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# A Histopathological Study to Evaluate Lactic Acid Bacterial Effects within Honeybee Larval Guts as a Control Agent of American Foulbrood Disease; In *vitro*.

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**ABSTRACT**

Honeybee specific lactic acid bacteria (LAB) were potentially demonstrated with their high biotic potential within honeybee gut. LABs are well known for their probiotic and treatment effects. In this study, honeybee Larvae were reared artificially in three groups: control group reared on an artificial diet only; an infected group with *Paenibacillus larvae larvae* (*P. l. larvae*), the causative agent of one of the popular honeybee diseases that infect brood; American foulbrood (AFB) disease. Larvae of this group were fed on an artificial diet mixed with pathogen spores. The third group; LAB treated larvae which were exposed to honeybee LABs and the pathogen spores mixed with larval food. The histological impact was determined upon infected and treated honeybee 5$^{th}$ larval instar midgut. Investigations of anterior midgut showed alterations in the histological integrity between groups. However, control and LAB treated groups showed a complete health status of midgut epithelial cells and their components. On the other hand, the infected group with *P. l. larvae* revealed a dramatic highly damaged midgut epithelial cells. Cells lost their shape and their boundaries with complete lysis of cell membranes. Aggregation of nuclei and a complete distortion of chromatin material. The results demonstrated that honeybee LAB could help in reducing and limiting of this lethal bacterial disease, AFB.

## INTRODUCTION

Honeybees are the most important pollinators worldwide. They are considered as a key species of agriculture, in particular; and maintenance of almost all life on earth; in general (Corby-Harris *et al.* 2014). For that reason, the health status of honeybees has become of great interest.

Declining of honeybee numbers within colonies, in many countries all over the world, endangers pollination (Aizen and Harder 2009; Van Engelsdorp and Meixner 2010; van der Zee *et al*. 2012). Bees are constantly under threat due to the combined damage from bacteria, parasites, viruses, pesticides, insecticides, and artificial bee food (Cox-Foster *et al.* 2007; Oldroyd 2007; Stokstad 2007; Aliouane *et al.* 2008; Higes *et al*. 2008). Rearing of honeybee larvae in *vitro* is an important tool (i.e. in the laboratory and in the absence of nurse bees) because it allows researchers to control surrounding conditions compared to in *vivo* (i.e. in the hive by nurse bees). However,

here artificial larval rearing and application may also be said as "It is an in *vivo* application conducted in an in *vitro* system". Thus, testing of the antibiotics and toxicity of plant protection products on brood can be first done in a standard way in the laboratory. That is because food uptake containing the testing compound can be determined easily in vitro compared with hive condition (Wittmann and Engels 1981; Oomen *et al*. 1992; Schuur *et al*. 2003; Becker *et al*. 2009; Aupinel *et al*. 2007a).

American Foulbrood (AFB) is a serious bacterial disease that infects honeybee brood. It is caused by the spore-forming bacteria, *Paenibacillus larvae larvae* (*P. l. larvae*). It is lethal to infected brood individuals and leads to eliminate infected colonies (Ashiralieva and Genersch 2006). AFB control is a considerably difficult process because the spores can subsist for long periods of time bearing different environmental conditions (De Graaf *et al*. 2006; Hrabak and Martinek 2007; Genersch 2010). Burning of infected colonies used to be the most relevant method for control (Matheson and Reid 1992; Ratnieks 1992) and still in use in many countries. Antibiotics appeared as an alternative solution to the burning of beehives. The widespread application of antibiotics released new generations of resistant bacterial strains reduced the half-life expectation of honeybees and endangered the normally exist microbiota of the bee colonies. On the other hand, the risk of honey contamination which decreases its nutritional and commercial values (Charbonneau *et al*. 1992). Subsequently, there is a drift to find an alternative, safe and natural biocides for the control of AFB which is a great challenge to keep healthy colonies and improve the honey quality (Gonza´lez and Marioli 2010).

Recently, a special symbiotic LAB microbiota was found to be common inhabitants of honeybee guts (Olofsson and Va´squez 2008). Strains of these bacteria are generally recognized as safe (GRAS) food-grade microorganisms and employed as probiotics afford human health (FAO/WHO 2001 and 2002). LAB microbiota within *A. mellifera* is composed of 13 bacterial species within the genera *Lactobacillus and Bifidobacterium* (Olofsson and Va´squez 2008; Va´squez *et al*. 2009; Olofsson *et al.* 2011). They were mainly isolated from the honeybee worker's crop. The honeybee crop lies between the esophagus and ventriculus. It is a pivotal organ in food production; as it is used for the collection and transportation of nectar to the beehive. Also, plays an important role in honey and beebread production (Olofsson and Va´squez 2008; Va´squez and Olofsson 2009) and for long term storing food for both adult honeybees and larvae.

The purpose of this study is to keep tracking LAB within honeybee larval guts. It is a comparative histological study to understand the role of those symbiotic microbes as a control agent of AFB disease and give some information about LAB existence in the larval guts.

## MATERIALS AND METHODS

Worker honeybee larvae (*Apis mellifera carnica*) were obtained from the honeybee colonies at the Honeybee Research Department, Plant Protection Research Institute, Agricultural Research Centre, Ministry of Agriculture of Egypt, during summer 2018.

### *Paenibacillus larvae larvae* Strain:

A local strain of *P. l. larvae* of previously isolated and identified with KAT-PCR, designed by Allipi *et al*. 2002, was cultivated on J-agar according to (Gordon *et al.*, 1973). Obtained spores were suspended in sterile 0.9% NaCl. The microscopic count of the spore suspensions was made in a Thoma Counting Chamber

using a phase-contrast light microscope (100x). Spore suspensions with concentrations of $5x10^4$ spores/ml were prepared fresh for the experiment.

**LAB Strains:**

Five honeybee LABs were previously identified and tested for their inhibitory effects on *P. l. larvae*. They also were applied for field treatment and they gave promising results (Mahmoud *et al.* 2019). These strains were: Two strains of *Lactobacillus plantarum* (GenBank Accession numbers: MK780211 and MK780215), two of *Lactobacillus kunkeei* (Accession numbers: MK780216 and MK780218) and one strain of *Lactobacillus sp*. (Accession number: MK780212).

LAB strains were cultured anaerobically according to Mahmoud *et al.* 2019. A mixture of about $5x10^5$ bacteria/ml was prepared freshly for each experimental dose.

**Exposure Bioassays"**

Larvae were reared in vitro according to Aupinel *et al.* (2005) with minor modifications. Honeybee larvae were reared in forty artificial sterilized queen cups. The cups were arranged inside a 9 cm diameter Petri dish per treatment, which was placed inside a larger 15 cm diameter petri dish. The distance between the two petri dishes is filled with sterile water and the outer dish cover is used only (Fig.1). The surrounding environment and all the equipment were sterilized from the beginning until the end of the experiment. Larvae were reared on a diet consisting of 50% royal jelly (v/v), and 50% of an aqueous solution of D-glucose (12%) and D-fructose (12%). The sugar was dissolved in distilled water and the sugar solution was autoclaved at 121 ◦C for 20 minutes and kept frozen. The diet was prepared and then stored at +4 ◦C for the whole duration of the experiment. Before grafting, Fresh 300 µL of the pre-warmed diet were added daily using a pipette according to treatment. Experiment was divided into three groups:

First group was the control group which was provided with an uninfected diet. Second group was the infected one that was provided with larval diet mixed with $5x10^4$ of *P. larvae* spores. While the third group was the probiotic treated group and was supplied with a diet mixed with $5x10^4$ of *P. larvae* spores and $5x10^5$ of LAB mixture. First instar worker larvae (after 12 hours) were taken from brood combs and grafted on the larval diet of different treatments.

Twenty-four hours post-exposure to a diet containing spores; all larvae were transferred to new queen cups containing a diet without *P. larvae* spores. 300 µL of the diet was provided to the larvae once a day using a sterile pipette tip. Only the third group was provided with diet mixed with LAB for other four days post-infection. The Petri dishes were inserted into plastic boxes with a wire mesh inside. The boxes were filled with a thin layer of 15.5% glycerol and 0.45% methyl benzethonium chloride to prevent fungal growth. The larvae were kept in an incubator at 35 ◦C with 85 to 90% relative humidity through a plate of a saturated solution of K2SO4 and NaCl placed at the bottom of the incubator.

**Histological Studies:**

5th larval instars were collected from each group and dissected on petri dishes. Midgut was separated, and Transverse sections were compared for each group.

**Electron Microscopy:**

Midgut sections were immersed and fixed overnight in cold 3% glutaraldehyde. Specimens were washed with phosphate buffer (pH 7.2) for 3 times through an hour. Then transferred to initial fixative 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours. They were washed again in phosphate buffer (pH 7.2) for 3 times. Tissues were dehydrated

through an ethanol series, then specimens were infiltrated with Spurr's epoxy resin in a graded series of absolute alcohol-Spurr's resin mixtures, then embedded in freshly prepared Spurr's resin and harden at 70°C for 20 hrs. Semithin 5-6 µm sections were mounted on slides, stained with toluidine blue. Specimens were examined and investigated tissue sections were analyzed using the transmission electron microscope model JEOL JEM-1200EX II at 100 KV.

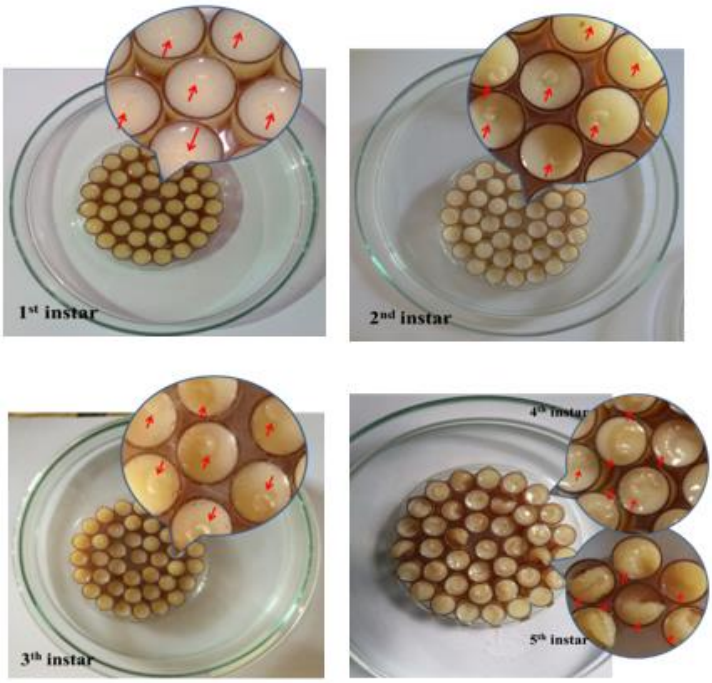


**Fig. 1:** Artificial rearing of the honeybee larvae showing different larval instars of honeybee workers in *vitro*.

## RESULTS

In vitro reared larvae showed healthy status in control group only 7.5% mortality. Also, LAB treated groups showed low mortality about 17.5% and the rest of larvae retain their health status. Infected larvae with *P. l. larvae* group not only suffer great larval lose about 80% mortality till reaching 5th instar but also survived 5th instar larvae showed AFB disease clear symptoms. Figure 2 showed honeybee 5th instar larvae from the three groups. First and third group larvae from control and AFB treated larvae respectively, showed brightly white, healthy larvae. While infected larva from group three turned its color into brown. By the end of the experiment, infected larvae turned into gelatinous ropy mass and their colors became very dark brown .

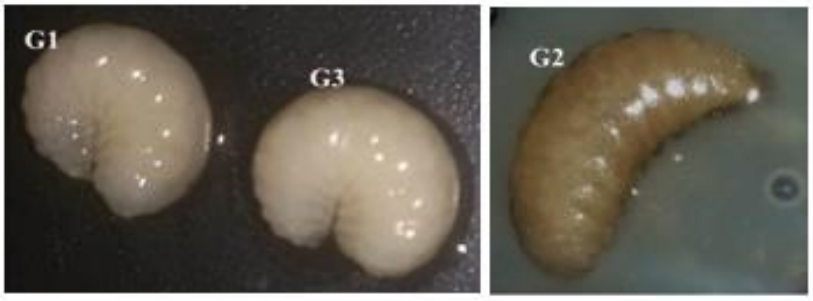


**Fig. 2:** In vitro reared 5th instar honeybee larvae. G1: larvae of 1st group (control); G3: larvae of 3rd group (treated with LAB) and G2: larvae from 2nd group infected with *P. l. larvae*.

**Histological Study:**

Histological investigations of anterior midgut showed alterations in the histological integrity between groups. Sections in control group (fed only on royal jelly in sugar syrup) revealed that the midgut epithelium cell was characterized by an oval nucleus surrounded with the normal nuclear membrane. Homogenous cytoplasm with abundant and dense mitochondria and appearance of some vacuoles. The epithelial cell is surrounded by a well-defined cell membrane and tightly attached with the basal membrane (Fig. 3A). Moreover, the apical border was regular bearing numerous, long filaments called microvilli extending into the midgut lumen. Microvilli were observed releasing vesicles among them (Fig. 4A, a).

Third group (LAB treated group) also showed a normal and stable cell status. Columnar digestive cells were laterally connected by long cell junctions, which were tight and intact. Nucleus and Nucleolus appeared normal. Cytoplasm kept its homogeneity with numerous mitochondria and the presence of cell vacuoles. Cells were closely adhering to the basal membrane (Fig. 3B). Spherocrystals are present in the apical cytoplasm of the digestive columnar cells and rough endoplasmic reticulum that is distributed among the cytoplasm (Fig. 5A, a). We observed normal, dense and long microvilli harboring LAB cell. The LAB cell forms extracellular polymeric biofilms by which they attach themselves to the gut (Fig. 4B, b).

The infected group with *P. l. larvae* (group 2) revealed dramatic highly damaged midgut epithelial cells. Cells lost their shape and their boundaries with complete lysis of cell membranes. Cytoplasmic proteolysis, aggregation of irregular nuclei with an abnormal distribution of heterochromatin patches. Also, fragmentation, complete degradation and distortion of nuclear chromatin of others as well as partial degradation of the nuclear membrane. The appearance of swollen and lacerated mitochondria exhibiting lysing matrix. A widely distributed huge number of vacuoles and appearance of autophagosomes. Finally, detachment of the digestive cells from basal membrane (Fig. 3C, D, E, F). Apical cell region showed an irregular and damaged cell membrane surface. Microvilli appeared irregular, withered, detached, and fragmented (Fig. 4C).

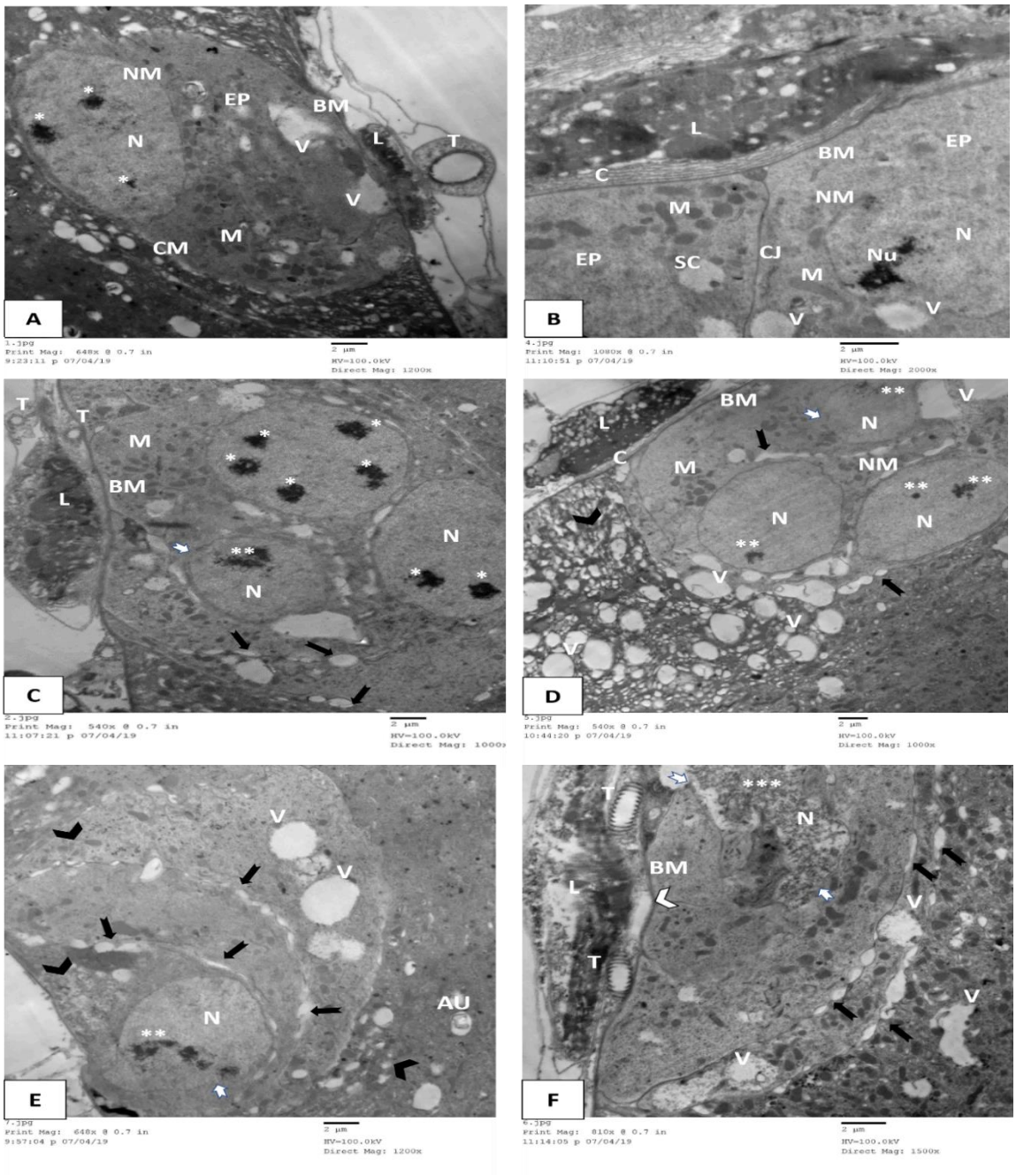


**Fig. 3:** Transmission electron photomicrographs of anterior midgut region cells of 5th instar honeybee workers'larvae, **A**: control group, showing typical epithelial cell (**EP**) , **B:**, LAB treated group also exhibited normal columnar cells laterally joined by cell junctions (**CJ**) . **C, D, E, and F:** *P. l. larvae* infected group. In **C** and **D** Note, Disappearance of cell membranes, cytoplasmic proteolysis (**black arrows**), some mitochondria were found swollen (**M**) and others were degradated and lacerated (**black arrowheads**), a lot of widely distributed large vacuoles (**V**), aggregation of irregular shaped nuclei (**N**) with partial degradation of their nuclear membranes (**white arrows**), and abnormal distribution of heterochromatin (*****) with gradual fragmentation and degradation (******). E and F total nuclear proliferation (***), appearance of autophagosomes (**AU**) filled with cell debris and finally cellular collapse and detachement of the cell basal membrane (white arrowhead). N: Nucleus; Nu: Nucleolus; NM: Nuclear Membrane; T: Tracheole; L: Longtudinal muscular cell; C: extracellular channels; BM: Basal Membrane; M: Mitochonderia; V: Vacuole; CM: Cell Membrane; CJ: Cell Junction; Sc: Spherocrystal; AU: Autophagosomes.

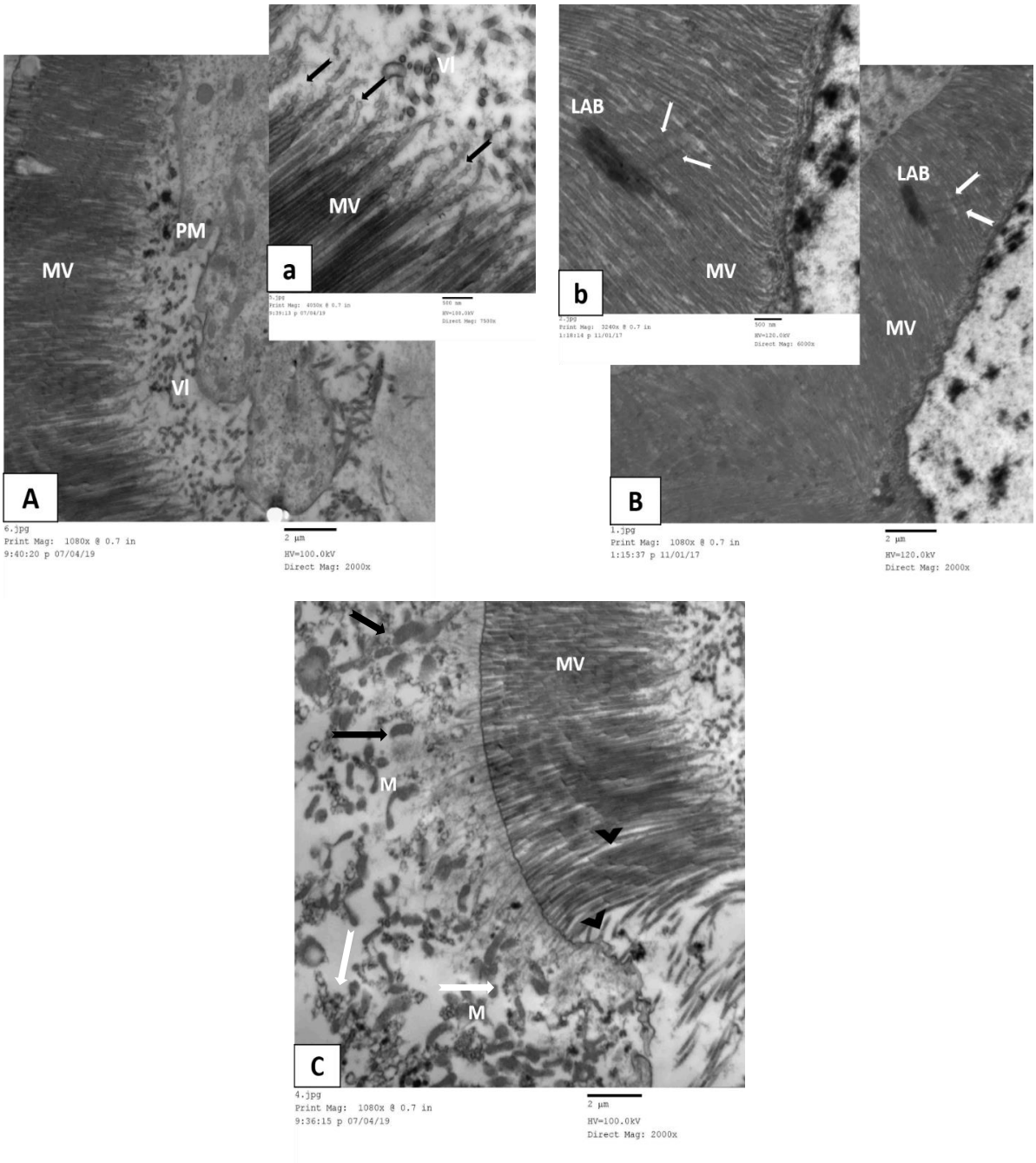


**Fig. 4:** Micrographs of the microvilli region within the anterior midgut of the fifth instar workers' larvae **A**: Control group; showing normally arranged microvilli (**MV**) end with vesicles (**Vl**) and normal peritrophic membrane (**PM**) **a:** A magnified detailed view of the microvilli (**MV**) exhibiting vesicle (**Vl**) releasing among them (**black arrows**) **B:** LAB treated group illustrating normally arranged microvilli (**MV**) harboring LAB bacterial cell (**LAB**) **b:** A magnified LAB bacterial cell attached to the gut with extracellular polymeric biofilm (**white arrows**) **C:** *P. l. larvae* infected group; showing the apical cell region with partially swollen mitochondria (**black arrows**) and others were lacerated and their matrix lysis (**white arrows**); Microvilli (**MV**) detachment (**black arrowheads**) and partial destruction of the digestive cell. **MV:** Microvilli; **Vl:** Vesicle; **PM:** Peritrophic membrane; LAB: Lactic Acid Bacteria **M:**Mitochondria.

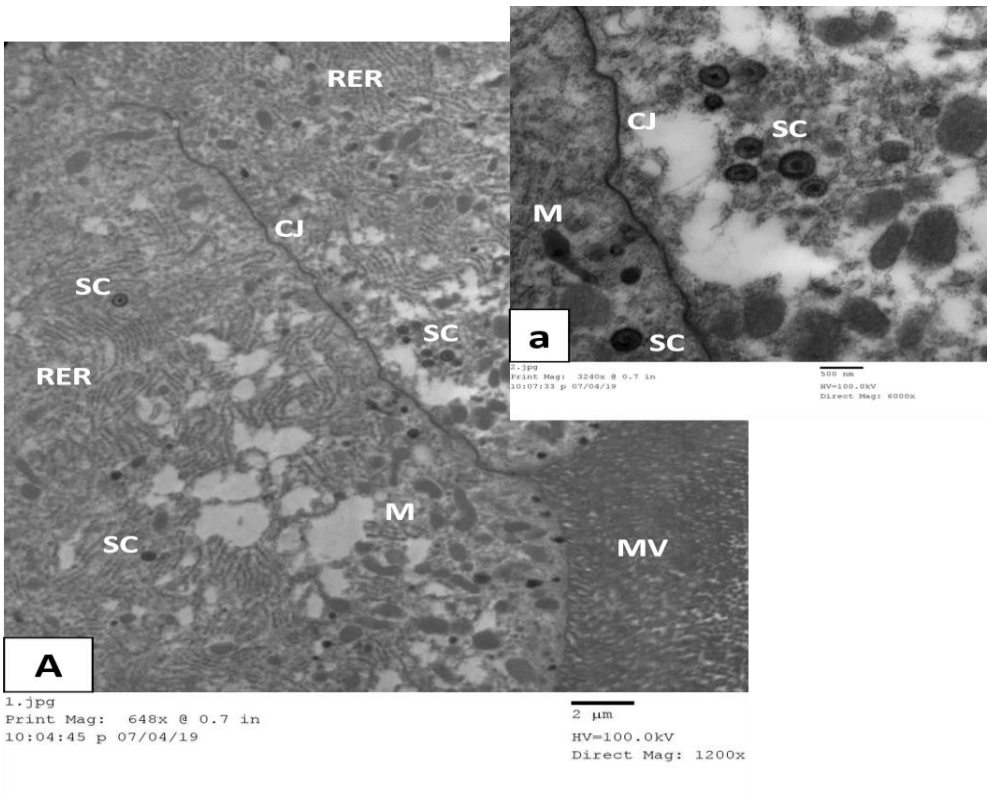


**Fig. 5: A:** Micrograph of the fifth instar larvae anterior midgut in LAB treated group showing normal columnar cells connected with cell junction (**CJ**), normal rough endoplasmic reticulum (**RER**), Mitochondria (**M**), Note, cytoplasm with spherocrystals (**SC**). **a:** enlarged spherocrystals. **CJ:** Cell Junction; **RER:** Rough Endoplasmic Reticulum; **M:** Mitochondria; **MV:** Microvilli; **SC:** Spherocrystal.

## DISCUSSION

Recent studies on *A. mellifera* subspecies have demonstrated that LAB microbiota is a dominant constituent of honeybee surrounding environment (Olofsson *et al.* 2011). Mahmoud *et al.* (2019) demonstrated by both in vitro and field applications that the honeybee endogenous LAB microbiota inhibits one important honeybee pathogen, *P. l. larvae* that is the main cause of AFB disease. In the current study, we investigate the presence of LAB microbiota in honeybee gut and give some information about its role in controlling of AFB disease within larval midgut.

To reach our research goal, we reared honeybee larvae in *vitro* to control all surrounding circumstances to be sure of diet constituents and that larvae have taken bacteria in the required doses. As in *vitro* rearing of honeybee larvae is very important mainly in researches on pathogens, risk assessment, effectiveness and validation of treatment. Since many recent studies have developed protocols that facilitate producing unambiguous workers with low mortalities (Vandenberg and Shimanuki, 1987; Aupinel *et al*., 2005).

Honeybee larvae of 24-30 h old are susceptible to infection with *P. l. larvae*, but third instar larvae and older do not show symptoms of the disease (Brødsgaard *et al*. 1998). In the current study, *P. l. larvae* were added to 1st instar (about 12h post-hatching) larval food to ensure oral infection of the infected group under investigation. The group of LAB treated larvae was fed with both pathogen and LAB probiotics at the same early larval stage. The histological alterations within the honeybee guts that might have been happened by invading of *P. l. larvae* and LAB treatment were

investigated in fifth instar larvae after complete treatment with LAB doses.

We targeted the digestive system mainly the midgut region to understand the effect of *P. l. larvae* bacterial pathogen and the role of LAB in AFB treatment within midgut cells. However, the digestive system is the main site of contact with pathogens and xenobiotics (Han *et al*., 2012; Johnson *et al*., 2009). The midgut epithelium is responsible for the detoxification of ingested foreign substances (Higes *et al*., 2013). Also, it is the only tissue of honeybees that can undergo cell proliferation (Ward *et al*., 2008). The midgut of honeybees is a tubular organ which bears a single epithelium layer. This layer consists of three types of cells: 1. Digestive cells responsible for the synthesis of digestive enzymes and absorption of nutrients (Serrão and Cruz-Landim 1996; Fialho *et al.* 2013); 2. Endocrine cells that synthesize hormonal peptides for controlling digestion, peristalsis, diuresis and development (Neves *et al.* 2002; Cruz *et al.* 2011). 3. Regenerative cells that play a role in the renewal of digestive and endocrine cells (Fernandes *et al.* 2012; Cruz *et al.* 2013). *P. l. larvae* spores germinate in the larval midgut about 12 h post-infection. Vegetative bacteria pass through the midgut epithelium where they colonize and proliferate within few days post-infection (Yue *et al.* 2008). Peritrophic membrane begins to develop in honeybee larval guts within 8 to 36h (Bamrick 1964; Davidson 1970). This membrane represents the main barrier against infections that may explain why elder larvae and adults can resist *P. larvae* infection (Sturtevant and Revell 1953).

Alterations of ultrastructure in honeybee larvae exposed to *P. l. larvae* have been reported to include swollen mitochondria, which are also documented as a response to toxification in both vertebrate and invertebrate cells (Cheville 2009; Vandenbulcke *et al.* 1998). Aggregation and degeneration of cell nuclei and their complete destruction as well as chromatin materials that were completely fragmented and collapsed. Severe proteolysis and intensive vacuolization of cytoplasm. Mitochondrial swelling and lysis, and detachment of microvilli from midgut epithelium. Some of our observations were also reported in honeybee larvae of *Apis mellifera jemenitica*, upon infection with *P. larvae* (Ayaad *et al.* 2018). Autophagosomes were observed also in infected midgut cells since they participate in the removal of damaged organelles and turnover of intracellular compounds (Grogan and Hunt 1984; Jimenez and Gilliam 1989; Serrão, and Cruz-Landim 2000). Finally, the detachment of the epithelial cells from its basal membrane. Subsequently, alterations in midgut epithelial cells could be considered as an indicator of the inevitable exposure to environmental stressors (Pawert *et al.* 1996; Zhong *et al.* 2012). These severe destructions may be due to *P. l. larvae* that produce proteases causes degradation of cell membrane as well as cell junctions. So that, the bacterial pathogen could invade the hemocoel to proliferate. It had been suggested that the possible mechanism of invasion of *P. larvae* into *Apis mellifera* gut is due to the activation of specific cell receptors on the cell surfaces that initiate phagocytosis (Gregorc and Bowen 1998; Antunez *et al*. 2011).

On the other hand, our results showed that peritrophic membrane and epithelial cells were stable and homogenous in control and LAB treated guts; compared to infected larvae that showed signs of degeneration. Despite, larvae treated with LAB had taken the same loads of *P. l. larvae* as infected group larvae, LAB treated larval gut retain their healthy status. Epithelial cells were normal columnar cells separated with cell junction, normal rough endoplasmic reticulum, numerous mitochondria,

and the appearance of spherocrystals in apical region of the digestive cells for regulation of ion concentration in the cytoplasm (Gouranton 1968; Martoja and Ballan-Dufrancais 1984). This normal status may be due to LAB probiotic effects through the production of antimicrobial peptides, organic acids, hydrogen peroxides and other antibacterial substances (De Vuyst and Vandamme, 1994).

Our investigation gives some information about LAB existence within honeybee guts. We observed the probiotic bacterial cell in larval gut treated with LAB. LABs attached themselves to gut through biofilms. Biofilm formation is known in LAB species that inhabit the human gut and vagina (Domingue *et al.* 1991; Lebeer *et al.* 2007; Macfarlane and Dillon 2007). As LAB produce exopolysaccharides which are responsible for biofilm formation and adhesion include the production of proteins, carbohydrates, enzymes, nucleic acids, lipids or membrane bound receptors.

Results strongly suggest that honeybee endogenous LABs play an important role in preliminary inhibition of *P. l. larvae* and retaining of health status of honeybees. Also, we recommend honeybee LABs as an alternative treatment for the severe AFB disease instead of conventional antibiotics, which will need more studies about the relation between LABs and bee health.

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## ARABIC SUMMARY

**دراسة الأنسجه المرضيه لتقييم تأثير بكتيريا حمض اللاكتيك داخل معي يرقات نحل العسل كعامل مكافح لمرض تعفن الحضنة الأمريكي؛ معمليا.**

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تعرف بكتريا حمض اللاكتيك المستخرجة من نحل العسل بقدرتها الحيوية العالية داخل أمعاء نحل العسل. فتشتهربكتيريا حمض اللاكتيك بخصائصها العلاجية وكونها مضاد حيوي أولي. في هذه الدراسة، تم تربية يرقات نحل العسل معمليا في ثلاث مجموعات: المجموعة الأولي، مجموعة اليرقات السليمه، والتي تم تربيتها على نظام غذائي مصنع فقط؛ المجموعة الثانية: هي مجموعة يرقات تم إصابتها ببكتيريا *البانيباسيللس لارفي لارفي*، العامل المسبب لأكثر الأمراض شيوعا في خلايا نحل العسل والتي تصيب الحضنة؛ مرض تعفن الحضنة الأمريكي. تم تغذية يرقات هذه المجموعة على نظام غذائي مصنع ممزوج بجراثيم البكتيريا الممرضة. المجموعة الثالثة: وهي مجموعة اليرقات التي تم علاجها ببكتيريا حمض اللاكتيك وهنا تعرضت اليرقات لبكتيريا حمض اللاكتيك وجراثيم البكتيريا الممرضه عن طريق مزجهما بغذاء اليرقات. تم رصد التغيرات النسيجيه في المنطقة الأمامية للمعي الأوسط ليرقات العمر الخامس في الثلاث مجموعات. وتبين من الفحص عدم ظهور أي أعراض ممرضه أو تغير في التركيب النسيجي في المجموعات السليمة والمعالجه ببكتيريا حمض اللاكتيك. فأظهرت بكتيريا حمض اللاكتيك سيطره علي المرض وسلامة نسيج المعي الأوسط وسلامة خلاياه. بينما المجموعة المصابة ببكتيريا *البانيباسيللس لارفي لارفي* فكانت خلايا المعي الأوسط لليرقات تالفة بشكل كبير. فقد فقدت الخلايا شكلها وحدودها مع تحلل كامل لأغشية الخلايا. كذلك ظهر تجمع للأنوية وتلف تام للمواد الكروماتينية. أظهرت النتائج أن بكتيريا حمض اللاكتيك المصاحبة لنحل العسل يمكن أن تساعد في علاج هذا المرض الجرثومي الفتاك والحد منه.