24-well cell culture plates for taking photographs. Arrowheads indicate larvae. B) Survival rate of larvae in control, typical strain DAT606-inoculated and atypical strain DAT561-inoculated groups. Thirty-five larvae were used to calculate survival rates in each group, and Log-rank test were used for statistical analysis. Atypical strain DAT561 reproduced EFB in experimentally reared larvae and survival rate of larvae in group atypical strain DAT561 were significantly lower than in both control and typical strain DAT606 groups ($P<0.05$) .

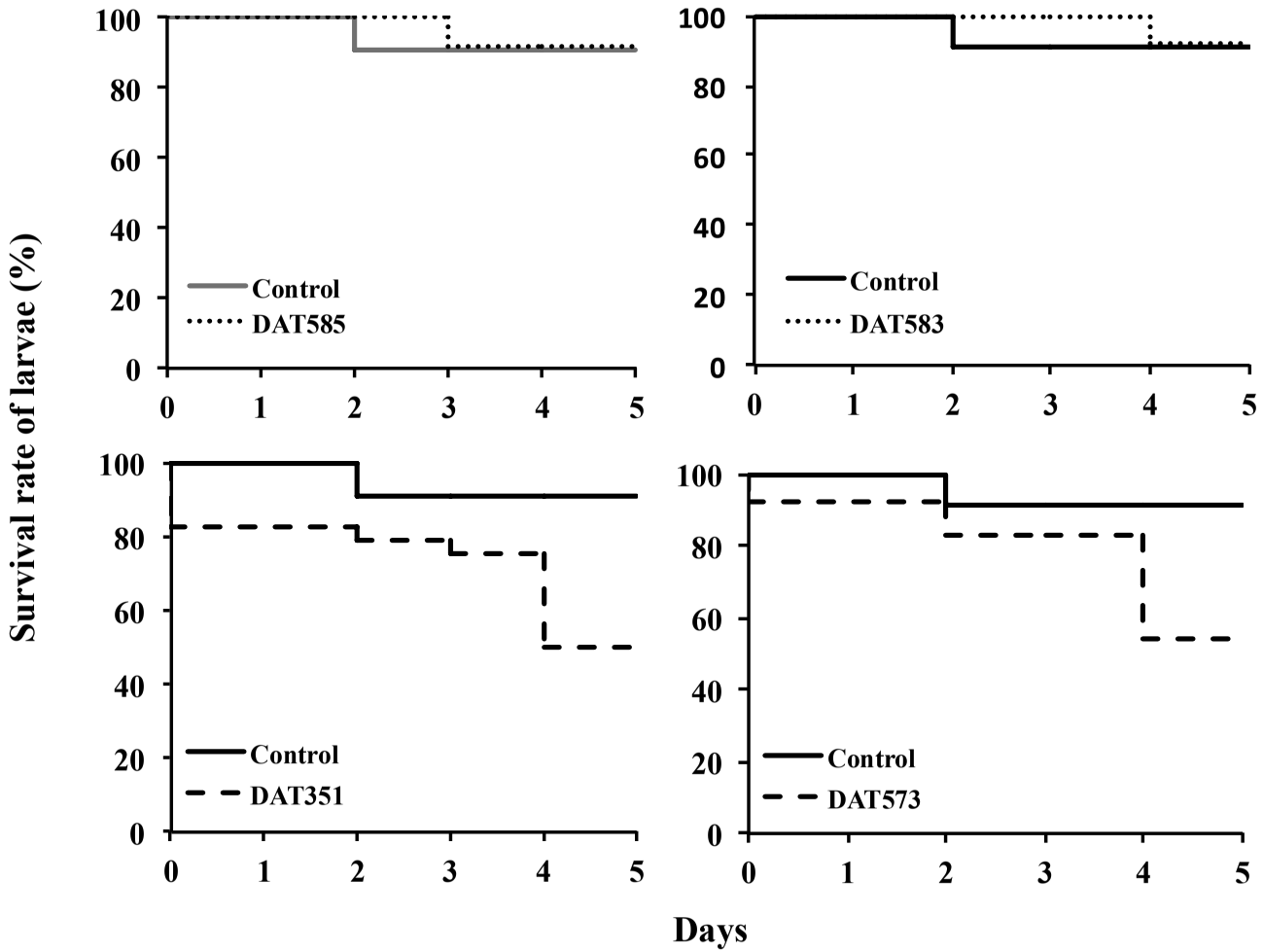

Fig. 8. Virulence confirmation of other *M. plutonius* typical and atypical strains. Survival rate of larvae in control, typical strains DAT583 or DAT585-inoculated, atypical strains DAT351 or DAT573-inoculated groups. Thirty-five larvae for control group and twenty-four larvae for inoculated groups were used to calculate survival rates, and Log-rank test was used for statistical analysis. Atypical strains DAT351 and DAT573 showed significant virulence to honeybee larvae under experimental conditions by increasing larval mortality ($P<0.05$) , whereas typical strains DAT583 and DAT585 maintained a higher survival rate of larvae similar to the control group.

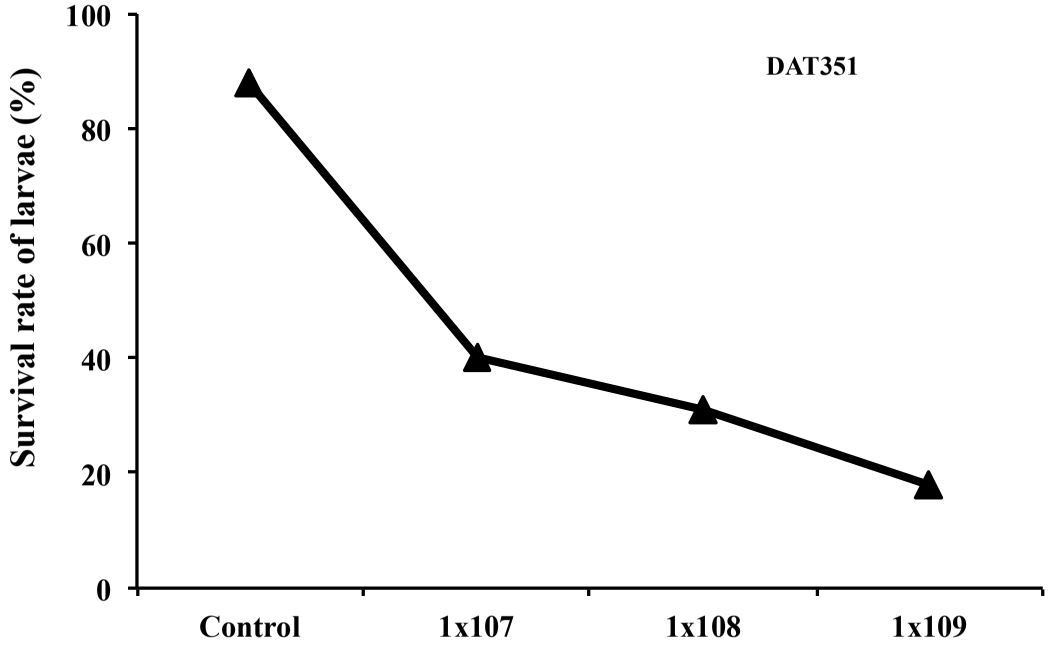

Fig. 9. Virulent ability of different concentrations of *M. plutonius* atypical strain DAT351 to honeybee larvae. Survival rate of larvae in control and 3 different concentrations of DAT351 (1×10^7CFU/ml; 1×10^8CFU/ml; 1×10^9CFU/ml)-inoculated groups at day five. Fifteen larvae were used to calculate the survival rate in each group. Higher concentrations of DAT351 bacterial cells increased mortality of larvae.

3.2.2.3. Inter-growth of *M. plutonius*

To understand the changes in the pathogen *M. plutonius* in larvae body after infection, the intestinal contents of infected larvae were examined at different times by plating method. As a result, the bacteria *M. plutonius* (atypical strain DAT351) mixed in the diets colonized and grew rapidly in the larvae digestive tract after ingestion. The concentrations of *M. plutonius* bacterial cells increased 2.5 times during seventy-two hours in the larval gut environment during this experiment (Fig. 10).

3.3. Antagonistic activities of gastrointestinal bacteria to *Melissococcus plutonius*

3.3.1 Materials and methods

3.3.1.1. *In vitro* antagonistic activity

Antimicrobial effects of the isolates obtained in this study on the growth of *M. plutonius* atypical strain DAT351 and DAT561 that cause EFB symptoms in infectious bioassays were evaluated by *in vitro* growth inhibition assays.

Bifidobacteria: All eleven bifidobacteria were cultured anaerobically on Wilkins-Chalgren liquid medium at 35°C for forty-eight hours. The cell-free supernatant (CFS) was recovered by centrifugation cultures at 4000 g for 4 min at 10°C and then filtered (pore size: 0.22 μm; Millex GS filters, Millipore, USA). The *M. plutonius* bacteria (atypical strains DAT351 and DAT561) were cultured on KSBHI agar plates at 35°C under anaerobic conditions using an AnearoPack System (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). *M. plutonius* bacterial suspension was prepared by suspending the colonies in saline water and the absorbance at 600 nm was adjusted to 0.01. Five microliters of the adjusted *M. plutonius* bacterial suspension was then added to 1 ml of KSBHI liquid medium, which was

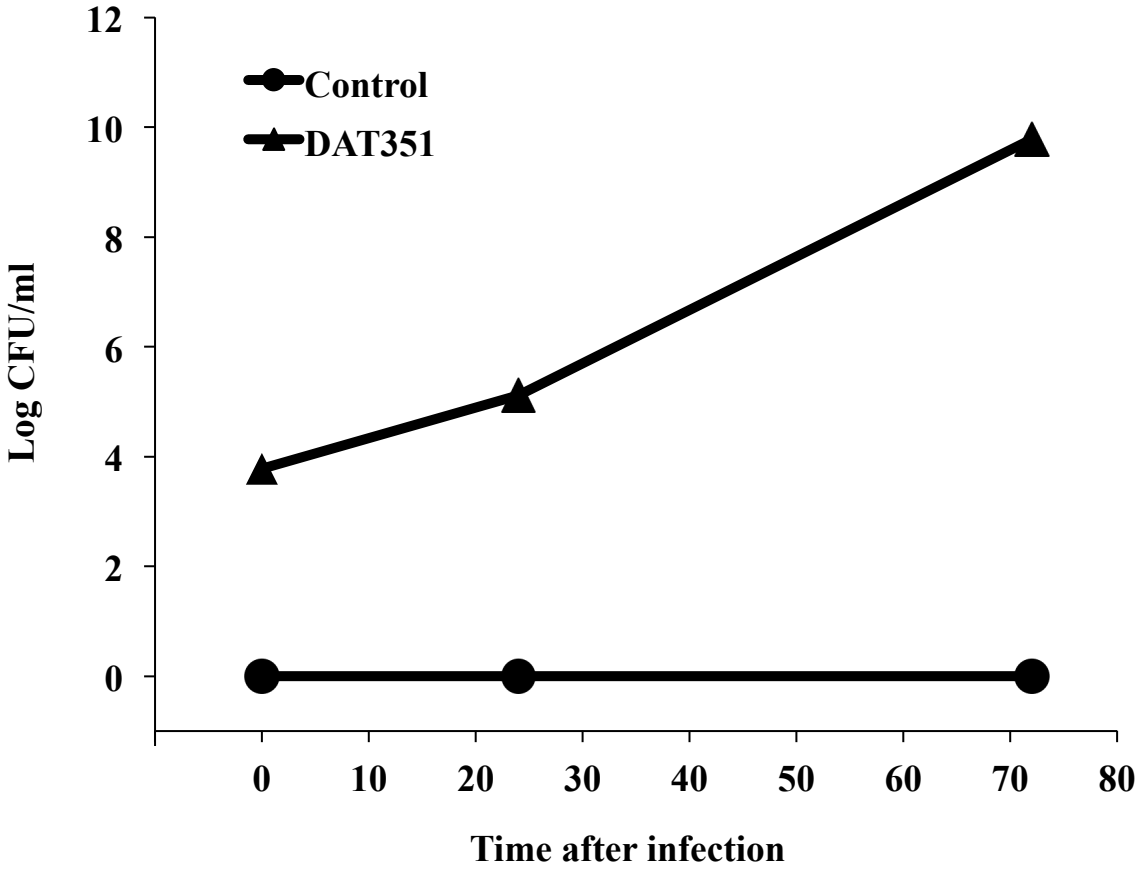

Fig. 10. The growth of *M. plutonius* atypical strain DAT351 in honeybee larvae gut. Three larvae/group were collected and homogenized at 0, 24 and 72 hours after feeding larvae for 1 hour with *M. plutonius* DAT351 for plating and calculating colony formation units of DAT351. Atypical strain DAT 351 grew rapidly in the environment of larval gut.

then mixed with 500 µl of each of the bifidobacterial CFS before incubation at 35°C for forty-eight hours under anaerobic conditions. The bacterial mixture added to Wilkins-Chalgren liquid medium was used as a control (i.e. no CFS was added). Inhibitory activity was confirmed by counting the colonies on the KSBHI agar plates. All experiments were performed in triplicates and the results expressed as log10 CFU/ml. The t-tests were performed for the statistical analyses of inhibitory activity.

Lactobacilli: All seventeen lactobacilli were cultured anaerobically in MRS liquid medium at 35°C for one week and the cell-free supernatant (CFS) was recovered by centrifugation of each culture at 4000 g for 4 min at 10°C and then filtered (pore size: 0.22 µm; Millex GS filters, Millipore, USA). The atypical *M. plutonius* strain DAT351 was cultured on KSBHI agar plates at 35°C under anaerobic conditions using an AnearoPack System as previously described. KSBHI liquid medium (900 µl) inoculated with 5 µl of DAT351 bacterial suspension in saline water (O.D=0.01) were mixed with 100 µl of each CFS of the lactobacillus bacteria before incubation at 35°C under anaerobic conditions. The DAT351 bacterial suspension mixed with 100 µl of MRS liquid medium was used as a control (i.e. no CFS was added). After forty-eight hours incubation, the absorbance at 600 nm of these DAT351 bacterial cultures was measured as inhibition activity. All experiments were performed in triplicates and the percentages of DAT351 growth compared to controls are presented as results. One-way ANOVA was used for the statistical analysis of inhibitory activity.

3.3.1.2. *In vivo* assay of gastrointestinal isolates

Lactobacilli: The affect of lactobacilli from adult guts in this study to larvae and royal jelly antibacterial activity to these isolates were evaluated by feeding first instar larvae under experimental conditions similar to those described above. Fifteen first instar larvae in

each group were fed with MRS liquid medium only for control and cell suspensions of AcjLac1 or AcjLac2 cultured in MRS liquid medium for experimental groups. After three hours, all groups of larvae were fed with fresh artificial diet for twenty-two hours. Three larvae from each group were collected when transferred to the fresh artificial diet and the same larval collections were performed for each group after twenty-four hours and seventy-two hours. Collected larvae were then homogenized in 100 µl MRS liquid medium individually for every assay and the extracts were cultured on MRS agar plates at 35°C under anaerobic conditions for recording the colony formation units after forty-eight hours. On the other hand, AcjLac9, AcjLac10, AcjLac11, AcjLac14 and AcjLac16 were also tested by similarly feeding larvae and photos of the MRS agar plates culturing the lactobacilli from three larvae guts after twenty-four hours and seventy-two hours feeding with fresh artificial diet were assayed as results.

3.3.1.3. *In vivo* antagonistic activity

To investigate whether the *in vitro* antagonisms of gastrointestinal isolates in this study can be sustained in larvae guts, *in vivo* antagonistic activities were analyzed by feeding bioassays.

Lactobacilli: Similarly, first instar larvae were collected and used in this experiment. To avoid negative influence from too multiple phylotypes of bacteria in larvae, all seventeen lactobacilli obtained in this study were divided into two groups and named as AcjLac mixture 1 (combinations of AcjLac1-AcjLac8) and AcjLac mixture 2 (combinations of AcjLac9-AcjLac18). Two experimental groups of larvae were pre-fed the bacterial suspension prepared from AcjLac mixture 1 or AcjLac mixture 2 cultures in MRS liquid medium (ca.$1x10^9$ cfu/ml). At the same time, the control and infectious control groups of larvae were fed MRS liquid medium only. After three hours, all of the groups, except the

control group, were infected with *M. plutonius* (DAT351; ca. $1x10^7$ cfu/ml) for one hour through the artificial diet. One hour after infection with *M. plutonius*, the control and infectious control groups were fed the artificial diet for all periods of the experiment. Conversely, the experimental groups were continuously fed the artificial diet mixed with the same combinations of AcjLac strains for an additional twenty hours before being provided with the artificial diet. Twenty-five larvae were used in each group and mortalities recorded for five days. Statistical significance of larval survival rate was determined through Log-rank tests.

3.3.1.4. Analysis of organic acid metabolites

Organic acids are important metabolites of lactic acid bacteria including bifidobacteria and lactobacilli and are suggested as one of the most important antibacterial mechanisms of probiotic lactic acid bacteria (Servin, 2004). To investigate such functional metabolites of bifidobacteria and lactobacilli obtained in this study, high performance liquid chromatography (HPLC) analysis was performed using cell free supernatants. Cell free supernatants (CFSs) of all eleven AcjBF strains were recovered from previously prepared cultures by centrifugation at 10°C, 4000 g for 4 min and filtering (pore size: 0.22 μm; Millex GS filters, Millipore, USA) after forty-eight hours incubation at 35°C under anaerobic conditions and Wilkins-Chalgren liquid medium used as control. On the other hand, cell free supernatants of *Lactobacillus* isolates were also recovered from each culture by the same methods described above. All seventeen AcjLac strains were sub-cultured in MRS liquid medium at 35°C under anaerobic conditions and then all cultures were centrifuged at 10°C, 4000 g for 4 min and then filtered (pore size: 0.22 μm; Millex GS filters, Millipore, USA) for obtaining the cell free supernatant after one week. MRS liquid medium was used for the controls. The filtered CFSs were injected into a LC-10AD (SHIMADZU, Kyoto, Japan)

HPLC system equipped with a Shim-Pack SCR-102 (H) column (SHIMADZU, Kyoto, Japan) at 45°C. p-toluensulfonic acid were used as mobile phase and lactic acid, formic acid, acetic acid, critic acid, malic acid, succinic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid were detected. Flow rate of samples was 0.8 ml/min. The results are shown as absolute values of organic acids concentrations in CFSs.

3.3.2. Results

3.3.2.1. *In vitro* antagonistic activity

Bifidobacteria: Although the results were not statistically significant, the CFS from all AcjBF isolates exhibited inhibitory activities against the *M. plutonius* strains DAT561 and DAT351. Slight differences in the inhibitory effects of the CFS on the pathogenic bacterial strains were observed with the CFS of isolate AcjBF10 exhibiting the strongest inhibitory activity. The antagonisms of bifidobacterial CFS to different strains of *M. plutonius* were also slightly variable (Fig. 11).

Lactobacilli: As shown in Fig. 12, all cell free supernatants from *Lactobacillus* isolates obtained in this study showed antagonistic activity against *M. plutonius* strain DAT351 in *in vitro* assay. The growth of DAT351 in controls that were not affected with CFS of lactobacilli were shown as 100, and compared to the controls, all CFSs significantly inhibited the growth of DAT351 by greater than 62% except one CFS from AcjLac3, which decreased the growth of DAT351 by 33% (Fig. 12) (One-way ANOVA, $P<0.05$). CFSs of lactobacilli showed variant inhibitions of *M. plutonius* strain DAT351 with the CFS of AcjLac17 exhibiting the strongest antagonism and the CFS of AcjLac3 showing the weakest inhibition.

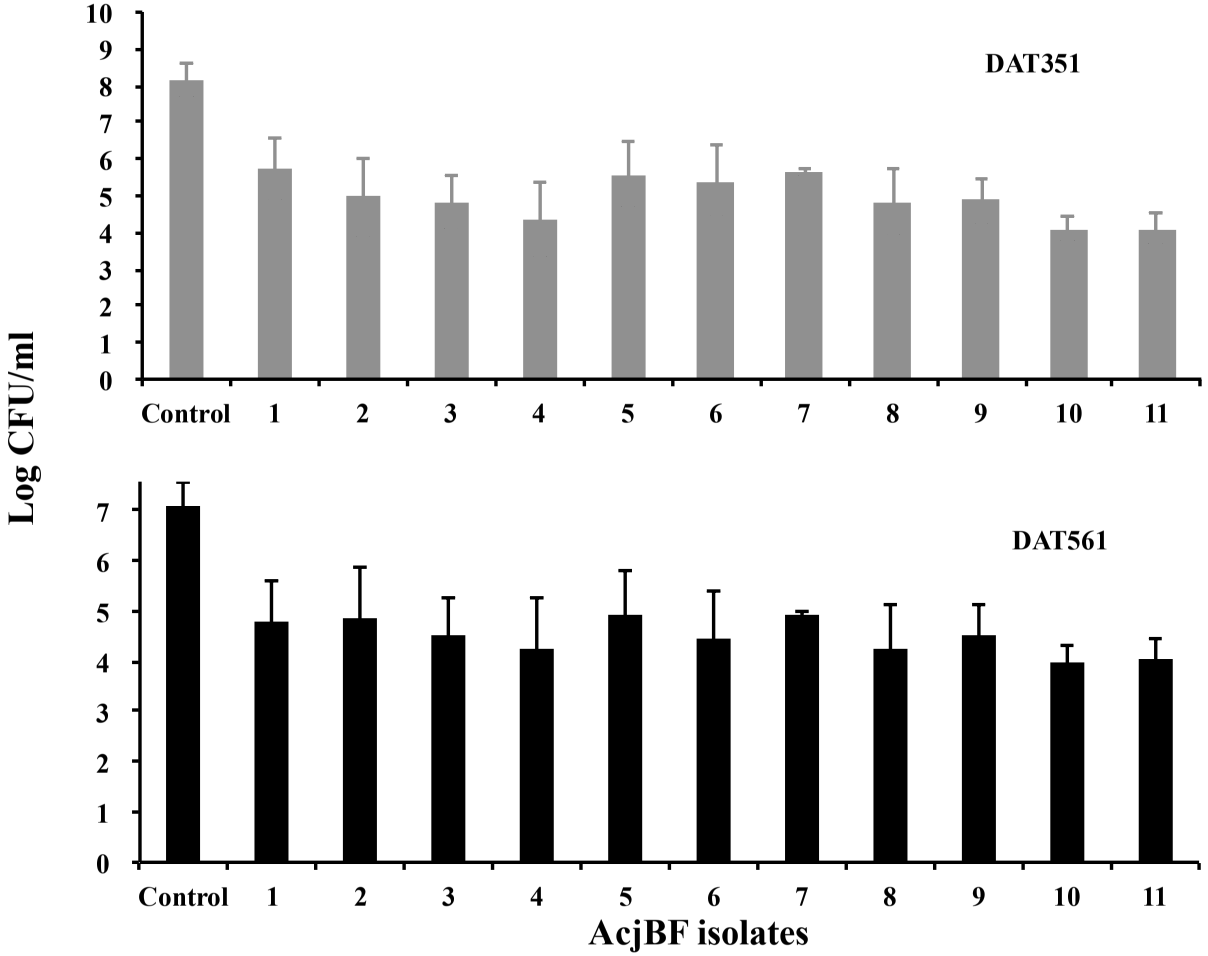

Fig. 11. The growth inhibitory effects of CFS produced by AcjBF isolates against *M. plutonius* atypical strain DAT351 (Gray) and DAT561(black). *M. plutonius* bacterial suspension was mixed with AcjBFs CFS and incubated at 35℃ for 48 h under anaerobic conditions. Bacterial mixture only with Wilkins–Chalgren liquid medium was used as control. All experiments were performed in triplicate and the results expressed as log10 CFU/ml. T-tests were used for statistical analysis. All AcjBF CFSs showed inhibition to both atypical strains of *M. plutonius* although not statistical significant.

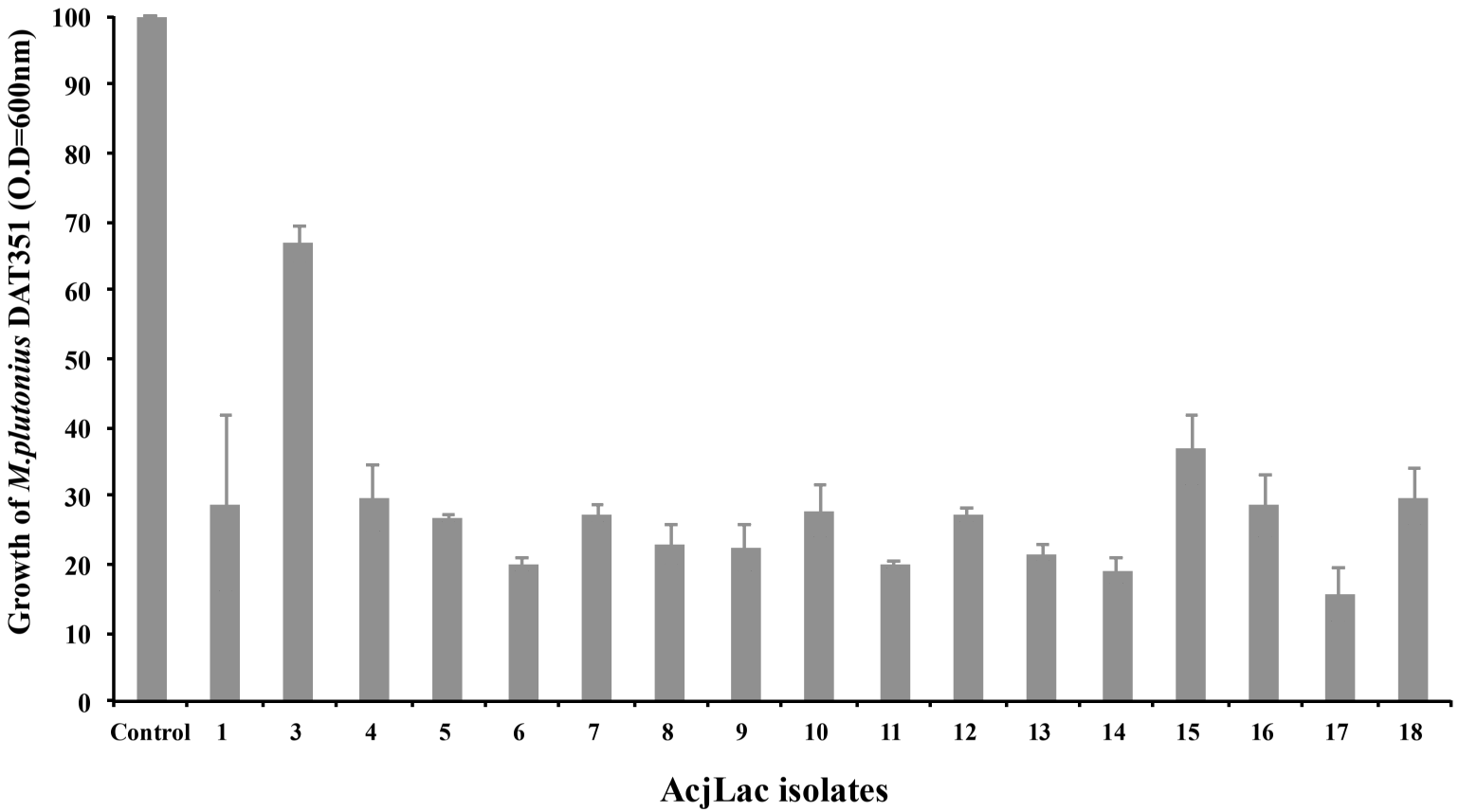

Fig. 12. The growth inhibitory effects of CFS produced by AcjLac isolates against *M. plutonius* atypical strain DAT351.
M. plutonius bacterial suspension was mixed with AcjLacs CFS and incubated at 35℃ for 48 h under anaerobic conditions. Bacterial mixture only with MRS liquid medium was used as control. All experiments were performed in triplicate and the inhibited percentage of the growth of *M. plutonius* (optical density absorbance at 600 nm) was expressed as results. One-way ANOVA was used for statistical analysis. All AcjLac CFSs showed significant inhibitoryactivity against the growth of DAT351 ($P<0.05$).

3.3.2.2. *In vivo* assay of gastrointestinal isolates

Lactobacilli: Effects of royal jelly antibacterial activity against isolates belonging to the genus *Lactobacillus* in this study were determined by *in vivo* feeding assay. The results showed that the amounts of *Lactobacillus* colonies isolated from larvae gut decreased after feeding, although the survival ability was different among AcjLac strains (Fig. 13, Fig. 14). For example, AcjLac1 decreased quickly in the gut of larvae by no colonies were detected from the larvae gut at 72 hours after feeding, whereas AcjLac2 decreased slowly and survived even after 72 hours. Results from *in vitro* co-incubation analysis of *Lactobacillus* isolates and artificial diets also revealed that all lactobacilli in this study were sensitive to royal jelly (data not shown). This demonstrates that the reason *Lactobacillus* isolates cannot survive in the gut of larvae for a long time may be due to the antibacterial activity of royal jelly in artificial diet.

3.3.2.3. *In vivo* antagonistic activity

Lactobacilli: *In vivo* antagonistic activity of *Lactobacillus* isolates against pathogenic bacteria *M. plutonius* strain DAT351 were evaluated by larvae infectious feeding assay. As shown in Fig. 15, larvae in the group treated with the combinations of AcjLac strains (AcjLac mixture 1) showed significant higher survival rate than larvae in the group just infected with DAT351 (Log-rank test, $P<0.05$). Despite Log-rank test, larvae in the group of AcjLac mixture 2 showed no statistically significantly higher survival rates in this experiment, these results revealed that *Lactobacillus* bacteria isolated from the Japanese honeybee gut in this study not only inhibit the growth of *M. plutonius* strain DAT351 in *in vitro* but also exhibit *in vivo* antagonistic activity in larval gut and improve the survival

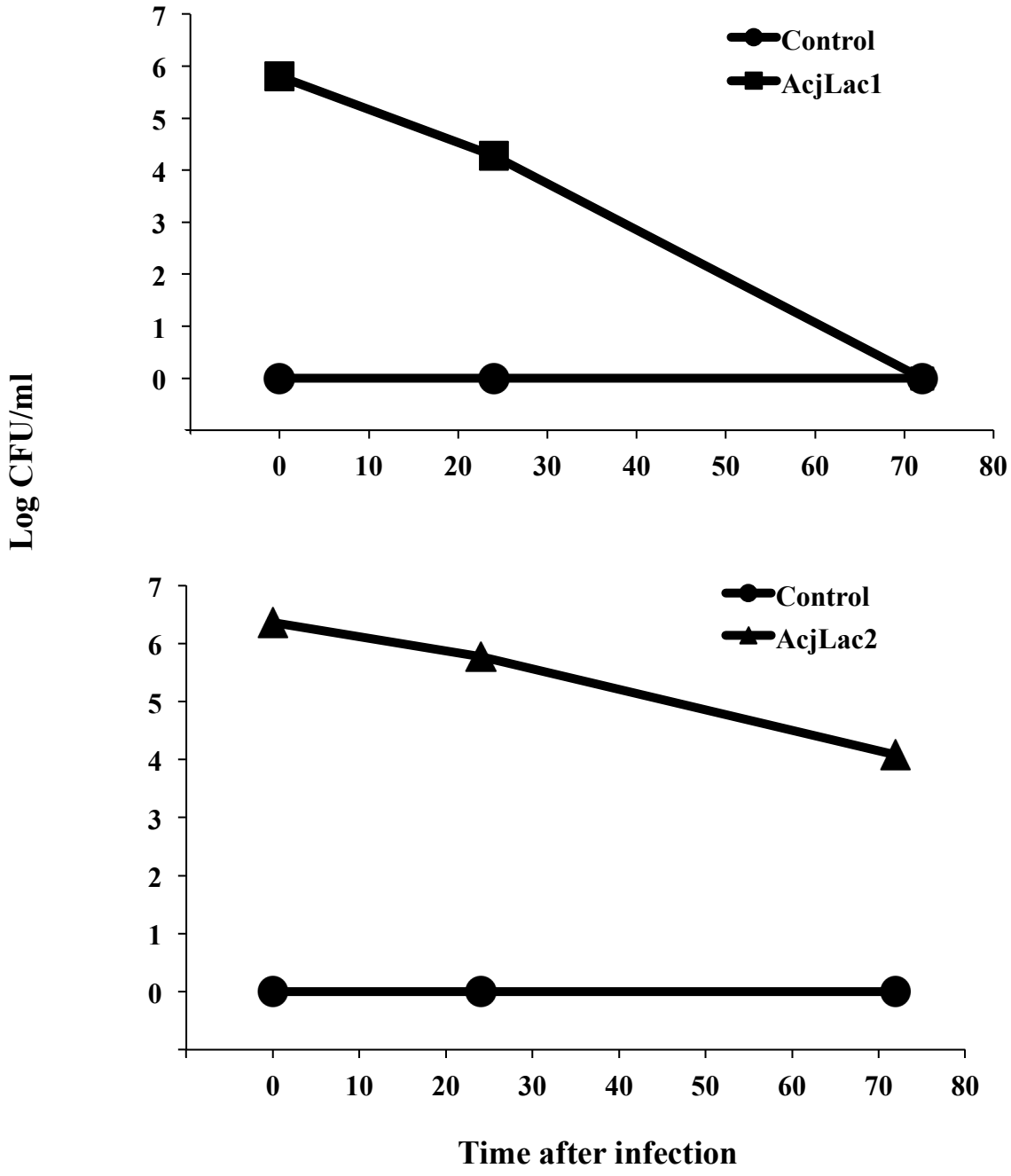

Fig. 13. The growth of isolate AcjLac1 and AcjLac2 in honeybee larvae gut. Three larvae were collected and homogenized at 0, 24 and 72 hours after stopping to fed with bacterial cell isolate AcjLac1 or AcjLac2 for plating and counting colony formation units. AcjLac1 and AcjLac2 isolates decreased at different rates in the environment of the larval gut.

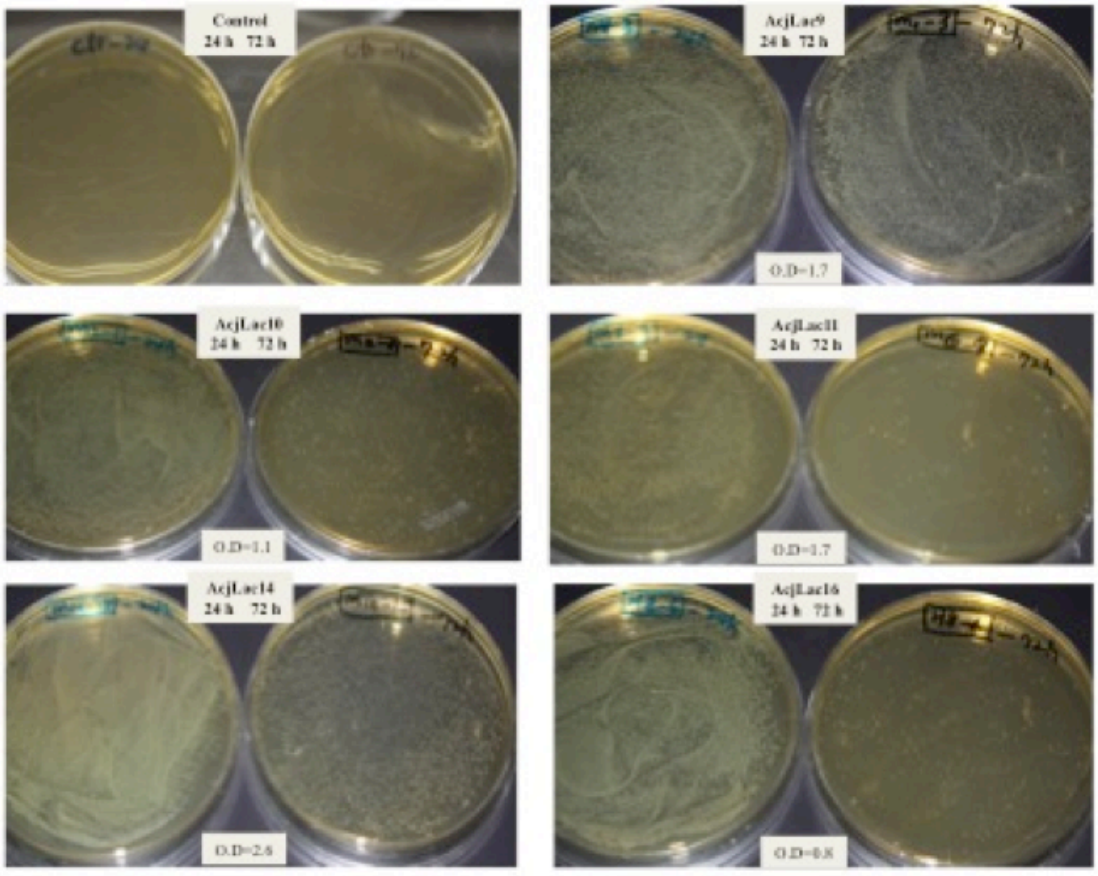

Fig. 14. The growth of isolates AcjLac9, AcjLac10, AcjLac11, AcjLac14 and AcjLac16 in honeybee larvae gut. Three larvae were collected and homogenized at 24 and 72 hours after stopping to fed with bacterial cell AcjLac isolates for plating. The agar plates grown colonies of AcjLac are expressed as results. The growth of all tested AcjLac isolates were inhibited in the larval gut environment.

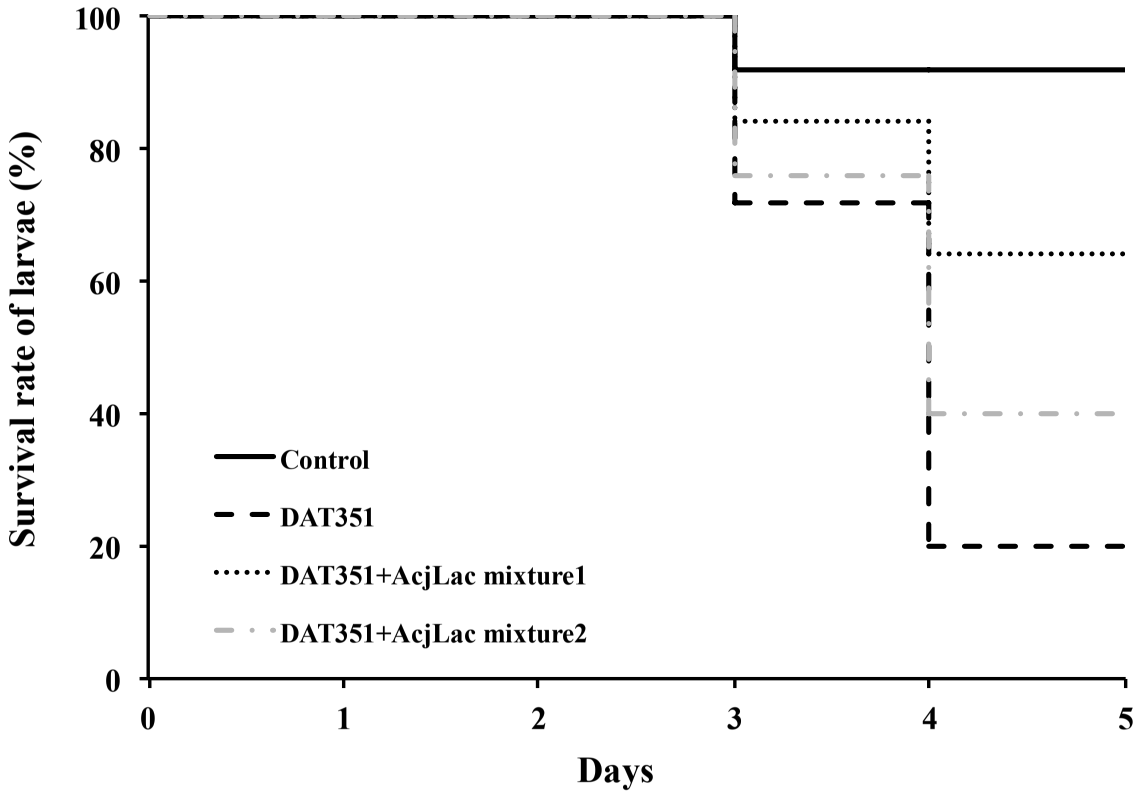

Fig. 15. *In vivo* antagonistic activity of AcjLac isolates to *M. plutonius* atypical strain DAT351. Survival rates of larvae in control, atypical strain DAT351-inoculated and atypical strain DAT351 plus mixture1 or mixture2 of AcjLac cell suspension-inoculated groups. Twenty-five larvae were used to calculate survival rate in each group and the Log-rank test was used for statistical analysis. Combinations of AcjLac isolates showed antagonistic activity *in vivo* by improving the survival of larvae in AcjLac-inoculated groups compared to the group inoculated only with DAT351. The survival rate of larvae in AcjLac mixture 1 group were significantly higher than in the DAT351 group ($P<0.05$), although group AcjLac mixture 2 did not show significant higher survival rate compared to group DAT351.

ability of infected larvae. The control groups of larvae maintained high survival rates throughout the experiments.

3.3.2.4. Organic acid production

Bifidobacteria: HPLC analysis was performed to investigate the functional organic acids produced by bifidobacterial isolates obtained in this study. All AcjBF isolates produced acetic acid, formic acid and lactic acid in cell free supernatants with no significant differences among the strains except AcjBF1 that produced higher concentrations of lactic acid and lower concentrations of formic acid than other strains (Fig. 16). This result revealed that bifidobacteria from the Japanese honeybee gut also similarly metabolize functional organic acids, especially acetic acid and formic acid, during growth.

Lactobacilli: Organic acids produced by each AcjLac strain were investigated using HPLC analysis and the results revealed that three kinds of organic acids: lactic acid, acetic acid as well as succinic acid were produced into the CFSs and organic acid production patterns were different among AcjLac strains (Fig. 17). All 17 AcjLac strains produced high concentrations of lactic acid during growth, especially strain AcjLac1. Moreover, all but one isolate, AcjLac3, also produced lower amounts of succinic acid, whereas production of acetic acid was not detected from strains AcjLac3, AcjLac17 and AcjLac18 (Fig. 17). These results demonstrate that Japanese honeybee intestinal lactobacilli also produce high concentrations of lactic acid and low concentrations of acetic or succinic acid during growth despite productive variability in organic acids among different isolates.

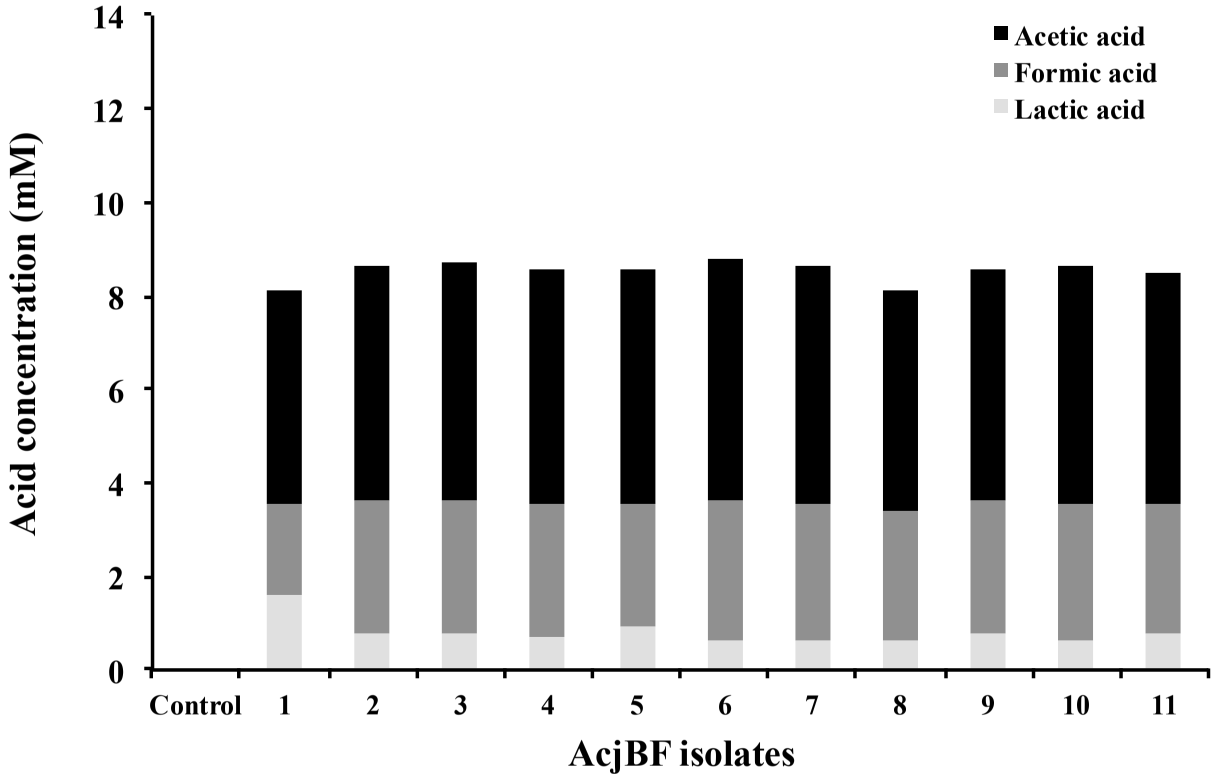

Fig. 16. The absolute concentrations of organic acids produced by AcjBF isolates. CFS obtained from cultures of AcjBF isolates incubated at 35℃ under anaerobic conditions for 48 hours were used for HPLC analysis. Wilkins–Chalgren liquid medium was used as control. All AcjBF isolates produced acetic acid, formic acid and lactic acid during growth.

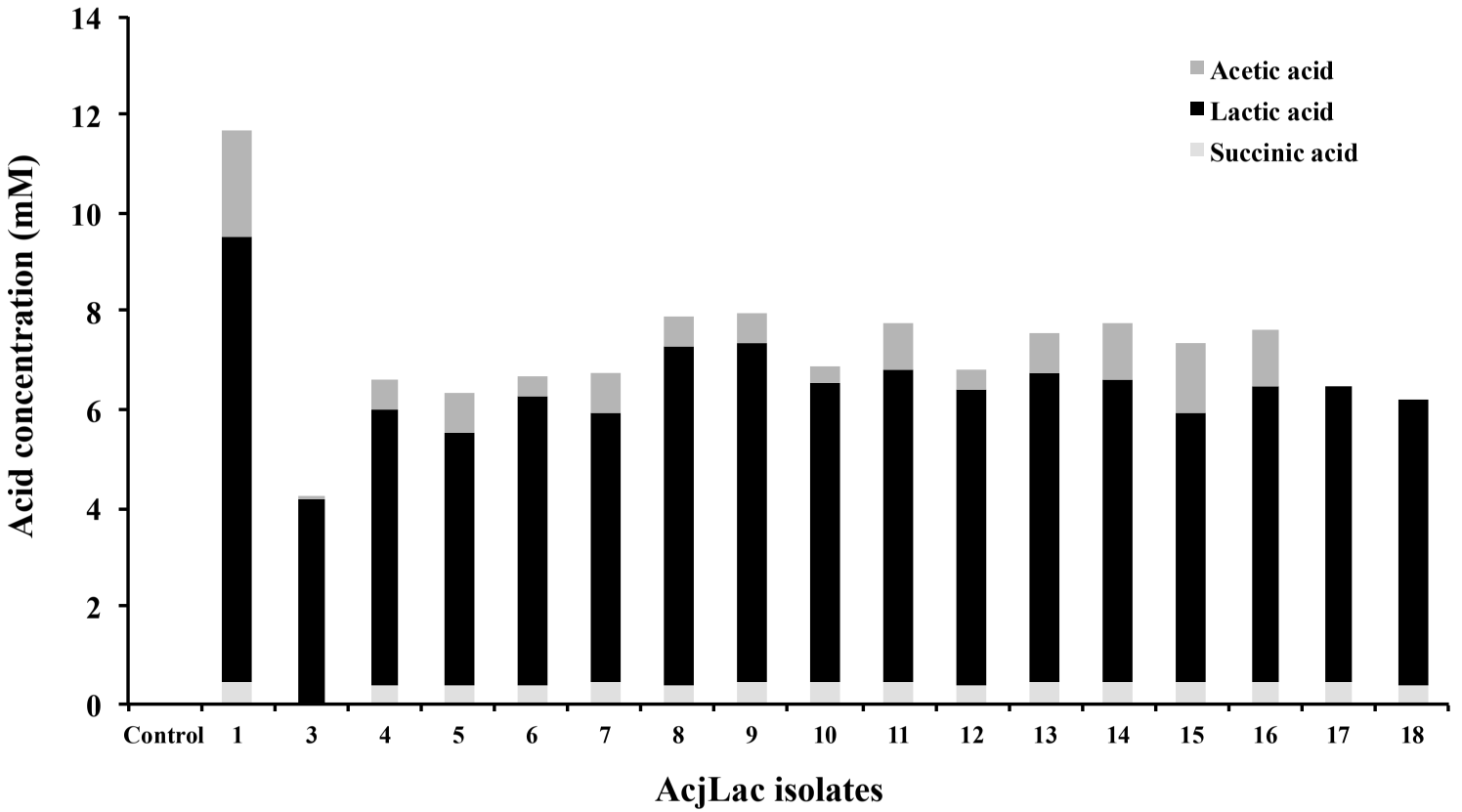

Fig. 17. The absolute concentrations of organic acids produced by AcjLac isolates. CFS obtained from cultures of AcjLac isolates incubated at 35℃ under anaerobic conditions for one week were used for HPLC analysis. MRS liquid medium was used as control. All AcjLac isolates produced high concentrations of lactic acid during growth, at the same time, some isolates also produced low concentrations of acetic acid or succinic acid.

3.4. Discussion

To investigate the potential of intestinal bacteria isolated from Japanese honeybee digestive tract for protecting honeybees from pathogens in this dissertation, one important honeybee pathogenic bacterium *M. plutonius* was used as an indicator organism. The bacterium *M. plutonius* is a gram-positive lanceolate coccus bacteria and the causal agent of EFB, a major infectious disease of honeybee larvae. Although the bacterium *M. plutonius* is well proven to be the causative agent of EFB (Bailey, 1983), experimental reproduction of EFB by *M. plutonius* is extremely difficult due to a decrease in virulence when they are cultured in artificial media (McKee et al., 2004). In accordance with previous results, cultured typical strains of *M. plutonius* did not cause larval EFB under experimental conditions in this study. However interestingly, cultured atypical strains of *M. plutonius* showed the ability to cause EFB in larvae reared *in vitro* and virulence was maintained even after repeated subculture in laboratory media (Fig. 7A and 7B; Fig. 8). This result revealed that the virulence of pathogenic bacterium *M. plutonius* is different between typical and atypical strains. In agreement with this result, different *M. plutonius* strains collected from different locations of Europe also varied in their ability to cause larval mortality (Charrière et al., 2011). Similarly, variable virulence to honeybee larvae among different strains of *A. apis*, which is causal agent of one other infectious disease Chalkbrood, were also confirmed by Vojvodic et al. (2011).

Dead larvae infected with atypical strains of *M. plutonius* showed typical clinical signs of EFB (Fig. 7A) demonstrating that atypical strains of *M. plutonius,* unlike typical strains, are causative agents of EFB in honeybee larvae and can maintain virulence under laboratory conditions. Coherent with previous research demonstrating that *M. plutonius* can sometimes be present in honeybee colonies without any symptoms of EFB (Forsgren et al., 2005), typical strains of *M. plutonius* in this study did not cause EFB symptoms in infectious

bioassays even though they can colonize the larval gut (Fig. 7; Fig. 8). Based on previous results and this study, EFB has at least two types of causal agents belonging to the same *M. plutonius* species. Some strains of *M. plutonius* (atypical strains) are able to cause larval EFB singly, whereas some strains of *M. plutonius* (typical strains) may require certain conditions such as secondary invaders to cause EFB. Earlier studies suggested that some secondary bacteria are associated with or necessary for *M. plutonius* to cause honeybee larval EFB (Bailey, 1956; Bailey and Ball, 1991).

Moreover, this study also confirmed that atypical strains of *M. plutonius* multiply rapidly in the larval gut environment under experimental conditions (Fig. 10) and virulent ability in larvae is positively related to the amounts of colonized bacteria in the larval gut (Fig. 9). These results are in accordance with previous studies indicating that digested bacterial cells of *M. plutonius* multiply vigorously within the gut of the larvae (Bailey and Ball, 1991) and the dose of *M. plutonius* is strongly correlated with larval mortality (Mckee et al., 2004). Bailey (1983) suggested that the pathogenic effect of *M. plutonius* infection may result from nutritional starvation because of competition between pathogenic bacteria and larvae. In this study, as well as the study by McKee et al. (2004), larvae subjected to excess diet during all experiments revealed that the pathogenesis of *M. plutonius* may not be only due to starvation. Conversely, typical strains of *M. plutonius* did not exhibit virulence to larvae despite growth in the gut environment were similar to atypical strains of *M. plutonius* (data not shown), suggesting strongly that other pathogenic mechanisms may attribute to the virulence of the bacterium *M. plutonius*. Although it is unknown how atypical strains of *M. plutonius* kill larvae, and the pathogenic mechanisms of EFB remain unclear, the results observed in this dissertation provide a important clues for elucidating the EFB pathogenesis and developing novel control technologies.

To improve novel EFB control strategies using honeybee gastrointestinal bacteria, the potential of Japanese honeybee gut bacteria were examined by *in vitro* and *in vivo* antagonistic activity against *M. plutonius* atypical strains (DAT351 or DAT561). In the present study, the *in vitro* growth inhibitory effects of CFSs from all lactic acid bacterial isolates AcjBF and AcjLac on *M. plutonius* revealed that all of the obtained bifidobacterial isolates and lactobacilli exhibited antagonistic activity against *M. plutonius* (Fig.11, Fig. 12). Bifidobacteria and lactobacilli are well known as lactic acid bacteria and their *in vitro* antimicrobial capabilities to broad spectrums of pathogens are intensively reported. Previous research indicates that multiple bacterial strains of the genera *Bifidobacterium* and *Lactobacillus* exhibit *in vitro* antagonistic activity against various human (Hütt et al., 2006) and animal such as fish (Balcázar et al., 2007), frog (Pasteris et al., 2011), dog (Fernández-Juri et al., 2011) as well as pig (Klose et al., 2010), associated pathogens. In addition, a recent study also reported the antibacterial properties of lactic acid bacteria to the honeybee pathogen *Paenibacillus larvae* (Forsgren et al., 2010).

Extensive studies indicate that multiple mechanisms are responsible for the antimicrobial effects of lactic acid bacteria including bifidobacteria and lactobacilli (Servin, 2004) and one of the proposed mechanisms is metabolic production of organic acids, H_2O_2 as well as antibacterial compounds like bacteriocins and low molecular weight peptides (Bermudez-Brito et al., 2012). Although further analyses are necessary to clarify the compound(s) responsible for inhibiting the growth of *M. plutonius*, organic acid dependent antimicrobial activity is a considerable candidate mechanism. To evaluate organic acid production of gut LAB obtained in this study, HPLC analysis was performed and the results showed that the concentrations of acetic acids, formic acid and lactic acids secreted from AcjBF bacterial cultures increased as the colonies grew (Fig. 16), and high concentrations of lactic acid were produced during the growth process of lactobacilli (AcjLac) (Fig. 17). In

addition, neutralization of CFS of AcjLac1 with alkali significantly decreased inhibition of growth in the pathogenic bacteria *M. plutonius* (data not shown). Although these studies could not confirm that compound(s) active in the antibacterial effects of these lactic acid bacterial strains, the organic acids produced by the lactic acid bacterial isolates are likely important candidates. Antagonistic functions of organic acids produced by probiotic lactobacilli have also been reported in a previous study (Fayol-Messaoudi et al., 2005). Recently several studies confirm that one of the main inhibitory mechanisms of probiotic LABs may result from production of organic acids and consequent lowering of culture pH (Tejero-Sariñena et al., 2012; Neal-McKinnery et al., 2012).

Moreover, combinations of some AcjLac strains also delayed the onset of larval mortality, although one group of larvae fed with mixture 2 (combination of AcjLac9-18) did not show a significantly higher survival rate than the infectious control group (Fig. 15). These results demonstrate that bifidobacteria and lactobacilli from the Japanese honeybee intestinal tract maintain antagonistic activity to the honeybee pathogen *M. plutonius in vivo*. The Genera *Bifidobacterium* and *Lactobacillus* are two main components of lactic acid bacteria and some strains have been extensively studied, particularly in so far as they can be used as probiotic agents (Ouwehand et al., 2002).

There is increasing evidence of successful prevention or cure of infectious diseases by application of lactic acid bacterial probiotics in humans and a variety of animals including insects. For example, lactic acid bacteria not only protect humans from a wide variety of pathogens including *Escherichia coli* (Medellin-Pena and Griffiths, 2009), *Clostridium difficile* (Segarra-Newnham, 2007), *Listeria monocytogenes* (de Waard et al., 2002) and *Staphylococcus aureus* (Bouchard et al., 2013), but also have been reported to improve the health status of honeybee colonies (Audisio and Benítez-Ahrendts, 2011), and can also protect honeybees from the pathogens *Paenibacillus larvae* (Forsgren et al., 2010)

and *M. plutonius* (Vásquez et al. 2012). Although the mechanisms of lactic acid bacterial *in vivo* antagonistic activities are unclear, lactic acid bacterial isolates obtained from the Japanese honeybee digestive tract may prevent honeybee larvae from EFB infection by administration as probiotics.

Examination of the effects of artificial diet on intestinal lactic acid bacteria revealed that the growth of all Japanese honeybee intestinal lactobacilli were inhibited in the larvae gut due to the antimicrobial activity of royal jelly in artificial diet, and the negative effect of royal jelly on the growth was strain specific (Fig. 13; Fig. 14). Such antibacterial effects of royal jelly are likely due to the presence of antimicrobial peptides and low pH (4.0). A previous study also demonstrated that royal jelly prevented the growth of lactic acid bacteria that had been provided as probiotics to feeding larvae (Forsgren et al. 2010). The inhibition activity of royal jelly on lactic acid bacteria may negatively affect survival ability of larvae by causing lose of their antagonistic activities during the long experimental periods post infection and the strain specific susceptibility to royal jelly may differ the antagonistic activity of lactobacilli strains in the larval gut and thus affect the survival rate of the AcjLac mixture 2 group (Fig. 15). Royal jelly negative influence on antagonism of lactic acid bacteria in experimental feeding assays may be depressed in nature, as the larvae of workers only fed on royal jelly for the first three days in natural honeybee colonies (Winston, 1991).

Members of the genus *Bacillus* inhabit a variety of environments including intestinal tracts of humans and animals as well as insects and play important roles for sustaining host health by inhibiting various pathogens. The beneficial associations between bacteria of the genus *Bacillus* and honeybees were also described in previous studies, *Bacillus subtilis* strains have been reported to inhibit the growth of two other major honeybee pathogens *P. larvae* (Evans and Armstrong 2005; Alippi and Reynaldi 2006) and *A. apis* (Sabaté et al. 2009). Porrini et al. (2010) also demonstrated anti-parasitic action of *B. subtilis* strains on *N.*

ceranae, the causal agent of *Nosema* disease in honeybees. In another study on the association between honeybees and *Bacillus* spp., the *Bacillus* strains negatively affected the health of the parasitic mite, *Varroa destructor* (Tsagou et al. 2004). Together with previous studies, the results in this dissertation indicate that different *Bacillus* strains possess extensive and distinct spectra of inhibitory effects on a variety of pathogenic bacteria.

Bacteria of the genus *Bacillus* were widely used as probiotics and previous studies have shown that administration of *B. subtilis* spores as probiotics can benefit the health of honeybees (Sabaté et al. 2012) and other animals such as pigs (Guo et al. 2006). Bacteria in the genus *Bacillus* are known to produce a wide variety of antimicrobial, antifungal, antiviral substances or metabolites. The production of various antimicrobial substances such as lipopeptides (Yakimov et al. 1995; Das et al. 2008), pumilacidin (Naruse et al. 1990) as well as mycosubtilin (Duitman et al. 1999) by *Bacillus* species has been well documented implying that the production of these substances is a well-established mechanism for inhibiting pathogens (Hong et al. 2005). Such antimicrobial metabolites of *Bacillus* strains have also been reported to inhibit a variety of honeybee pathogens, including *P. larvae, A. apis* (Sabaté et al. 2009) and *N. ceranae* (Porrini et al. 2010).

Honeybee larvae were protected from EFB infection when fed intestinal isolates obtained in this study, despite growth inhibition by royal jelly in the artificial diet, both *in vitro* and *in vivo* by antimicrobial properties (Fig. 13; Fig14). This demonstrates that these Japanese honeybee intestinal isolates can function as probiotics and multiple mechanisms may be active in enhancing survival of larvae infected with pathogenic bacteria *M. plutonius* (Fig. 15). Potential mechanisms by which probiotics confer enhanced resistance in hosts are not only production of antimicrobial substances, but also include enhancement of epithelial barriers, increases in adhesion to intestinal mucosa, inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms and modulation of the immune system

(Bermudez-Brito et al., 2012). Bouchard et al. (2013) revealed a strain specific ability of live *Lactobacillus casei* to reduce adhesion and internalization of *Staphylococcus aureus* in MAC-T cells. Other studies also suggestes that oral administration of lactic acid bacteria facilitate protection of mice from influenza viruses (Kobayashi et al., 2011) and reduce the Salmonella invasion and inflammation of chicks (Chen et al., 2012) by enhancing immune responses. Except for producing antimicrobial metabolites as discussed above, stimulating innate immune system is also expected as one important mechanism of action by honeybee intestinal isolates to increase their resistance against the pathogenic bacteria *M. plutonius*. Evans and Lopez (2004) also described that application of probiotics to honeybees can induce their immune responses. Although further intensive studies are needed to clarify mechanisms of antagonistic activity observed in this dissertation, Japanese honeybee intestinal isolates are capable of inhibiting the growth of *M. plutonius in vitro* and preventing larvae from EFB infection and enhance their survival, this implies that these isolates are potentially well suited for use as probiotics in apiculture for controlling diseases and maintaining honeybee health.

Chapter 4. General Discussion

Humans and animals including insects harbor a wide diversity of bacteria in their digestive tract and such gastrointestinal bacteria perform different functions for maintaining the host health such as contributing to nutrition, promotion of gut maturation and integrity, antagonism against pathogens and immune modulation (Tappenden and Deutsch, 2007). To date, there is increasing research attention on development of novel biological disease control strategies for preventing human and animals including insects. Intestinal bacteria have become important prospective candidates for such biological agents. For better understanding of the potentiality of honeybee gut bacteria in developing novel sustainable disease control strategies as biological control agents, gastrointestinal bacteria of the Japanese honeybee, which are more resistant to pathogens, were isolated by culture-dependent methods and subjected to characterization and antagonistic assays against one of the most important honeybee pathogenic bacterium *M. plutonius*.

The pollen or nectar foraging behavior and colony living properties of honeybees give rise to associations with a wide range of gastrointestinal microbes. Together with previous studies and this study, it can be expected that honeybees obtain the following two types of gastrointestinal bacteria by contact with workers of the colony or the environment. The first type of bacteria are resident bacteria that build up commensal or symbiotic relationships with honeybees during long terms of evolution due to vertical transmission between generations by worker contacts. For example, bacteria of the genera *Bacillus, Lactobacillus* and *Bifidobacterium* may be resident bacteria within honeybee gut bacterial communities. Symbiotic relationships between bacteria of the genus *Bacillus* and honeybees have been revealed and it suggested that bacteria of *Bacillus spp.* play important roles in preserving food stores in honeybee hives (Gilliam, 1979). Frequently the presence of

Bacillus bacteria in the gut of the European honeybee *A. mellifera* have been intensively reported (Rada et al., 1997; Kacániová et al., 2004; Evans and Armstrong, 2006) and bacteria belonging to the genus *Bacillus* have also been cultured from the gastrointestine of Japanese honeybee *A. cerena japonica* (Chapter 2) suggesting that *Apis-Bacillus* relationships may have co-evolved from ancient times.

Furthermore, honeybee gastrointestinal lactobacilli, well known as lactic acid bacteria (LAB), have been widely studied to confirm associations with honeybees and these studies have obtained similar phylotepes of *Lactobacillus* bacteria from all honeybees regardless of sampling from various honeybee species (Vásquez et al., 2012; Disayathanoowat et al., 2012), subspecies (Olofsson et al., 2011) and different geographical locations including Germany, Sweden, Switzerland, United States and Africa (Mohr and Tebbe, 2006; Olofsson and Vásquez, 2008; Babendreier et al., 2007; Vásquez et al., 2009; Jeyaprakash et al., 2003). Results described above suggest that bacteria of the genus *Lactobacillus* are resident bacteria of honeybee gut bacterial communities and originate from the honey stomach (Olofsson and Vásquez, 2008), indicating co-evolved symbiotic relationships between honeybees and the genus *Lactobacillus*.

Bacteria belonging to the genus *Bifidobacterium*, one other species of LAB, have also been identified intensively from honeybee digestive tracts together with bacteria of the genus *Lactobacillus*. Similar to the results of lactobacilli, phylotypes of bifidobacteria from different species (Vásquez et al., 2012; Disayathanoowat et al., 2012) and different locations (Olofsson and Vásquez, 2008; Vásquez et al., 2009; Ahn et al., 2012) were also closely related to each other, demonstrating that bacteria of the genus *Bifidobacterium* are also resident bacteria colonizing the honeybee gastrointestine with symbiotic associations. LAB, lactobacilli and bifidobacteria, were also isolated from the Japanese honeybees, subspecies

of *A. cerana* (Chapter 2), confirming the existence of long term symbiosis between *Apis* and LAB.

On the other hand, another type of gut bacteria are transient bacteria that are horizontally transmitted accidently within honeybee individuals or between individuals and environments but not inherited among generations. For example, bacteria of the genus *Pantoea, Stenotrophomonas* and *Streptomyces,* which were not detected in a previous study (Yoshiyama and Kimura, 2009) even from the same Japanese honeybee, were obtained for the first time from the digestive tract of honeybee in this study (Chapter 2), demonstrating that these gastrointestinal bacteria are transient bacteria that are accidently present in the gut of honeybees. It is possible that these bacteria were acquired from the environment by horizontal transmission when foraging because these genera of bacteria are well known to be associated with soil and plants (Deletoile et al., 2009; Hagemann et al., 2008; Song et al., 2004).

A recent non-culture dependent study suggested that a set of bacteria simpler than other species of insects inhabit honeybee digestive tracts (Martinson et al., 2011). Earlier culture dependent research indicates the diversity of gut bacteria due to differences in species, age, season and geographical location (Gilliam, 1997). Differences of culture and non-culture dependent methodologies may also contribute to such differences, culture dependent methodologies can only observe few of the bacteria in the gut when compared to non-culture molecular technology. Although more intensive investigations are required to improve our knowledge of the bacterial communities in the honeybee gut, it can be concluded based on previous and current studies that all honeybees possess resident bacteria such as the genera *Bacillus* and *Lactobacillus* as well as *Bifidobacterium* and also harbor some common phylotypes of these bacteria. Some distinct phylotypes of these genus bacteria also exist in the honeybee gut and the gastrointestinal bacteria are diverse and

dynamic not only due to differences in species or subspecies, but also dependent on different life stages, seasons, geographical locations even within the same species or subspecies.

In this dissertation, distinct phylotypes of LAB, which have not been isolated from the European honeybee, were observed from the Japanese honeybee gut (Chapter 2). In accordance with previous results from a study by Disayathanoowat et al. (2012), some phylotypes of LAB in *A. cerana* from Thailand were not detected from *A. mellifera* in Thailand. Together with current and previous studies, it can be suggested that in two species of honeybees, *A. meliffera* and *A. cerana*, except of sharing some closed phylotypes of LAB, the compositions of gastrointestinal LAB are distinct. Results from another previous study on symbiosis of honeybees and LAB also provides strong evidence supporting this suggestion, because some lactobacilli from *A. cerana* construct different clusters from that of *A. mellifera* in the phylogenetic tree, except the cluster of Lkun (*L. kunkeei)* and fhon13 (Vásquez et al., 2012).

One possible explanation for these differences may be genetic divergence of the two honeybees species, *A. mellifera* and *A. cerana*. Important influences of host genes on the structure of gut bacterial communities were also highlighted in studies on wasps (Brucker and Bordenstein, 2012) and termites (Hongoh et al., 2005). Japanese honeybees *A. cerana japonica* are normally wild bees, whereas European honeybees *A. mellifera* are commonly domesticated for breeding. Such different life styles of these two species of honeybees may also be an important contribution that greatly affects the bacterial communities colonizing their gastrointestinal tracts (Dillon and Dillon, 2004). In addition to the above reasons, some breeding and honey foraging activities of humans may affect the bacterial communities of *A. mellifera* worldwide, whereas the wild species *A. cerana* may harbor species-specific phylotypes because of a lower influence from human activities (Disayathanoowat et al., 2012).

The results described in this study also provide further evidence for gut bacterial diversity among individuals of the same subspecies, as the compositions of gut bacteria from same subspecies Japanese honeybee of *A. cerana* show significant differences (Table 7 of Chapter 2). Although the exact reasons for such differences among Japanese honeybee gut isolates observed in two similar studies are unclear, a thoughtful possibilities is seasonal or age differences during sampling. Structure of the gastrointestinal tract, conditions of pH, redox conditions, presence of digestive enzymes and the type of food ingested may also affect the diversity of insect gut microbiota (Dillon and Dillon, 2004). Honeybee gut environments vary with age, season, function of diet and other environmental conditions (Evans and Armstrong, 2005), and as a habitat niche for gastrointestinal bacteria, such changes in the gut environment may lead to individual, seasonal and locational diversity in honeybee gut microflora. Other studies indicate seasonal changes in enterobacterial dynamics within the gastrointestinal tract of *A. mellifera* (Lyapunov et al., 2008) and that variability of gut bacterial communities depends on life stages of the honeybee (Moran et al., 2012).

Differences in gut bacteria even at strain level may lead to functional diversity (Engle et al., 2012), variability in gut bacterial components among different *Apis* species discussed above may result in variable contributions to honeybee health. To evaluate the beneficial effects of isolates obtained from Japanese honeybee in this study, antagonistic assays were conducted using the pathogenic bacterium *M. plutonius*. Important infectious larval disease, EFB, has not been systematically studied until recently, because of difficulties in establishing EFB with the bacterium *M. plutonius* due to a decrease in virulence during experimental culturing (McKee et al. 2004) or due to absence of secondary invaders (Bailey and Ball, 1991). However, single artificially cultured *M. plutonius* atypical strains isolated from EFB infected larvae of Japan recently resulted in symptoms of EFB and

high mortality of *in vitro* raised larvae (Chapter 3). This provides a possibility for further investigations on antibacterial activities against the pathogenic bacterium *M. plutonius* and EFB resistant effects of the Japanese honeybee gut isolates by *in vitro* analysis and *in vivo* bioassays using atypical strains of *M. plutonius.*

In this study, all isolates of bifidobacteria and lactobacilli showed *in vitro* inhibition of *M. plutonius* (Chapter 3). Antibacterial properties of LAB, including bifidobacteria and lactobacilli inhabiting different environments, against various pathogens are indicated in many previous studies (Neal-Mckinney et al., 2012; Fayol-Messaoudi et al., 2005; Smaoui et al., 2010; Kim et al., 2007). Morover, LAB isolated from gastrointestinal tract of honeybee *A. mellifera* were recently reported to exhibit antimicrobial activities against honeybee pathogenic agents such as *P. larvae* (Forsgren et al., 2010) and *M. plutonius* (Vásquez et al., 2012). Similarly, bacteria of *Bacillus* have also been examined intensively for their antimicrobial effects against a wide range of pathogens (Risøen et al., 2004), including the honeybee pathogens *P. larvae* (Evans and Armstrong 2005; Alippi and Reynaldi 2006) and *A. apis* (Sabaté et al. 2009). Results in this dissertation provide further evidence of the *in vitro* antibacterial effects of LAB isolated from the Japanese honeybee gut to the honeybee pathogen *M. plutonius*.

The results of *in vivo* bioassays of this study revealed that isolates possessing *in vitro* inhibitory activities against *M. plutonius* also showed *in vivo* antagonistic activity by improving survival of larvae infected with EFB causal agent *M. plutonius* (Chapter 3). This implies that these isolates from the Japanese honeybee digestive tract may be used as biological control agents to prevent honeybees from EFB, but further intensive studies are required. Bacteria of the genera *Bifidobacterium, Lactobacillus* and *Bacillus* are considered as beneficial microorganisms and administered broadly as probiotics, one important biological control agent for preventing humans and various animals from various diseases

(Nikoskelainen et al., 2001; Ouwehand et al., 2002; Sorokulova et al., 2008; Cutting, 2011). Based on the extended knowledge on benefits of probiotics to hosts, protecting insects with application of probiotics is also recently expected. Honeybee researchers also hope to protect honeybees by developing honeybee probiotics. Several studies report the beneficial activities of probiotics or their metabolites on honeybee health with accelerating death of honeybee mites (Tsagou et al., 2004), reduction in microsporidian *N. ceranae* (Porrini et al., 2010), influences on intestinal microflora (Pătruică and Mot, 2012) and improvements in colony performance (Sabaté et al., 2012). In addition, some studies also indicate the potentiality of probiotic effects of honeybee gastrointestinal bacteria (Sabaté et al., 2009; Forsgren et al., 2010; Vásquez et al., 2012). Although the antagonistic effects of isolates obtained from the Japanese honeybee gut against other pathogenic bacterium in this study need to be confirmed with further studies, combined with the above previous studies, results in this dissertation indicate that honeybee gastrointestinal bacteria are a good source for developing safe biological control agents to prevent honeybees from diseases.

It is unclear how Japanese honeybee gut isolates inhibit the pathogenic bacteria *M. plutonius in vitro* and what mechanisms improve the larval resistance to EFB infection, but the results of antagonistic activity of CFSs proposes that one most presumable mechanism of *in vitro* inhibitory may be antimicrobial compounds such as organic acids and their metabolites. In addition, given intestinal isolates can not survive for a long time in larvae gut after feeding, enhanced survival of larvae infected with *M. plutonius* is observed when such intestinal isolates are applied as probiotics implying that other multiple mechanisms, such as enhancement of immune responses, may function in *in vivo* resistance. Similar immune responses induced by probiotics have also been reported previously on honeybees (Evans and Lopez, 2004). Mechanisms of probiotic actions have not been elucidated fully but are considered as multifactorial. It is considered that major mechanisms, in addition to

producing antimicrobial substances such as H_2O_2, organic acids, bacteriocins and strain-specific metabolites (Servin, 2004), include enhancement of epithelial barrier integrity, increased adhesion to intestinal mucosa, inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms and modulation of immune system (Bermudez-Brito et al., 2012). The mechanisms of the Japanese honeybee gut bacterial *in vitro* and *in vivo* antagonistic activity against *M. plutonius* should be studied further for improving the application of probiotics.

It is considered that honeybee larvae are usually free of internal microorganisms. Food acquirement from nurse bees is one important route to expose larvae to various microbes including the pathogenic bacteria *M. plutonius*. Therefore, excluding pathogen *M. plutonius* from adults and honeybee hives by applying probiotics to adults to cut off the infection route is one essential method for reducing EFB infection of larvae. In addition, administration of these intestinal isolate as probiotics to adult workers in honeybee hives may enhance their antagonistic activity against pathogen *M. plutonius* in the adult gut. Antibacterial compounds, such as royalisin, of royal jelly (Fujiwara et al., 1990) or higher pH conditions of larval gut environment (pH>7) (Yoshiyama et al., 2010) may attribute to their royal jelly susceptibility and incapability of colonization in the larvae gut. The explanations above indicate, such negative affects from royal jelly and a high pH gastrointestinal environment to antagonism of gut isolates against *M. plutonius* should be avoided when feeding adult bees.

The bacterium *M. plutonius* is thought to be homologous and a clonal bacteria, but two phenotypically and genetically different strains (typical and atypical strains) were reported recently (Arai et al., 2012). As described in Chapter 3, atypical strains of *M. plutonius* caused larval EFB infection. On the other hand, *M. plutonius* typical strains used in this study lost virulent ability similar to *M. plutonius* strain used in the previous study

(McKee et al., 2004) when artificially cultured. Such typical strains of *M. plutonius* may exhibit virulence to larvae only when influenced by secondary invaders. Given the mechanisms of pathogenesis of atypical strains are unknown, we can predict the presence of distinct virulence mechanism in different types of *M. plutonius* strains. Recent determination of complete genome sequence of two representative strains of *M. plutonius* (Okumura et al., 2011) may provide understanding of the phenotypic and genetic diversity of *M. plutonius* typical and atypical strains. This may help with studies on the differences of pathogenic mechanisms of *M. plutonius* in honeybee larvae and explain the pathogenesis of EFB. Elucidation of EFB pathogenesis then may contribute to better understanding of the functional mechanisms of probiotic isolates reported in this dissertation.

Conclusions

In conclusion, the Japanese honeybee harbors diverse bacteria in its digestive tract and some bacteria are transient due to horizontal transmission among individuals of a hive or between honeybees and the environment. However, some bacteria, such as lactic acid bacteria and bacilli, are resident because of vertical transmission between honeybee generations. In addition, Japanese honeybees, a subspecies of the Asian honeybee, harbor distinct phylotypes of gastrointestinal bacteria, including some *Apis* species-specific bacteria shared with the European honeybee. This suggests that the structure of honeybee gut bacterial communities is different depending on species, subspecies, season, age and location. Such diverse strains of Japanese honeybee gastrointestinal bacteria are antagonistic to pathogenic bacterium *M. plutonius*, and application of these intestinal bacteria as probiotics can also enhance larval resistance to EFB infection. To our knowledge, this is the first report to demonstrate an antagonistic effect of gut bacteria isolated from the Japanese honeybee on *M. plutonius,* the causative agent of EFB. Together with previous studies, I conclude that intestinal bacteria of honeybee play important roles in maintaining honeybee health by defending against pathogens including *M. plutonius* and may potentially be applied as biological agent to control EFB. Although further studies are required to confirm whether this antagonistic activity can be observed in individuals and colonies in the natural environment, this dissertation reports a possible means by which novel disease management techniques can be developed in apiculture.

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