J. Plant Prot. and Path., Mansoura Univ., Vol.6 (1): 203- 217, 2015

INHIBITION EFFECT OF SOME NEW TYPES OF LACTIC ACID BACTERIA (EXTRACTED FOR GUT OF THE HONEYBEE *Apis mellifera*) AGINST *Paeni bacillus larvae larvae*

Shireen A. M. Ma'moun[1]; M. I. Imam[1], E. A. Nafea[2]; Rabia A. Anan[1]; M. S. Salama[1]; Akila M. El Shafai[1] and A. H. Kaschef[1]

[1]Department of Entomology, Faculty of Science, Ain Shams University, Cairo, Egypt.

[2]Honeybees' Research Center, Plant Protection Institute, Cairo, Egypt.

ABSTRACT

The probiotic lactic acid bacterium (LAB) was reported in the gut of the honeybee *Apis mellifera*. Partial 16S rRNA gene sequences of the bacterial flora found in the gut of the honeybee workers, revealed the presence of seven novel strain of LAB. Five of the anaerobic LABs are closely related to five different strains of the lactic acid bacteria *Lactobacillus kunkeei* species, one is closely related to a strain of *Lactobacillus plantarum* species and the last one is identical to a strain of species *Fructobacillus fructosus.* We used inhibition assays on agar plates to evaluate the antagonistic effects of the newly identified honeybee LAB on *Paenibacillus larvae larvae* (*P. l. larvae*), American foulbrood (AFB) bacterial pathogen, growth *in vitro*. The individual LAB phylotypes showed different inhibition properties against ***P. l. larvae*** growth on agar plates, whereas a combination of all seven LAB phylotypes resulted in a total inhibition (no visible growth) of ***P.l.larvae***. The results demonstrate that honeybee specific LAB possess beneficial properties for honeybee health. Enhancing growth of LAB or applying LAB to honeybee colonies should be further investigated.

American foulbrood / *Paenibacillus larvae* / Lactic Acid Bacteria / *Lactobacillus* / inhibition

INTRODUCTION

The honeybees *Apis mellifera* is an extremely beneficial insect due to its role in pollination and for its products (honey, wax, propolis, pollen, royal jelly and venom). Hence, apicultural economic development strongly relies on the health status of honeybee colonies. Honeybees face many diseases and consequently rely on a diverse set of individual and group-level defenses to prevent disease. One route by which honeybees and other insects might combat disease is through the shielding effects of their microbial symbiotic.

Bees are constantly under threat due to combined damage from bacteria, parasites, viruses, pesticides, insecticides, and artificial bee food (Cox-Foster *et al.*, 2007, Stokstad, 2007, Aliouane*et al.*, 2008, Higes*et al*., 2008).

The most common bacterial disease which is lethal at the honeybees' larval stages, is the American foulbrood (AFB) disease. It is caused by an endospore-forming, Gram-positive rod-shaped bacterium, *P. l. larvae* that infects young larvae through ingestion of contaminated food (Shimanuki, 1997).

AFB is the most virulent brood disease known in honeybees (*Apis mellifera* L.). It is one of the few bee diseases capable of killing a colony and possess unique problems for prevention and control because the bacterial spores can remain viable for long periods of time (35 years or more) and survive adverse conditions (Matheson and Reid, 1992).

The traditional methods of AFB disease control through killing and burning of affected bee colonies were in use until several years ago and the prophylactic treatments of the other bee samples with antibiotics and sulfonamides were a real hazard with regard to the accumulation of drug residues in honey bee products. That is why, since 2003, the use of antibiotics and sulfonamides in most European apiculture is prohibited by the law (Law on Apiculture, 2003) (Parvanov *et al.*, 2006).

Bacteria belonging to lactic acid bacteria (LAB) are functionally related by their ability to produce lactic acid during their fermentative metabolism (Klaenhammer *et al.*, 2002). A novel flora of LAB composed of *Lactobacillus* and *Bifidobacterium* has recently been identified in the honey stomach of honeybees, *A. mellifera* (Olofsson and Vásquez, 2008; Vásquez *et al*., 2009). Furthermore, the findings revealed that honeybees and the novel LAB flora evolved in mutual dependence of one another; the LAB obtaining a niche in which nutrients are available, the honeybees and the honey in turn being protected by the LAB from harmful microorganisms (Olofsson and Vásquez, 2008). LABs are known to be good producers of antimicrobial substances such as organic acids, hydrogen peroxide and antimicrobial peptides (de Vuyst and Vandamme, 1994).

Forsgren *et al.* (2010) suggested that the newly identified LAB genera *Lactobacillus* and *Bifidobacterium*, exhibit strong inhibitory effects on *P.l. larvae* growth, and can to some extent prevent symptom development of AFB in honeybee larvae infected by *P.l. larvae*.

It will be very useful to find a natural, safe and cheap method for AFB control in Egypt in its early stages. The purpose of the present study was to assess the prevalence of the probiotic bacterium *Lactobacillus* in the honeybee's gut and evaluate the newly identified lactic acid bacteria as a control agent of *P. l.larvae larvae* and its effect on American foulbrood disease.

MATERIALS AND METHODS

Bees were obtained in an apiary yard of the Apiculture Research Department, Plant Protection Research Institute, Agricultural Research Center, Dokki, Egypt, during the summer 2011.

Lactic acid bacteria isolation procedure

Worker honeybees were obtained in an apiary from colonies maintained using standard beekeeping practices. Collected worker bees were freshly manipulated. Whole guts were dissected out by separating the abdomen from the thorax, cutting open the abdomen with a scissor along both sides, removing the ventral cuticle and transferring the gut to a sterile 1.5 ml Eppendorf tube containing 0.9 ml physiologic saline (0.9% NaCl,

0.1% Tween 80, and 0.1% peptone). All instruments used in the dissection process were flame-sterilized between each individual.

Each tube was shaken vigorously and cultured immediately on MRS agar (oxoid). The isolates were cultivated both aerobically and anaerobically (using a gas pack system and a gas pack kit, oxoid) at 37 °C for 2 to 3 days. White, small, round colonies were picked randomly from the media involved and were sub cultured to obtain pure isolates (Olofsson and Vàsquez, 2008).

Identification of different LAB were carried out by DNA isolation, 16S rRNA gene partial amplification and sequencing.

Lactic acid bacteria DNA extraction

For bacterial DNA preparation from cultured colony, a part of the colony was resuspended in 50 µl TE buffer (10 mMtris-HCl and 1 mM EDTA, pH8) and subsequently incubated at 90 °C for 15 min. Probes were centrifuged at 5000 g for 10 min (Kilwinski *et al.*, 2004). The supernatant containing the DNA was transferred to a new tube and directly used for PCR analysis.

PCR amplification of 16S-rRNA gene:

A pair of flanking universal sequences was used for primer binding sites to partially amplify target 16S-rRNA genes from the bacterial isolates:

16S-1F : 5'-AGAGTTTGATCCTGGCTCAG-3'

16S-517R: 5'- ATTACCGCGGCTGCTGG -3'

The reaction mixture (25 µl) contained 2xPremix *Taq* (Ex Taq Version, Takara, Japan), 10 µM of each forward and reverse primers; 1U Taq DNA polymerase and 50 ng of bacterial DNA obtained as described above. DEPC water was added to a final volume of 25 µl.

The optimal temperature cycling conditions were adjusted according to Arturo *et al.,* (1995). The cycling program consisted of a 94 °C (5min) step, 30 cycles of 94 °C (1min), 54 °C for annealing (1min), 72 °C (1min), and a final step of 72 °C (5min). Amplifications were performed in a thermal cycler Techne (TC 312). The PCR products were separated electrophoretically in a 1% agarose gel at 100 V for 30 min, stained with ethidium bromide and photographed on an u. v. transilluminator using an Olympus D-760 16 megapixels digital camera.

Sequence analysis:

The amplified lactic acid bacterial DNA (16S rRNA partial sequence) was purified and sequenced in Macrogen lab (Macrogen Inc., Korea). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Obtained sequences were identified and compared directly to all 16S rRNA sequences deposited in GenBank using Basic Local Alignment Search Toolprograms for nucleotide databases search using a nucleotide query (BLASTN search program), National Center for Biotechnology

Information (NCBI), National Library of Medicine, USA (Altschul *et al.*, 1997).

The NCBI, BLASTN, homepage is:(http://www.ncbi.nlm.nih.gov/BLAST/)

Sequence alignments were performed by ClustalW 2.1 XP software and phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei, 1987) using ClustalX 2.1 XP software.

Isolation and preparation of the*, P. l. larvae*, AFB bacterial pathogen:

The bacterium, *P. l. larvae* was isolated from ropy larval remains collected from AFB infected honeybee colonies present in Agriculture Research Center; Plant protection institute; Department of Apiculture Research. The collection took place during summer 2011 from highly infected colonies.

Ropy larval remains of died honeybee larvae were suspended in 10 ml sterile distilled water and kept at room temperature for 10 min, then the suspension is heat-shocked at 80 °C for 10-15 min (effective time to kill non-spore-forming bacteria).

For the isolation of *P. l. larvae*, after vortex mixing, 1ml of the bacterial stock suspension is directly inoculated onto J-agar (5.0 gmtryptone, 15.0 gm. yeast extract, 3.0 gm. K_2HPO_4, 2.0 gm. glucose, 20.0 gm. agar, 1000 ml of distilled water "Adjust pH to 7.3-7.5" (Shimanuki and Knox 1988) by using the pour-plate technique. The plates were incubated in an inverted position at 35 ± 2 °C for 48 hours.

A number of individual colonies were randomly selected from the inoculated plates, depending on the individual colonies characteristics. The selected colonies then inoculated onto J-agar plates by using the streak-plate technique, following the same incubation conditions (Piccini and Zunino, 2001). Initial identification assessing colony characteristics, microscopic characterization and standard biochemical tests (Alippi, 1992). For accurate identification,Bacterial DNA from *P.l.larvae* sub-cultured colonies was extracted and purified. Then, polymerasechain reaction (PCR) of 16S rRNA gene was carried out, using KAT-PCR described byAlippi *et al*. (2002).

Inhibition bioassays against *P. l. larvae* bacterial spores

Antibacterial activity of the isolated honeybee lactic acid bacteria, Oxytetracycline (Terramycine) antibiotic and royal jelly were tested and compared on the AFB bacterial pathogen, *P. l. larvae*, by inhibition zone assay.

Lactic acid bacteria (LAB) inhibition bioassay

According to Forsgren *et al.* (2010), agar media was prepared for LAB (MRS agar pH 6.2, supplemented with 0.5% L-cysteine) and poured in petri-dishes. LAB phylotypes were impregnated in the center of the prepared agar individually at a final concentration of 10^7. In addition, a suspension containing mixture of all LAB types at a concentration of 10^7 of each was also impregnated. The agar media were incubated anaerobically at 35 °C for 12 hours. Agar media of J-agar was prepared as previously described. The temperature of the agar was adjusted to 42 °C. *P. larvae*spores were mixed with the J- agar, poured into the agar media containing the grown LAB and incubated at 35 ◦C for 3 days. Inhibition of *P.*

*l. larvae*strain by LAB was defined as the diameter of the inhibition zone (zone around LAB without *P. larvae* growth).

Oxytetracycline (Terramycine) antibiotic inhibition bioassay

Terramycine was tested against *P. l. larvae*by standard disk diffusion method (Shimanuki and Knox, 1991). Terramycine antibiotic was purchased from Sigma and a stock solution (200 mg/L in 50% methanol) was prepared. Successive dilutions were prepared to yield 20, 6 and 0.6 mg/L. A layer of the J- agar mixed with *P. larvae* spores was poured in a large Petri-dish (15cm in diameter). Three antibiotic test disks (6.35 mm) were done apart within the agar layer, using sterile large blue tips. Those test disks had been shown to absorb 20 µL of water. 20 µL of each antibiotic solution was poured to saturation and allowed to dry. This resulted in a series of disks containing 4, 1.2 and 0.12 µg of each antibiotic/disk (Kochansky *et al.*, 2001). Petri-dish was incubated at 35 ◦C for 3 days.

Royal jelly inhibition bioassay

A layer of the J- agar mixed with *P. larvae* spores was poured in a large Petri-dish (15cm in diameter). 3 small susceptibility test discs were done apart on the agar plate for different concentrations of royal jelly. 20 µl of each concentration (1000, 333, and 200 mg/ml) of royal jelly which corresponded with 20, 6.66 and 4 mgper 20 µl of royal jelly respectively (Eshraghil and Seifollahi, 2003) were added in themiddle of every marked small discs and were incubated at 35 ◦C for 3 days.

Following incubation, the plates were examined andthe clear zones of inhibition surrounding the discswere measured and compared with establishedzone size ranges for royal jelly, Terramycin and LAB antimicrobial inhibitory zones.

RESULTS

Isolation of lactic acid bacteria

16 White, small, round colonies were picked randomly from the MRS agar media involved and were sub cultured to obtain pure isolates. Only 3 colonies were selected from MRS agar incubated aerobically (Fig. 1a) while 13 colonies from aerobic incubation of cultured media (Fig. 1b).

Figure (1): (a) 3 Aerobic subcultures of Lactic acid bacteria isolated on MRS agar media.
(b): 13 Anaerobic subcultures of Lactic acid bacteria isolated on MRS agar media.

PCR amplification of 16S-rRNA gene

The PCR product was found to be about 560 bp long (Fig. 2), which was the same size as the region on the LAB 16S rRNA gene between the two primers.

Figure (2): 16S rRNA amplified region (560 bp) from the 16 LAB sub culltured colonies. Lanes (from L1 to L13): 16S rRNA PCR products from anaerobic sub cultured LAB. Lanes (L14 to L16): 16S rRNA PCR products from aerobic sub cultured LAB. Lanes C1 and C2 are positive controls for PCR.

Sequencing results

Alignments of the LAB16S rRNA gene partial sequence showed that sequences are closely identical to seven lactic acid bacteria. Five of the anaerobic LABs results are closely related to 5 different strains of lactic

acid bacteria of *Lactobacillus kunkeei* species (Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus*), a Fructophilic bacteria isolated from fresh flowers, bees and bee-hives. One aerobic lactic acid bacterial sequence is closely related to a strain of *Lactobacillus plantarum* species (Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus*) extracted from fresh fruits and vegetables and the last one is anaerobic, it is identical to a strain of species *Fructo bacillus fructosus* lactic acid bacteria (Bacteria; Firmicutes; Bacilli; Lactobacillales; *Fructobacillus*) found in honeybees and beehives, they are rich sources for fructophilic lactic acid bacteria.

The BLAST result of the sequence of LAB 1 showed 99% identity with *Lactobacillus kunkeei* strain 100-20 16S ribosomal RNA gene, partial sequence (Accession number: JQ009337.1). LAB 2 gave 99% identity with *Lactobacillus kunkeei* strain 103-20 (Accession number: JQ009348.1). LAB 3 was closely related to*Lactobacillus kunkeei* strain B11-1 (Accession number: JQ009352.1) with also 99% identity. LAB 4 gave 99% identity to the strain *Lactobacillus kunkeei* strain C1-20 (Accession number: JQ009351.1). LAB 6 was 99% related to *Lactobacillus kunkeei* strain 53-1 16S (Accession number: JQ009342.1). For LAB 11, it gave a 99% identity to the strain: FF30-3 of genus *Fructobacillus fructosus* 16S ribosomal RNA, partial sequence, (Accession number: AB793268.1). And finally LAB 16 (LAB 3a aerobic cultured bacteria) has a closely related sequence, 99% identity, to *Lactobacillus plantarum* species, strain AU5-800R (Accession number: KF023220.1). All the sequences found were of total query coverage of 100% and E-Value of 0.0.

Inhibition bioassay results:

Lactic acid bacteria (LAB) inhibition bioassay

The combination of all seven Lactic acid bacteria (Lab) phylotypes resulted in total inhibition (no visible growth) of the investigated *P. l. larvae* strain. Four Lab phylotypes (Lab 2, Lab 3, Lab 4 and Lab 11) individually completely inhibited the represented *P. l. larvae* genotype. Lab 1 and Lab 6 showed only partial inhibition by giving 1.9 and 1.5 cm inhibition zones, respectively whereas there was no growth inhibition detected by the isolate Lab 16 (Fig. 3). Results were compared and verified by +ve and -ve control plates.

Oxytetracycline (terramycine) and Royal jelly inhibition bioassays

P. l. larvae was highly susceptible to terramycine with values ranging from 4, 1.2 and 0.12 µg giving inhibition zones 1.3, 1.1 and 1 cm, respectively (Fig. 4a). The royal jelly showed different inhibitory effects against *P. l. larvae*, Its 20, 6.66 and 4 *mg* concentrations tested have inhibitory zones 1, 0.5 and 0.3 cm, respectively (Fig. 4b).

The antibacterial activity of the extracted honeybee LAB, Oxytetracycline (terramycine) and royal jelly were compared and summarized in Table 1.

Figure (3): Inhibition activity of honey bee LAB phylotypes (Lab 1, Lab 2, Lab 3, Lab 4, Lab 6, Lab 11, Lab 16 and their combination) on *P. l. larvae*.

Figure (4): The inhibitory effects of the (a) Oxytetracycline (terramycine) in different concentrations (4, 1.2 and 0.12 µg) against *P. l. larvae*. (b) Royal jelly in different concentrations (20, 6.66 and 4 mg) against *P. l. larvae*.

Table (1): Inhibition of *P. l. larvae* bacterial spores, American Foulbrood (AFB) bacterial pathogen,by seven honeybee lactic acid bacteria (LAB) phylotypes and their combination, oxytetracycline (Terramycine) antibiotic and royal jelly.

Inhibition substances		Inhibition zone (cm)
Honeybee Lactic Acid Bacteria (LAB) Phylotypes	Lab 1	1.9
	Lab 2	Total inhibition
	Lab 3	Total inhibition
	Lab 4	Total inhibition
	Lab 6	1.5
	Lab 11	Total inhibition
	Lab 16	No inhibition zone
	Combination of all phylotypes	Total inhibition
Oxytetracycline (Terramycine) Concentrations	4 µg	1.3
	1.2 µg	1.1
	0.12 µg	1
Royal Jelly Concentrations	20 mg	1
	6.66 mg	0.5
	4 mg	0.3

DISCUSSION

Our study aimed to assess the gut of honeybee adults, PCR results and direct sequencing revealed the presence of seven lactic acid bacteria. Five anaerobic lactic acid bacterial strains belong to *Lactobacillus kunkeei* species, one aerobic LAB is closely related to *Lactobacillus plantarum* species and the last one is anaerobic, it is close to a strain of species *Fructobacillus fructosus.*Lactic acid bacteria inhibition assays on agar plates were investigated, to evaluate the effects of the extracted honeybee LAB on *P. l. larvae* growth in vitro.

Bacteria belonging to lactic acid bacteria (LAB) are functionally related by their ability to produce lactic acid during homo- or heterofermentative metabolism (Klaenhammer *et al*., 2002). *Lacto bacillus* and *Bifido bacterium*, two of the most important genera within LAB, are commonly found as commensals and are used as probiotics for humans and animals (Ouwehand *et al*., 2002). A novel flora composed of *Lactobacillus* and *Bifidobacterium* has recently been identified in the honey stomach of honey bees, *A. mellifera* (Olofsson and Vásquez, 2008; Vásquez *et al*., 2009). The phylogenetic analyses performed in both studies showed the LAB flora in the honey stomach to be composed of twelve different phylotypes. Furthermore, the findings revealed that honeybees and the novel LAB flora evolved in mutual dependence of one another; the LAB obtaining a niche in which nutrients are available, the honeybees and the honey in turn being protected by the LAB from harmful microorganisms (Olofsson and Vásquez, 2008).

Forsgren *et al.* (2010) suggested that the newly identified LAB genera *Lactobacillus* and *Bifidobacterium*, exhibit strong inhibitory effects on *P. larvae* growth,

American Foulbrood (AFB) bacterial pathogen, *Paenibacillus larvae* ssp. *larvae*spores are highly resistant to desiccation, high temperatures (100°C for more than 10 min), exposure to UV light and also survive contact with classical disinfectants like 10% formaldehyde solutions for longer than 5hours.

The first medications tested were synthetic antibacterials: the sulfa drugs, particularly sulfathiazole (Haseman and Childers, 1944; Eckert, 1947; Reinhardt, 1947; Johnson, 1948; Katznelson and Gooderham, 1949; Katznelson, 1950). They were effective against AFB, but their stability and consequent residues in honey caused problems, and the registration was allowed to lapse in the 1970s (Shimanuki and Knox, 1994).

Oxytetracycline (OTC, Terramycin1), usually as its hydrochloride, has been used since the early 1950s for the prevention and control of AFB and EFB (Gochnauer, 1951; Katznelson *et al*., 1952). It remains after many years the only approved drug treatment for the foulbrood diseases in the United States.

Although Oxytetracycline hydrochloride (OTC) and sodium sulfathiazole have been used in many countries for the control of AFB (Hitchcock *et al.,*1970; Peng *et al.,* 1996; Alippi *et al.,* 1999), strains of *P. l.larvae* resistant to oxytetracycline (OTC) and to sodium sulfathiazole antibiotics have been reported in Poland (Glinski and Rzedzicki, 1977a,b), Germany (Plagemann, 1991) and Argentina (Alippi, 1996). In addition, OTC has been reported to be toxic for larvae (Peng*et al.,* 1992) and for adult bees (Alippi*,* 1996).

Lactic acid bacteria is known to be good producers of antimicrobial substances such as organic acids, hydrogen peroxide and antimicrobial peptides (de Vuyst and Vandamme, 1994). However, there is a clear variation in the production of antimicrobial substances and other beneficial qualities between the different species and genera within LAB (Ouwehand *et al*., 2002; Pfeiler and Klaenhammer, 2007). Our results strongly suggest that the probiotic LAB bacteria linked to the honeybee stomach have important implications for honeybee pathology in general and for AFB tolerance in particular.

Our results demonstrated a strong inhibitory effect of the combined honeybee stomach LAB flora. The combination of all seven Lactic acid bacteria (Lab) phylotypes resulted in total inhibition (no visible growth) of the investigated *P. l. larvae* strain. Four Lab phylotypes (Lab 2, Lab 3, Lab 4 and Lab 11) individually, completely inhibited the represented *P. l. larvae* genotype. Evans and Armstrong (2005, 2006) have previously shown antagonistic interactions between honeybee symbionts such as *Bacillus* spp. against *P. larvae*. However, in their studies no interaction was shown for any of the honey stomach LAB flora.

Our results also demonstrate that not all LAB inhibit *P. larvae*, as the Lab 1 and Lab 6 strains showed only partial inhibition of the *P. l. larvae* by giving 1.9 and 1.5 cm inhibition zones, respectively. LAB possess

different qualities at the species and strain level and some of these antagonistic qualities may be situated on unused genes or plasmids. It is well known that *Lactobacillus*spp., produce antimicrobial substances (Kim *et al*., 1997). These facultative anaerobes tolerate the acidic environments and ferment sugars to produce lactic or acetic acid. The production of lactic acid makes the environment acidic which inhibits the growth of some harmful bacteria. These bacteria could be cultured as microbial food supplement, which benefits the host by improving its intestinal microbial balance (Fuller, 1989).

We additionally evaluated the results by comparing the antibacterial activity of the extracted honeybee LAB, Oxytetracycline (terramycine) and royal jelly, it was found that inhibitory effect of honeybee LAB against the *P. l. larvae* bacteria is clearly stronger than the Oxytetracycline (terramycine) and royal jelly by giving either total inhibition activity or clearly larger inhibitory zones.

The bacteria belonging to the genus *Lactobacillus*, are normal inhabitants of the gut of honeybees and are GRAS (Generally Regarded As Safe).Strains of this genus have been shown to have important metabolic and protective functions in the gastrointestinal tract, interfering with enteric pathogens and maintaining a healthy intestinal microflora. These bacteria are considered beneficial gut inhabitants of humans and other animals and are involved in immunomodulation, interference with enteric pathogens and the maintenance of a healthy microbiota (Mitsuoka, 1992).

In addition, there is evidence that probiotics induce an immune response in honeybees (Evans and Lopez, 2004). Bacteria inhabit the intestines and protect against some unhealthy organisms. Thus *Lacto bacillus* are also known to inhibit the growth of major honeybee pathogens (Promnuan *et al*., 2009; Forsgren *et al*., 2010). Treatment by formic, lactic, and acetic acid is widely employed by beekeepers to guard against such honeybee pathogens as *Varroa destructor* and *Nosema apis*. Organic acids such as formic acid, which is produced by bifidobacteria(Van der Meulen *et al.,* 2006), and both lactic and acetic acid, which are produced by LAB discovered in the honey stomach are antimicrobial substances, meaning that these bacteria may be of considerable importance in protecting honeybees against pathogens.

CONCLUSIONS

We have provided the first information about a novel nonpathogenic LAB bacteria in the honeybee gut of *Apis mellifera* found in Egypt.

LAB are beneficial bacteria have a role to play in disease control/prevention, enhancement of growth rates, and nutrition.

These right microbes could be used to inoculate the hive and the bee gut to aid colony health and diet. The colonization of bee intestine by bacteria such as *Lactobacillus* sp. is a very important process; the micro-organisms participate not only in food digestion but also in the production of

vitamins and antibiotic substances which eliminate pathogenic micro-organisms.

Recommendations:

Thus, the preliminary studies showed that *Lacto bacillus* endosymbiont were invariably present in populations of *Apis mellifera*. The frequent presence of native *Lactobacillus* strains associated with Egyptian honeybees confirmed in this research study could encourage research related to the development of probiotic treatments for bee colonies.

Information about the interactions that occur between different bacterial species inside the hive and the dynamics of the bacterial community could be important in order to develop new approaches for disease control, avoiding the use of commercial antimicrobial substances such as antibiotics.

REFERENCES

Aliouane, Y; Kacimi El Hassani, A; Gary, V; Armengaud, C; Lambin, M. and Gauthier M. (2008).Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. Environmental Toxicology and Chemistry:1.

Allipi, A. M. (1992). Detection de *Bacillus larvae*en poblacionesmixtasdeesporasbacterianas a partir de restoslarvales.Micta-obiologia SEM, 8: 115-118.

Alippi, A. M. (1996). World News: International Workshop on AFB. Bee World,77: 112-115.

Alippi, A. M.; Albo, G. N.; Leniz, D.; Rivera, I.; Zanelli, M. L. and Roca, A. E. (1999).Comparative study of tylosin, erythromycin and oxytetracycline to control AFB of bees.Journal of Apicultural Research 38: 149-158.

Alippi, A. M.; Lòpez, A. C. and Aguilar, O. M. (2002). Differentiation of *Paenibacillus larvae* subsp. *larvae*, the cause of American foulbrood of honeybees, by using PCR and restriction fragment analysis of genes encoding 16S rRNA. Applied and Environmental Microbiology 68: 3655-3660.

Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Arturo, A. M.; Oldelson, D. A.; Hichey, R. F. and Tiedje, J. M. (1995).Bactrial community fingerprinting of amplified 16-23S ribosomal DNA gene and restriction endonuclease analysis p. 1-8. *In* Akkermans, A.D.L., van Elsas, J.D., and Bruijn, F.J. (ed.), Molecular Microbial Ecology Manual eds. Kluwer Academic Publisher

Cox-Foster, D. L.; Conlan S.; Holmes, E. C.; Palacios, G.; Evans, J. D.; Moran, N. A.; Quan, P. L.; Briese, T.; Hornig, M. and Geiser, D. M. (2007). A metagenomic survey of microbes in honey bee colony collapse disorder. Science, 318(5848):283-287.

deVuyst, L. and Vandamme, E. J. (1994).Bacteriocins of lactic acid bacteria, Blackie academic & professional, Chapman & Hall, printed in Great Britain by the Alden Press, Oxford, ISBN 0751401749.

Eckert, J. E. (1947).Use of sulfa drugs in the treatment of American foulbrood disease of honeybees. Journal of Economic Entomology 40: 41–44.

Eshraghi, S. and Seifollahi, F. (2003).Antibacterial Effects of Royal Jelly on Different Strains of Bacteria.Iranian JournalofPublic Health 32 (1): 25-30.

Evans, J. D. and Armstrong, T. -N. (2005). Inhibition of the American foulbrood bacterium, *Paenibacillus larvaelarvae*, by bacteria isolated from honey bees, Journal of Apicultural Research 44: 168-171.

Evans, J. D. and Armstrong, T.-N.(2006). Antagonistic interactions between honey bee bacterial symbionts and implications for disease. BMC Ecology 6: 4.

Evans, J. D. and Lopez, D. L. (2004). Bacterial probiotics induce an immune response in the honey bee (Hymenoptera: Apidae). Journal of Economic Entomology 97: 752-756.

Forsgren, E.; Vásquez, A.; Olofsson, T. C. and Fries I. (2010). Novel lactic acid bacteria inhibiting *Paenibacilluslarvae*in honey bee larvae, Apidologie 41: 99–108.

Fuller, R. (1989).Probiotics in man and animals. Journal of Applied Bacteriology 66: 365-378.

Glinski, Z. and Rzedzicki, J. (1977a).Evaluation of *Bacillus larvae*to antibiotics and sulphonamides isolated in Poland in 1962-1971.PolskieArchiwunWeterynarijne 20(1): 9-16.

Glinski, Z. and Rzedzicki, J. (1977b). A comparison of the activity of certain new sulphonamide preparations against *Bacillus larvae*and other bacilli isolated from honey bees. Polskiearchiwumweterynaryjne20(3): 9-19.

Gochnauer, T. A. (1951). Drugs fight foulbrood diseases in bees. Minnesota Farms and Home Science9: 15.

Haseman, L. and Childers, L. F. (1944).Controlling American foulbrood with sulfa drugs.AgriculturalExperimentStationAnnual Bulletin 482: 3–16.

Higes, M.; Martin-Hernandez, R.; Botias, C.; Bailon, E. G.; Gonzalez-Porto, A. V.; Barrios, L.; Del Nozal, M. J.; Bernal, J. L.; Jimenez, J. J. and Palencia, P. G. (2008). How natural infection by *Nosemaceranae* causes honeybee colony collapse. Environmental Microbiology 10(10):2659-2669.

Hitchcock, J. D.; Moffet, J. O.; Lackett, J. J. and Elliot, J. R. (1970).Tylosin for the control of AFB disease in honey bees.Journal of Economic Entomology63: 204-207.

Johnson, J. P. (1948). Sulfa drugs for American foulbrood of honey bees: third report. Journal of Economic Entomology 41: 314–318.

Katznelson, H. (1950).*Bacillus pulvifaciens* (n. sp.), an organism associated with powdery scale of honey-bee larvae. Journal of Bacteriology 59: 153-155.

Katznelson, H. and Gooderham, C. B. (1949).Sulfathiazole in relation to American Foulbrood.Scientia Agricola29: 340–344.

Katznelson, H.; Arnott, J. and Bland, S. E. (1952).Preliminary report on the treatment of European foulbrood of honey bees with antibiotics.Scientia Agricola 32: 180–184.

Kilwinski, J, Peters, M, Ashiralieva, A and Genersch, E (2004). Proposal to reclassify *Paenibacillus larvae* subsp. *pulvifaciens* DSM 3615 (ATCC 49843) as *Paenibacillus larvae*subsp. *larvae*. Results of a comparative biochemical and genetic study. Veterinary Microbiology 104: 31-42.

Kim, D. S.; Cook, R. J. and Weller, D. M. (1997).*Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology 87: 551-558.

Klaenhammer, T.; Altermann, E.; Arigoni, F.; Bolotin, A.; Breidt, F.; Broadbent, J.; Cano, R.; Chaillou, S.; Deutscher, J.; Gasson, M.; van de Guchte, M.; Guzzo, J.; Hartke, A.; Hawkins, T.; Hols, P.; Hutkins, R.; Kleerebezem, M.; Kok, J.; Kuipers, O.; Lubbers, M.; Maguin, E.; McKay, L.; Mills, D.; Nauta, A.; Overbeek, R.; Pel, H.; Pridmore, D.; Saier, M.; van Sinderen, D.; Sorokin, A.; Steele, J.; O'Sullivan, D.; de Vos, W.; Weimer, B.; Zagorec, M. and Siezen, R. (2002) Discovering lactic acid bacteria by genomics, Antonie van Leeuwenhoek 82: 29–58.

Kochansky, J.; Knox, D. A.; Feldlaufer, M. and Pettis, J. S. (2001).Screening alternative antibiotics against oxytetracycline-susceptible and -resistant *Paenibacillus larvae*.Apidologie 32: 215–222

Matheson, A. and Reid, M. (1992).Strategies for the prevention and control of AFB.Parts I, II and III. American Bee Journal 132(6): 399-402; 133(7): 471-475; 134(8): 534-547.

Mitsuoka, T. (1992).The human gastrointestinal tract. In Wood, B.J.B. (ed.). The lactic acid bacteria in health and disease. Elsevier London 1: 69-114.

Olofsson, T. C. and Vásquez, A. (2008). Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apismellifera*. Current Microbiology 57: 356–363.

Ouwehand, A. C.; Salminen, S. and Isolauri E. (2002). Probiotics: an overview of beneficial effects, Antonie van Leeuwenhoek 82: 279–289.

Parvanov, P.; Russenova, N. and Dimov, D. (2006).Control of American foulbrood disease without antibiotic use. Bee Journal 8:97-103.

Peng, C.Y-S.; Mussen, E.; Fong, A.; Cheng, P.; Wong, G. and Montague, M. A. (1996). Laboratory and field studies on the effects of the antibiotic tylosin on honey bee *Apismellifera*L. (Hymenoptera: Apidae). Development and prevention of AFB disease. Journal of invertebrate Pathology 67: 65-71.

Peng, C.Y-S.; Mussen, E.; Fong, A.; Montague, M. A. and Tyler, T. (1992). Effects of chlortetracycline on honey bee worker larvae reared in vitro. Journal of invertebrate Pathology 60: 127-133.

Pfeiler, E. A. and Klaenhammer, T. R. (2007).The genomics of lactic acid bacteria.Trends Microbiology 15: 546-553.

Piccini, C. and Zunino, P. (2001).American foulbrood in Uruguay: Isolation of *Paenibacillus larvae larvae* from larvae with clinical symptoms and adult honeybees and susceptibility to oxytetracycline. Journal of Invertebrate Pathology 78: 176-177.

Plagemann, O. (1991).Sensitivity of *Bacillus larvae*to antibiotics and sulphonamides.TieraztlicheUmschau,46: 24-27. (In German.)

Promnuan, Y.; Kudo, T. and Chantawannkul, P. (2009).Actinomycetes isolated from beehives in Thailand. World Journal of Microbiology and Biotechnology 25: 1685-1689.

Reinhardt, J. F. (1947). The sulfathiazole cure of American foulbrood: an explanatory theory. Journal of Economic Entomology 40: 45-48.

Saitou, N. and Nei, M. (1987). The Neighbor-Joining Method – a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

Shimanuki, H. (1997).Bacteria. In: Morse, R.A. &Flottum, K. (eds.) Honey Bee Pests, Predators, and Diseases. 3:rd ed. A.I. Root Company, Medina, Ohio, U.S.A. 718 pp.

Shimanuki, H. and Knox, D. A. (1988). Improved method for the detection of *Bacillus larvae*spores in honey. American Bee Journal 128: 353–354.

Shimanuki, H. and Knox, D. A. (1991).Diagnosis of Honey Bee Diseases.USDA Agricultural Hand book No.AH-690, Washington, DC.

Shimanuki, H. and Knox, D. A. (1994).Susceptibility of *Bacillus larvae*to Terramycin. American Bee Journal134: 125–126.

Stokstad, E. (2007). Puzzling decline of U.S. bees linked to virus from Australia. Science 317(5843):1304-1305.

Van der Meulen, R.; Adriany, T. and Verbrugghe, K. (2006). Kinetic analysis of bifidobacterial metabolism reveals a minor role for succinic acid in the regeneration of NAD + through its growth associated production. Applied and Environmental Microbiology 72: 5204-5210.

Vásquez, A., Olofsson, T. C. and Sammataro, D. (2009).A scientific note on the lactic acid bacterial flora in honeybees in the USA – A comparison with bees from Sweden.Apidologie 40: 26-28.

التأثير المثبط لأنواع جديدة من بكتيريا حمض اللاكتيك (المستخرجه من امعاء نحل العسل) (أبيس ميلليفرا) ضد بكتيريا البانيباسيلاس لارڤي لارڤي

شيرين أحمد محمود مأمون[1]، محمد إبراهيم إمام[1]، عماد الدين أحمد نافع[2]، رابية عبد الوهاب عنان[1]، محمد سيد سلامة[1]، عقيلة محمد الشافعي[1] و أحمد حسن كاشف[1]

[1]قسم علم الحشرات - كلية العلوم - جامعة عين شمس - القاهرة - مصر

[2]مركز البحوث الزراعية- معهد بحوث وقاية النباتات- قسم بحوث النحل- القاهرة- مصر

في هذه الدراسة تم استخراج سبعة سلالات من بكتيريا حمض الاكتيك، المعروفة بخصائصها كمضاد حيوي أولي، من أمعاء شغالات نحل العسل (*ابيس ميلليفرا*). و قد تم تعريف هذه البكتيريا عن طريق معرفة التتابع النيكليوتيدي (DNA Sequencing) لمنطقة محددة من جين الـ (16S-rRNA) المكبرة بإستخام تفاعل البلمرة المتسلسلة. وقد أوضحت النتائج أن خمس سلالات من هذه البكتيريا تنتمي الي بكتيريا حمض الاكتيك من النوع (*لاكتوباسيلاس كوينكياي*) وواحدة من نوع (*لاكتوباسيلاس بلانتارام*) والنويع الأخير من نوع (*فراكتوباسيلاس فراكتوساس*). تم تقييم التأثير المثبط لبكتيرياحمض اللاكتيك، السبعة سلالات المستخرجة مجمعة ومنفردة كل علي حدة، في مقاومة بكتيريا الـ(*بانيباسيلاس لارڤي لارڤي*) المسببه لمرض تعفن الحضنة الأمريكي معمليا علي أطباق الأجار. أثبتت نتائج التقييم أن السبعة سلالات مجمعة وأيضا هناك أربعة أنواع منفردة تعمل كمثبط كامل، وغياب نمو بكتيريا الـ (*بانيباسيلاس لارڤي لارڤي*).

وبذلك نكون قد توصلنا الي طريقة جديدة لمقاومة آمنة لمرضتعفن الحضنة الأمريكي بإستخدام بكتيريا حمض الاكتيك وهو ما يحتاج الي دراسات أخري حقلية حتي يمكن تطبيقها وتعميمها كعلاج معتمد للمرض.