Original Article

Histopathological Impact in the Larval Gut of the Honeybee, *Apis mellifera jemenitica*, Upon Infection with the American Foulbrood Bacterium, *Paenibacillus larvae*

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ABSTRACT

Background: American foulbrood (AFB), caused by *Paenibacillus larvae* (*P.larvae*) bacterium, is one of the major threatening disease to the global apiculture industry. This Apiary-threatening bacterium has recently been detected in some of the Saudi Apiaries. The current study herein was conducted to investigate the impact of the locally isolated *P. larvae* infection on the histological integrity of the alimentary tract of the indigenous Saudi honeybee brood, *Apis mellifera jemenitica (A.mellifera jemenitica).* **Methods**: The 1st instar larvae were orally infected with the *P. larvae.* Non-infected (control) or infected (at 48 and 72h post-treatment) alimentary canals were dissected for histological investigations. Alimentary canals were separated into three parts; foregut (proventriculus), midgut (ventriculus) and hind gut (intestine) prior to histological investigation. Gut sections were subject to light and electron microscopy investigations at 48 and 72h post-infection. **Results**: Data showed histopathological alterations in the infected alimentary canal as early as 48h post-*P. larvae* infection. Infected proventriculus showed many vacuoles, separation of the epithelial layer and damage of circular muscles. Lacerations were observed in the basement membrane surrounding the midgut in the infected larvae. Vacuoles of different sizes were also observed in the hindgut of infected larvae. Moreover, aggregations of *P. larvae* bacterial cells were detected in the gut epithelium of infected larvae. At 72h post-*P. larvae* infection, infected broods showed separation and elongation of the midgut epithelial cells. These histological alterations may have led to the severe laceration observed in the layers of infected stomach. Finally, deterioration of muscular and epithelial layers of infected stomach was observed at 48h post-infection. In addition, increased vacuoles were also observed in the epithelial cells of the hindgut of infected larvae. These histological alterations have been confirmed on the subcellular level *via* transmission electron microscopy as it showed subcellular degradation in terms of degraded mitochondria and nuclei. **Conclusion**: These histopathological deteriorations are similar to that globally recorded in the AFB, and hence, provides warning alarm to the local authorities for taking immediate actions towards restricting the epidemics of this disease within the Saudi Apiaries. In parallel, utilizing the larval immune system against *P. larvae* infection is currently under investigation for producing natural pharmaceutical treatment of the AFB.

Key words: Histopatology, *P.larvae*, *A.mellifera jemenitica*, Saudi apiaries.

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INTRODUCTION

Honey bees and other eusocial insects comprise more than half of the insect biomass in the world.[1] Apiculture are recognized as high valued industry worldwide for their honey production and plant crop pollination. It has been assessed that one-third of the human diet is linked directly or indirectly to bee-pollinated crops.[2] Hence, honeybee's health has a great impact on economy and biodiversity worldwide. Consequently, the decline in honey bee population is currently considered as a global threat to the apiculture industry which is resulting in economic losses worldwide. This decline could be as a results of environmental stress, pollution, parasites and infectious diseases. Of them, AFB is the most threatening infectious, notifiable disease to the global apiculture industry, that is caused by the gram-positive, spore-forming bacterium, *Paenibacillus larvae* subs p. *larvae.*[3] Disorders which aid in the transmission of *P. larvae* include congestion in the bee's hive, warm temperatures, high concentrations of resources and periods of quarantine in the nest.[4] AFB postures a major threat to the beekeeping industry due to its contagious nature and its ability to destroy affected colonies.[3] AFB affects only the early larval stages (brood) of honey bee, i.e. 12–36h after hatching, are the most susceptible larval stage to *P. larvae* endospores infection.[5] In Saudi Arabia, beekeeping is an important industry in the rural communities as it contributes significantly to the country incomes.[6] The indigenous Saudi honey bee, classified as, *A. mellifera jemenitica,*[7] plays a role in honey production in the country. They show significant morphological and biological differences from other populations of same African race also classified as *Apis mellifera*.[7] In fact, candidate approaches toward the challenges facing the honey apiculture industry in Saudi Arabia are being implemented. On one hand, a comprehensive survey has been performed throughout Saudi Arabia which has detected not only AFB but also European Foulbrood (EFB) in some apiaries of the honey producing cities.[8,9] Based on these studies, we are currently probing innovative approaches for tackling these diseases (unpublished data). In the same trend forward, the current study herein was conducted to explore the histopathological alterations in the larval gut integrity of the indigenous honey bees, *A. mellifera jemenitica* brood upon infection with the AFB causative bacterium, *P. larvae*. To the best of our knowledge, this is the first study on *A. mellifera jemenitica,* as AFB has currently recorded in the Saudi Arabian Apiaries for the first time.

MATERIALS AND METHODS

Rearing experimental bee's brood

The honeybee, *A. mellifera jemenitica*, larvae were collected from an apiary maintained at Bee Research Chair, Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia. The method described by Aupinel *et al.*[10] was used with some modifications to obtain larvae of similar age. A frame was taken out from a healthy bee colony for fixing Jenter comb box (Jenter System, Figure 1A),[11] an excluder cage, (Hammann, Hassloch-Germany). Jenter comb box was disinfected for 30 min in 0.4% methyl benzethonium chloride (MBC) in water prior to fixation in the frame by cutting an equal part of the wax comb (Figure 1B). Jenter comb box was designed to permit free movement of the worker bees across the cage, which is essential for stimulating queen for egg laying, and nursing bee larvae. The queen was confined in the excluder cage and the frame was placed in the bee colony to allow the queen to lay eggs. After approximately 30 h, when a large number of eggs was laid in the cage cells, the queen was uncaged. The frame, along with the Jenter comb box containing the eggs, was kept in the middle of the bee colony in their hive for three days for natural incubation. The excluder cage allowed the worker bees to feed the newly hatched larvae, but prevented the queen from entering and laying new eggs. Jenter comb box was monitored daily until hatching. In the day of hatching, Jenter comb box containing the 1st instar larvae were brought to the laboratory and placed in an incubator (Binder, Tutlingen, Germany) adjusted at 34ºC and 80% relative humidity. Larvae were then supplied with normal (control group) or *P. larvae*-infective (test group) freshly prepared pre-warmed artificial diets [(50% royal jelly (v/v), and 50% autoclaved aqueous solution of D-glucose (12% w/v) and D-fructose (12% w/v)]**[10]** for carrying out the experiment. Fresh royal jelly from a specialized professional Saudi beekeeper was used. This royal jelly provision was split into 5g doses which were frozen at -18°C until used.

Preparation of *Paenibacillus larvae*

The local isolate of *P. larvae* bacterium[9] was used in this study. An individual colony of the bacterium was picked up from brain heart infusion (BHI) (Oxoid Ltd., Hampshire, UK) agar plate, inoculated in a tube containing 10 ml of BHI broth, and incubated at 36ºC overnight to serve as pre-culture. After incubation, the bacterial suspension was heat-shocked at 80ºC for 10 min to kill non-spore forming bacteria[12] and was cultured on BHI agar plates at 36ºC for 10 to 14 days for sporulation. Spore suspensions of *P. larvae* were made by harvesting bacterial

colonies in sterilized distilled water. Microscopic count of spore suspension was made in a cell counting chamber (Thoma) under light microscope (40×). Spore suspension was adjusted to approximately 1×10^6 spores per ml for bioassay.

Oral infection of bees' brood

In the day of hatching, Jenter comb box containing the 1st instar larvae (within 12h post-hatching) were brought from bee's hive and kept in the laboratory as described above. The 1st instar larvae were categorized into 2 groups (20 larvae each) in Jenter comb box, incubated at 34ºC and 80% RH, and adequate artificial diet was added as described above until the end of experiments. Before any experiment, the required amount of diet was freshly prepared and stored at +5°C during the experiment. Pre-warmed at 34°C diets were provided to the larvae with a micro pipette once a day. The test group of larvae was provided with *P. larvae*-infective diet (containing 1×10^6 spores per ml) in the first day of the experiment.[13] Then, in daily manner, both control and test groups were provided with normal (un-infective) diet until the end of experiment. After 48 or 72 h, control and infected larvae (3rd instar) were grafted, separately.[10] Three random samples of the digestive tracts of each group of larvae were dissected out under dissecting microscope. Each larval gut was separated into three parts; foregut (honey crop), midgut (ventriculus) and hindgut (intestine), and immersed in 0.1 M cacodylate buffer, pH 7.3 containing 2% glutaraldehyde for carrying histological investigations. The rest of infected larvae were left until showing the symptoms of AFB (Figure 2).

Histological examination of brood midgut integrity

Light microscopy

Fore, mid and hindguts of the digestive tracts of control and *P. larvae*-infected larvae were examined under light microscope.[14] Briefly, midgut sections were fixed overnight in cold 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) and post-fixed for 1h at room temperature in 1% OsO4 in the 100 mM phosphate buffer. Sections were then dehydrated through an ascending ethanol series, treated with propylene oxide and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thin 10 µm sections were stained with hematoxylin/eosin (Sigma-Aldrich), mounted with Paramount (Fisher) then examined using Olympus BX50 microscope. Images were recorded with an Olympus DP11 photo camera, which were imported into Adobe Illustrator CC, 2017 Software and adjusted for contrast and suitable qualities.

Electron microscopy

Control and *P. larvae*-infected midguts were prepared for transmission electron microscopy investigations at 48 and 72 h post-infection. Midguts were sectioned, fixed and stained routinely as described previously[15] with some modifications. Briefly, midgut sections were fixed overnight in cold 0.8% glutaraldehyde, 4% Paraformaldehyde in 0.1 M sodium cacodylate (pH 7.0) and post-fixed for 4h at 4°C in 1% OsO_4 in 0.1 M sodium cacodylate (pH 7.0). Tissues were dehydrated through an ethanol series, treated with propylene oxide and embedded in Epon-Araldite resin (1:1). Ultrathin 4 µm sections were mounted on slides, stained with 2% uranyl acetate for 30 min, and then incubated in lead citrate for 10 min. Investigated tissue sections were analysed using transmission electron microscope model JEOL JEM-100CX II at 80 kV.

RESULTS

In the current study, Jenter comb box (Figure 1) was used for queen egg laying inside the hive to ensure production of broods of the same age in natural conditions. However, brood infection with *P. larvae* was carried out outside the hives in a sterile precocious laboratory conditions. The resulting data demonstrate that oral infection of *A. mellifera jemenitica* 1st instar larvae with *P. larvae* spores resulted in AFB disease symptoms prior to pupation (Figure 2). AFB Symptoms were clearly obtained with 100% infection at the end of experiment (6 day- post-infection). Thus, the histological integrity of the alimentary canal was investigated 48 and 72h post-infection. At 48h post-infection, histological investigation

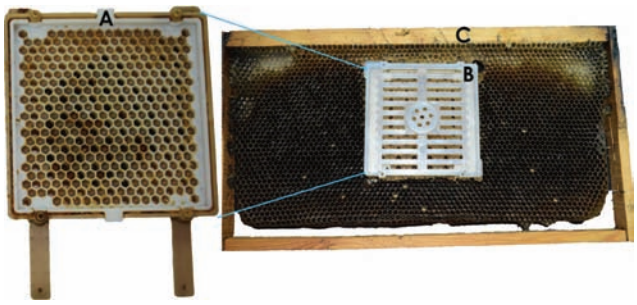

Figure 1: Jenter comb box used for egg laying and hatching within the hive. A: without the cover; B: with cover inserted within the wax comb (C). The system was installed as shown and each queen was added to it and entombed with the cover (B) which has openings smaller than its body size, while workers were allowed to freely get in and out for serving the queen and eggs. After egg laying, the queen was allowed to be free, the system was kept as it is in the hive (for 3 days later), then was relocated to the laboratory for experimental purposes.

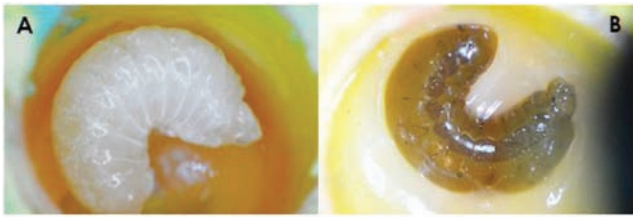

Figure 2: *In vitro* reared brood (of the same age) from an experimentally control (A) and *P. larvae* oral infection (B) 4th instar larvae (6 days post-hatching). Uninfected larvae developed normally until last larval instar. Infected larvae were perished as engorged totally black larvae, that were commended by *P. larvae* into the characteristic ropy mass by the end of experiment Olympus stereo microscope X200.

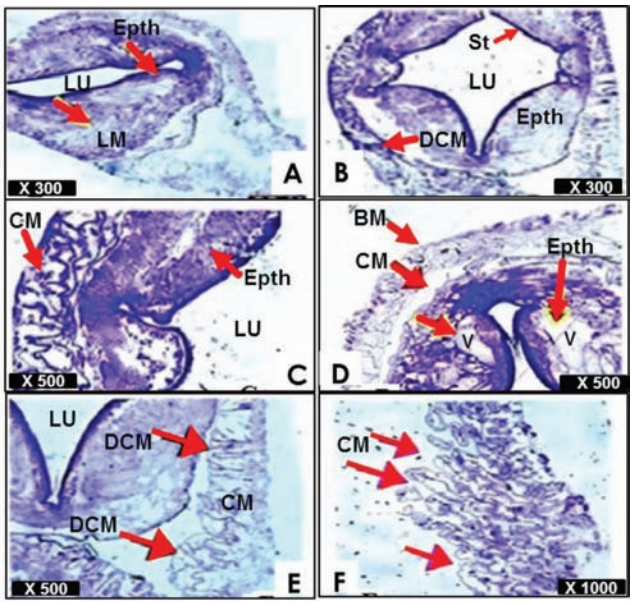

Figure 3: Light micrograph of hematoxylin/eosin stained transverse sections in the foregut (proventriculus) of *A. mellifera jemenitica* brood. A: shows Non-infected (control) with normal histological integrity. B: shows Histolpathological alterations of the infected proventriculus 48h post-infection with detached circular muscle layer (DCM). C: Normal epithelium and muscle layers. D: Separated muscle layers and degraded epithelium with clear vacuoles (V). E and F: Whole circular muscle layer proliferation with severe impairment 72h post-infection with *P. larvae*.
Epth: Epithelial layer; CM: Circular muscles; LU: Lumen; V: Vacuoles; BM: Basement membrane.

of foregut showed alteration in the histological integrity (Figure 3). Many vacuoles of different sizes within the epithelial layer were observed (Figure 3D). Moreover, laceration in the surrounding basement membrane, damage of circular muscle and separation from epithelial layer were observed (Figure 3D). At 72 h post-infection, foregut showed severe degradation of the circular muscle layer and the striated border (Figure 3E and F).

Infected midguts showed signs of histological alterations by 48h post-*P. larvae* infection as degradation in peritrophic membrane and epithelial layer (Figure 4). Vacuoles of different sizes were also observed in the epithelial layer, as well as separation and degradation of the circular muscle layer (Figure 4D). Increased number of vacuoles and degradation were observed in all the components of muscles and epithelial cells at 72h post-infection (Figure 4E and F).

Infected hindgut showed detachment of longitudinal muscular layer and vaculation at 48h post-infection (Figure 5C and D). Damages were observed with heavy deployment and increased vacuoles within the epithelial cells 48 post-infection (Figure 5E and F). Infected hindgut epithelium also showed the presence of intra- and intercellular aggregation communities of *P. larvae* bacteria at 72h post-infection (Figure 5G and H).

In order to examine the impact of *P. larvae*-infection on the sub-cellular organelles, TEM was employed. Strikingly, TEM data clearly showed severe damage at the mitochondrial and nuclear levels (Figures 6 and 7) in the

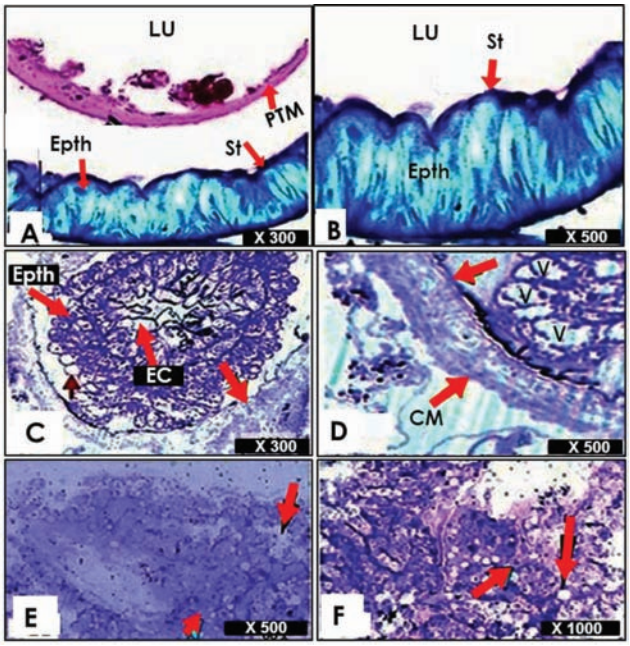

Figure 4: Light micrograph of hematoxylin/eosin stained transverse sections of midgut (ventriculus) of *A. m. jemenitica* brood. A and B: Non-infected (control) midguts with normal histological integrity. C: Histological integrity of control midgut with its epithelium (Epth) and enzyme cells (EC). D: Infected midgut showing histological alterations 48h post-infection with clear vacuoles (V) within the epithelium and detachment of circular muscles (CM). E and F: Severe histological deterioration and vacuolation of the epithelial cells 72h post-infection with *P. larvae*.
Epth: Epithelial layer; PTM: Peritrophic membrane; St: Striated border; CM: Circular muscles.

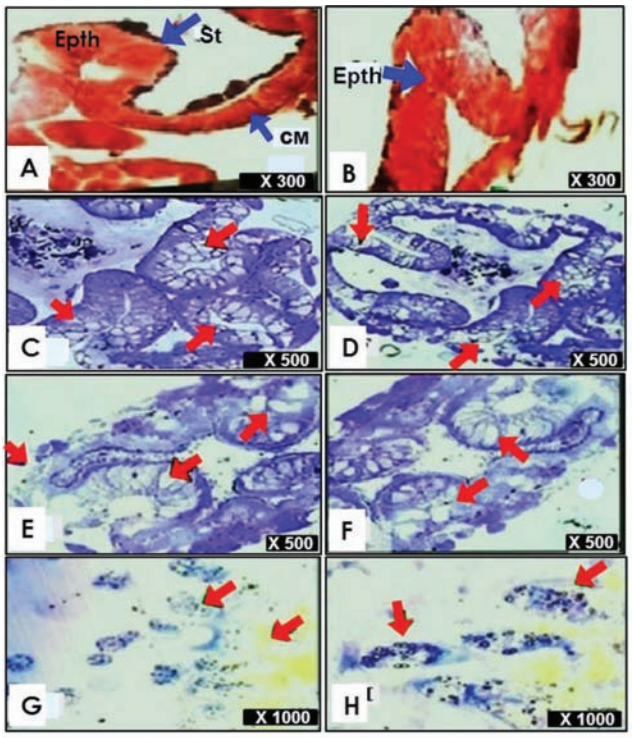

Figure 5: Light micrograph of hematoxylin/eosin stained transverse sections of *A. mellifera jemenitica* brood hindguts. A and B: Non-infected (control) sections with normal histological integrity. C and D: Histopathological alterations with of different vacuoles (arrows) 48 h post-infection. E and F: 72h post-infection showing heavy deployment and increased vacuoles (arrows) within the epithelial cells. G and H: Intra and intercellular aggregation of *P. larvae* bacterial cells 72 h post-infection.
St: Striated border, Epth: epithelial cells, CM: circular muscles.

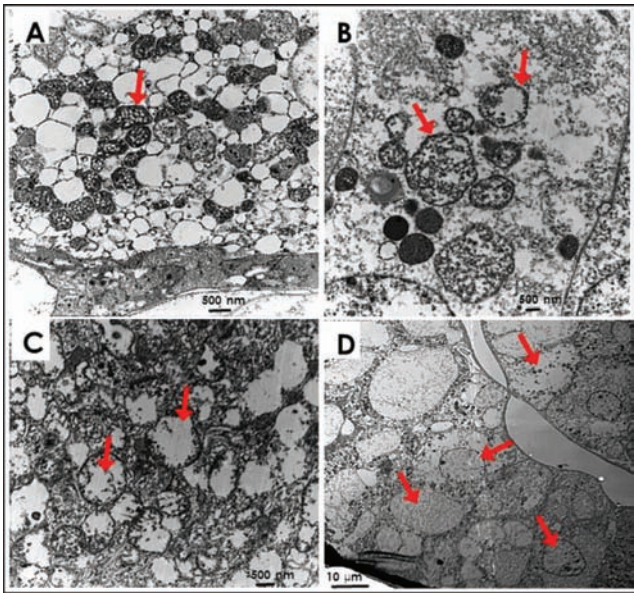

Figure 6: Electron microcopy panel of transverse sections of *A. mellifera jemenitica* brood midgut. Mitochondrial degradation and laceration 48h post-infection with *P. larvae* (B) compared to control ones (A). Complete degradation 72h post-infection (C and D).

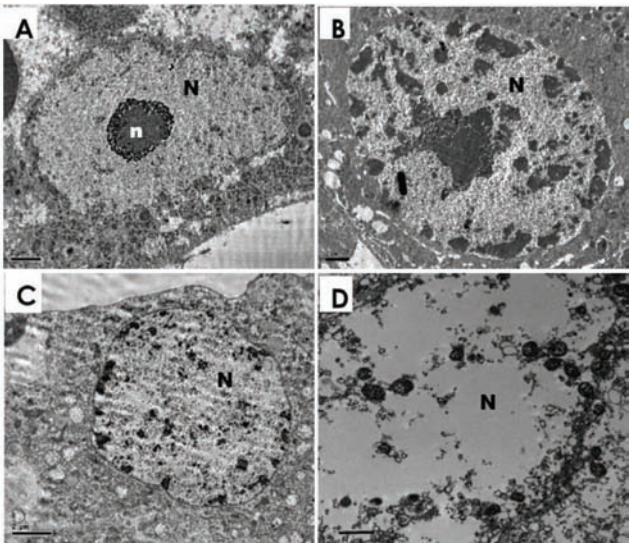

Figure 7: Electron microcopy panel of transverse midgut sections of *A. mellifera jemenitica* brood. Nuclear proliferation of epithelial cells 48h post-infection with *P. larvae* (B) compared to control one (A). Complete degradation and distortion of the nuclear chromatin contents 72h post-infection (C), and finally, cellular collapse (D).
N: nucleus; n: nucleolus.

midgut epithelium by 48h post-infection. The observed mitochondrial damage, visualized as clear white spots (Figure 6B), as early as 48 h post-treatment compared to untreated control (Figure 6A). These white spots increased as by time until mitochondria become empty-like vacuoles (Figure 6 C and D) 72 h post-infection. On the other hand, nuclear degradation in the epithelial cells was also observed as early as 48h post-infection compared to control ones (Figure 7 A and B). This deficiency was clear in the nuclear compartments, including nucleoli and chromatin materials, which increased by time until complete vanishing (Figure 7 C), and finally, epithelium collapse was reached at 72 h post-*P. larvae* infection (Figure 7D).

DISCUSSION

Honeybees are of great ecological and economic importance as they are the major pollinators of many cultivated crops and plants.[16] On the other hand, some significant challenging factors are limiting beekeeping worldwide. The first of these factors is the devastating beekeeping-threatening disease, the AFB, which infect honeybees larval stage (brood) by *P. larvae*.[5] Hence, in the presented study herein, it is important to clarify six points; a) beekeeping is an important economic subsector in the rural communities of Saudi Arabia. This is because beekeeping is an important source of income for most of the rural households, b) the local honeybee, *A. mellifera jemenitica,* is the only indigenous and smallest

honeybee race of *A. mellifera mellifera* in Saudi Arabia, well adapted to the local harsh environmental conditions including high temperatures and lack of rainfall, and can resistant *Varroa* mites[17,18] and tolerate hunger for long periods of time compared to the imported *A. mellifera carnica,*[19] c) we have currently recorded AFB in a Saudi apiary for the first time,[9] which might have been invaded the Saudi apiaries through the exchange of hive materials among bee colonies, handling numerous hives in a limited area, and trading of packaged bees and bee's products including honey.[5] This, in our opinion, is the current main challenge facing the beekeeping industry in Saudi Arabia, d) we are urgently in demands for strong efforts to keep this newly emerged honeybee food-borne disease under control, e) the current study was conducted as a first step towards controlling this disease by investigating whether this locally isolated *P. larvae* destroys the brood of the Saudi bee, *A. mellifera jemenitica* via the pathogenicity mechanism recorded in other honeybees[5] and finally f).[13] It was-reported that larvae of 24-30 h old (after hatching) are very susceptible to *P. larvae* infection, however, larvae infected in the third instar and beyond do not show signs of the disease.[13] Thus, in the current study, the 1st instar larvae (within 12h post-hatching) were orally infected with *P. larvae*, and the histological impact that might have been occurred by the gut invading *P. larvae* bacteria, were investigated 48 and 72h post-infection.

It has been established that AFB is a contagious disease, in which infection starts in an individual 1st instar larva, then, can ultimately cause an entire colony collapse. *P. larvae* infection is transmitted to larvae by two ways; by nurse bees or by remnant spores at the base of a brood cell. After passing through the fore gut, the ingested spores germinate in the mid gut of the infected *P. larvae* around 12 h post-ingestion.[20] In the midgut, vegetative bacteria proliferate and colonize the midgut epithelium within few days post-infection.[20] It has been reported that peritrophic membrane develops in honeybee larvae within 8 to 36h.[21,22] This membrane presents an effective barrier against infection. It is due to this characteristic it has been, postulated that older larvae and adult possess resistance to *P. larvae* infection.[23] Thus, peritrophic membrane of the infected larvae helps to retain the bacterial mass in the lumen of the midgut during the period of bacterial proliferation. Data of the current study recorded aggregation of *P. larvae* in the gut epithelium of *A. mellifera jemenitica* larvae within 48 h post-infection, which may indicate that bacterial proliferation and infection could have happened within this period. Then *P. larvae* penetrate and destroy this protective layer[24] that attack the epithelium during later stages of infection. Breaching of the epithelium occurs *via* the paracellular space to enter the hemocoel where they migrate and proliferate.[25] Finally, rupturing the epithelial layer results in larval death.[5]. This is in agreement with Yue *et al*.[20] who investigated that *P. larvae* spores germinate12h post-ingestion, colonize and proliferate massively in the midgut of infected larvae. And subsequently, destroy the epithelium to enter the hemocoel and cause larval death within days post-infection. Data of the current study proved that oral infection of *A. mellifera jemenitica* 1st larvae (within 12h post-hatching) resulted in strong evident symptoms of AFB in the engorged 3rd – 4th instars (Figure 2). It has been also reported that diseased pupae are an extremely rare event, because infected larvae normally die as engorged larvae. He also investigated that *P. larvae* use their proteases for disrupting the midgut epithelium and for the subsequent degradation of the larval remains to the ropy stage (a brownish, semi-fluid, glue-like colloid). It was also recorded that the ropy stage dries down to a hard mass (foulbrood) tightly adhering to the lower cell wall.[5] Consequently, the resulting foulbrood masses are highly infectious since they contain large amounts of spores which drive disease transmission within and between colonies and hence complete collapse of the hive.[26] Digestive tract of the vast majority of insects is divided into foregut, midgut and hindgut. In bees, in particular, the foregut consists of the pharynx, oesophagus, crop and proventriculus, the midgut corresponds to the ventriculus and the hindgut is divided into ileum and rectum.[27] Data of the current study showed that peritrophic membrane and epithelial cells were homogenous in control guts, compared to infected larvae that showed signs of degeneration. These observations are in agreement with those reported by[28] Furthermore, the current study showed histological destruction of the different parts of the alimentary canal which was clearly observed in the midgut of infected larvae. This severe destruction[5] *P. larvae* use their proteases for disrupting epithelial barrier integrity by degrading cell–cell as well as cell–matrix junctional structures, thereby, *P. larvae* can invade the hemocoel, where they further proliferate. In this context, it has been proposed that the possible mechanism of invasion of *P. larvae* into *Apis mellifera* gut could be due to the activiation of specific receptors on the cell surfaces that initiate phagocytosis. These disruptions may be attributed to the proteases secreted by *P. larvae*.[29,30]

The observed gut epithelium vacuoles in the current study could be attributed to the generalized separation and detachment of the epithelial cells, which increased laceration and vacuole formation as described by *mellifera*.[31] These histological alterations have been

shown on the sub-cellular level in terms of mitochondrial degradation leading to a complete degradation of each single mitochondrion. Further, nuclear deprivation was also observed which started as ruin of the nuclei as well as chromatin materials that completely collapsed.

Taken all together, results of the current study provide solid evidence that *A. mellifera jemenitica* brood showed vulnerability to *P. larvae* infection via the same histopathological mechanism recorded in previous similar studies on *Apis mellifera*. These findings may shed light on the demand to achieve more intensive researches towards controlling and limiting this apiary-threatening disease from escalating through the country. Hence, Immune responses of *A. mellifera* larvae are currently under investigation as to purify anti-*P. larvae* immune peptides from challenged indigenous honey bee strains aimed at usage as insect-derived natural antibiotic towards AFB.

CONCLUSION

The primary objective of the current study was to investigate the vulnerability of the Saudi honeybee, *A. m. jemenitica* to the AFB disease newly emerged in some Saudi apiaries, and to shed some light towards histopathological mechanisms of the locally isolated *P. larvae* bacterial infections in newly hatched honeybee broods. Thus, oral infection of 1st instar larvae resulted in AFB symptoms in the 3rd-4th instars. Histopathology results investigated using light and transmission electron microscopy revealed that oral infection brought about a massive destruction of the larval midgut epithelium as compared to untreated control one. Moreover, this observed histological damage was associated with severe distortion on the sub-cellular organelle level to the mitochondria and nuclei. These finding may indicate that *A. mellifera jemenitica* brood are via the same mechanism known in other honeybees and that this observed cellular destruction might take place by means of the breach of *P. larvae* proteases.

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CONFLICT OF INTEREST

None of the authors of this article has any conflict of interest.

CONTRIBUTION OF THE AUTHORS

The project was conceived and designed by Tahany H. Ayaad and Ashraf M Ahmed. Experiments were carried out by Mariam S. Ghamdi and Tahany H. Ayaad. The manuscript was written by Ashraf M. Ahmed, Nikhat J. Siddiqi and Tahany H. Ayaad. Honeybee rearing was carried out in the laboratory of Bee Research Centre under supervision of Ahmad A. Al-Ghamdi, bacterial culturing and maintenance was carried out by Mohammad J. Ansari, and finally, grafting and supply of bee's larvae was carried out by Abdelsalam A. Mohamed.

ABBREVIATION USED

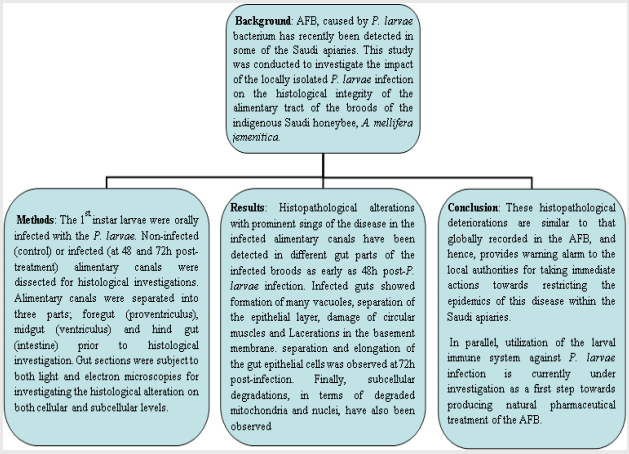
AFB: American foulbrood; ***P. larva:*** *Paenibacillus larvae*; ***A.mellifera larvae:*** *Apis mellifera jemenitica*; **EFB:** European foulbrood; **MBC:** methyl benzethonium chloride; **BHI:** Brain heart infusion; **RH:** Relative humidity; **DCM:** Detached circular muscle; **V:** Vacuoles; **Epth:** Epth: Epithelial layer; **CM:** Circular muscles; **BM:** Basement membrane; **TEM:** Transmission electron microscopy; **Epth:** Epithelial layer; **PTM:** Peritrophic membrane; **EC:** Enzyme cells; **N:** Nucleus; **n:** nucleolus; **1st:** first; **MAARIFAH:** National Plan for Science, Technology and Innovation.

REFERENCES

1. Kay AD, Bruning AJ, Alst A, Abrahamson TT, Huges WO, Kaspari M. A carbohydrate rich diet increases social immunity in ants. Proc Biol Sci. 2014;281(1778):20132374.
2. Engelsdrop D, Meixner MD. A historical review of managed honey bee populations in Europe and United States and the factors that may affect them. J Invert Pathol. 2010;103:S80-S95.
3. Genersch E, Ashiralieva A, Fries I. Strain- and genotype-specific differences in virulence of Paenibacillus larvae subsp. larvae, a bacterial pathogen causing American foulbrood disease in honeybees. Appl Environ Microbiol. 2005;71(11):7551-5.
4. Evans JD, Spivak M. Socialized medicine: individual and communal disease barriers in honey bee. J Invertebra Pathol. 2010;103(Suppl 1):S62-S72.
5. Genersch E. Honeybee pathology: current threats to honeybees and beekeeping. Appl Microbiol Biotech. 2010;87(1):87-97.
6. Adgaba N, Al-Ghamdi A, Sammoud R, Shenkute A, Tadesse Y, Ansari MJ, *et al*. Determining spatio-temporal distribution of bee forage species of Al-Baha region base on ground inventorying supported with GIS application and Remote Sensed Satellite Image Analysis. Saudi J Biol Scie DOI. (2017): http://dx.doi.org/10.1016/j.sjbs.2017.01.009
7. Alqarni AS, Hannan MA, Owayss AA, Engel MS. The indigenous honeybees of Saudi Arabia (Hymenoptera, Apidae, *Apis Mellifera jemenitica* Ruttner): Teir natural history and role in beekeeping. ZooKeys. 2011;134:83-93.
8. Al-Ghamdi A, Ansari MJ, Nuru A, Ahmed AM, Tahany HA, Khan KA, *et al*. Diagnosis and molecular detection of *Paenibacillus larvae*, the causative agent of American Foulbrood in honeybees in Saudi Arabia. Int J Trop Insect Sci. 2017;(In Press).
9. Ansari MJ, Al-Ghamdi A, Nuru A, Ahmed AM, Tahany HA, Al-Qarni A, *et al.* Survey and molecular detection of Melissococcus plutonius, the causative agent of European Foulbrood in honeybees in Saudi Arabia. Saudi J Biol Sci. 2017;(In Press).

10. Aupinel P, Fortini D, Dufour H, Tasei J, Michaud B, Odoux J, *et al*. Improvement of artificial feeding in a standard *in vitro* method for rearing *Apis mellifera* larvae. Bull Insectology. 2005;58(2):107-11.
11. Beespoke.info (2014). http://beespoke.info/2015/06/06/jenter-kit-instructions/
12. Shimanuki H, Knox DA. Diagnosis of honeybee diseases. Agri Handbook USDA. 2000;690:1-57.
13. Brødsgaard CJ, Ritter W, Hansen H. Response of *in vitro* reared honey bee larvae to various doses of *Paenibacillus larvae larvae* spores. Apidologie Springer Verlag. 1998;29(6):569-78.
14. Villalon JM, Ghosh A, Jacobs-Lorena M. The peritrophic matrix limits the rate of digestion in adult *Anopheles stephensi* and *Aedes aegypti* mosquitoes. J Insect Physiol. 2003;49(10):891-95.
15. Ahmed AM, Abdel Megeed AM, Al-Qahtaney HM. A Novel Mosquitocidal Bacterium as a Biocontrol Agent in Saudi Arabia: I - A Promising Larvicide Against *Aedes caspius* Mosquito. Pak J Zool. 2014;46(1):191-201.
16. Free J B Insect Pollination of Crops. Academic Press, 1993, London.
17. Ahmad AA, Hoopingarner R. Studies on different resistant traits of honeybees against *varroa* mites using model simulation. Pak Entomol. 2002;24(2):2002.
18. Khanbash MS. Study the tolerance mechanisms of Yemeni honeybee *Apis mellifera jemenitica* to *Varroa jacobsoni* Oud. J Nat Appl sci. 2002;6:109-18.
19. Abou-Shaara HF, Al-Ghamdi AA, Mohamed AA. Tolerance of two honey bee races to various temperature and relative humidity gradients. Environ Exp Biol. 2012;10:133-8.
20. Yue D, Nordhoff M, Wieler LH, Genersch E. Fluorescence in situ hybridization (FISH) analysis of the interactions between honeybee larvae and *Paenibacillus larvae*, the causative agent of American foulbrood of honeybees (Apis mellifera). Environ Microbiol. 2008;10(6):1612-20.
21. Davidson EW. Ult rastructure of peritrophic membrane development in larvae of the worker honey bee (*Apis mellifera*). J Invertebr Pathol. 1970;15(3):451-4.
22. Bamrick JF. Resistance to American foulbrood in honey bees. Comparative pathogenesis in resistant and susceptible larvae. Insect Pathol. 1964;6(3):284-304.
23. Sturtevant AP, Revell IL. Reduction of *Bacillus larvae* spores in liquid food of honey bees by action of the honey stopper, and its relation to the development of American foulbrood. J Econ Entomol. 1953;46(5):855-60.
24. Garcia-Gonzalez E, Generesch E. Honeybee larva peritrophic matrix degradation during infection with *Paenibacillus larvae*, the etiological agent of American Foulbrood of honeybees, is a key step in pathogenesis. Environ Microbiol. 2013;15(11):2894-901.
25. Djukic M, Brzuszkiewicz E, Fűnfhaus A, Voss J, Gollnow K, Poppinga I, *et al.* How to kill the honeybee larva: Genomic Potential and Virulence Mechanisms of *Paenibacillus larvae*. 2014;PloS One:93-e90914.
26. Lindström A, Korpela S, Fries I. The distribution of *Paenibacillus larvae* spores in adult bees and honey and larval mortality, following the addition of American foulbrood diseased brood or spore-contaminated honey in honey bee (*Apis mellifera*) colonies. J Invertebr Pathol. 2008;99(1):82-6.
27. Santos CG, Serrao JE. Histology of ileum in bees (HYMENOPTERA, APOIDEA). Braz j morphol sci. 2006;23(3,4):405-13.
28. Dussaubat C, Brunet JL, Higes M, Colbourne JK, Lopez J, Choi JH, *et al.* Gut pathology and responses to the microsporidium *Nosema ceranae* in the honey bee Apis mellifera. PLoS One. 2012;7(5):e37017.
29. Gregorc A, Bowen ID. Histopathological and histochemical changes in honeybee larvae *Apis mellifera* L. after infection with *Bacillus larvae*, the causative agent of American foulbrood disease. Cell Biol Int. 1998;22(2):137-44.
30. Antunez K, Arredondo D, Anido M, Zunino P. Metalloprotease production by *Paenibacillus larvae* during the infection of honeybee larvae. Microbiol. 2011;157(5):1474-80.
31. Szymas B, Je druszuk A. The influence of different diets on haemocytes of adult worker honey bees, *Apis mellifera*. Apidologie. 2003;34(2):97-102.

PICTORIAL ABSTRACT



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SUMMARY

- American foulbrood (AFB), caused by Paenibacillus larvae (P.larvae) bacterium has recently been detected in some of the Saudi apiaries.
- The impact of this locally isolated P. larvae infection on the histological integrity of the alimentary tract of the indigenous Saudi honeybee brood, A.mellifera jemenitica, has been investigated.
- Histopathological alterations with prominent sings of the disease in the infected alimentary canals as early as 48h or 72h post-P. larvae infections have been detected in
- different gut parts of orally infected brood viz. pro-ventriculus , ventriculus(midgut) and hindgut .
- These histopathological deteriorations have also been evaluated and confirmed on the subcellular level via TEM that revealed complete degradation and distortion of nuclei and mitochondrial compartments.
- In conclusion AFB has been detected for the first time in certain Saudi apiaries and its impact on the indigenous A. mellifera jemenitica brood gut has been confirmed with prominent deterioration of brood health leading to complete perish .
- Further investigation of this local honeybee race immune response towards such serious beekeeping threatening disease is currently under investigation.

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