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Original article

Probing the interaction of *Paenibacillus larvae* bacteriophage as a biological agent to control the american foulbrood disease in honeybee

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ABSTRACT

American foulbrood (AFB) is a harmful honeybee disease primarily caused by *Paenibacillus larvae*. The study aims to isolate and identify the AFB causative agent *P. larvae* and their specific phages to use as a new biological method for AFB disease control. Eight apiaries were inspected for AFB infections. Symptoms of diseased brood comb, were odd brood cells with soft brown decayed brood amongst healthy brood, were identified in the field and demonstrated the prevalence of AFB in every apiary. Three *P. larvae* isolates were identified using traditional techniques using a 452-bp PCR amplicon specific to the bacterial 16SrRNA gene and was compared between *Paenibacillus* isolates. Additionally, specific phages of *P. larvae* strains were applied to examine their efficiency in reducing the infection rate under the apiary condition. The infection rate was reduced to approximately 94.6 to 100 % through the application of a phage mixture, as opposed to 20 to 85.7 % when each phage was administered individually or 78.6 to 88.9 % when antibiotic treatment was implemented. Histological studies on phage-treated bee larvae revealed some cells regaining normal shape, with prominent nuclei and microvilli. The gastrointestinal tract showed normal longitudinal and circular muscles, unlike bee larvae treated with bacterial strains with abnormal and destroyed tissues, as shown by the basement membrane surrounding the mid-gut epithelium. Phage techniques exhibited promise in resolving the issue of AFB in honeybees due to their ease of application, comparatively lower cost, and practicality for beekeepers in terms of laboratory preparation.

1. Introduction

Maintaining honeybee numbers as high as possible is a major topic due to their most significant role in crop pollination and their products. So, the control of diseases that reduce their populations, such as the American foulbrood (AFB), is caused by *Paenibacillus larvae* and European foulbrood (EFB), which is caused by *Melissococcus pluton* is crucial (Genersch, 2010a). AFB, one of the most detrimental bee illnesses, is responsible for colony collapse disorder. After hatching, honeybee larvae are most vulnerable to attack 12–36 h later. Upon ingestion, *P. larvae* spores sprout in the midsection of the gut and swiftly proliferate, causing epithelial cell injury (Ghorbani-Nezami et al., 2015). It eliminates infected colonies as well as contaminated larvae. Dead larvae decompose upon drying into a ropy mass comprising scales comprising millions of spores readily transmissible between colonies (Genersch, 2010b). During early infection, the highly adapted *P. larvae* kill brood through secretion of secondary metabolites as well as chitin-degrading enzymes that allow breaching of the midgut epithelium and decomposition of the larva to a ropy mass (Daisley et al., 2020).

Previous comparative genome analysis and, more recently, the elucidation of the bacterial genome provided evidence that this bacterium harbors putative functional nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) and, therefore, might produce nonribosomal peptides (NRPs) and polyketides (PKs). Such biosynthesis

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products have been shown to display a wide range of biological activities, such as antibacterial, antifungal, or cytotoxic activity (Garcia-Gonzalez et al., 2014; Daisley et al., 2023). Spores of the causative agent in extreme conditions, including heat and dehydration, do not affect *P. larvae*, which can retain their infectiousness for up to 35 years, making controlling AFB difficult (Ghorbani-Nezami et al., 2015).

Meanwhile, the spores can be transmitted via different honeybee products like honey, pollen, royal jelly, wax debris, and adult workers (Erban et al., 2017). The symptoms of infected bees are represented in an irregular capped brood pattern, gummy larval cells that "rope" out upon insertion of a toothpick, foul, unpleasant odor, and a hard black scale that is difficult to remove with a pupal tongue (Ya et al., 2023).

Traditional and advanced methods were utilized to identify *P. larvae*. PCR methods for determining and genotyping the pathogen have been extensively developed, as described by Govan et al., (1999) who exploited molecular techniques for identifying *P. larvae* isolated from different apiary sources. In addition, Dobbelaere et al., (2001) reported using PCR to rapidly identify *P. larvae* using primers derived from gene regions encoding 16SrRNA (rDNA). They selectively amplified a segment of the 16SrRNA gene of *P. larvae* from pure culture spores in experimentally contaminated honey samples and larvae exhibiting clinical symptoms using polymerase chain reaction (PCR) (Piccini et al., 2002). Generally, molecular techniques such as PCR and 16SrRNA sequencing provided rapid and reliable methods for identifying *P. larvae* (Ashiralieva and Genersch, 2006).

Different techniques are widely applied to AFB control. Burning colonies and contaminated hive material are expensive for the beekeeping community (Mosca et al., 2023; Nilsson et al., 2024). It results in the loss of both hive materials and productive hives, and the shook swarm method, which involves transferring the adult bees to a disease-free hive without drawn combs and destroying the brood combs of the infected colony (Genersch, 2010a; Higes et al., 2014). Another common strategy for the prevention and treatment of affected colonies is the usage of antibiotics; however, antibiotics use is not permanent and lacks sufficient recovery due to many reasons: i) increasing antibiotic resistance among *P. larvae* strains (Tian et al., 2012); ii) accumulating of chemical residues in honey and honeybee products which decreasing the quality and hindering the marketing (Oliveira et al., 2015); iii) and having the potential to disrupt the normal microbial ecology of the honeybee gut microorganisms (Olofsson and Vasquez, 2008).

The prevalence of AFB, for which no effective and secure treatments, necessitates alternative and critical therapeutic strategies. One of these is the use of bacteriophages (Oliveira et al., 2015). They are abundant in nature and self-propagating, and Viruses specific to their hosts can infect and lys bacteria to target specific strains (Sheflo et al., 2013). Although numerous studies have investigated the properties of phages extracted from lysogenic strains of *P. larvae*, there is currently no report on phage therapy that targets AFB specifically. Consequently, phage therapy as a potential treatment for AFB should be investigated (Mosca et al., 2023; Nilsson et al., 2024). Phage-mediated interventions targeting *P. larvae* could reduce reliance on destructive treatments for infected colonies. Due to their specificity, they would not damage pollinators, humans, or essential microbes. Therefore, applying phage therapy to control AFB is economically and environmentally beneficial (Yost et al., 2016). The current study aims to isolate and identify the AFB causative agent Paenibacillus larvae and their specific phages to use as a new biological and advanced method for AFB disease control.

2. Materials and methods

2.1. Collection of honeybee brood combs infected with AFB

Infected honeybees (*Apis mellifera* L.) brood combs with AFB were collected from eight apiaries from different locations. Holst milk test was performed on all collected combs to differentiate between *P. larvae* and *M. pluton,* the causative agents of AFB and EFB diseases, respectively, using the method described by Genersch (2010a).

2.2. Isolation of P. larvae the cause of AFB

To isolate causative AFB, honeybee larval remained from the brood comb were collected with a sterile swab and suspended in 10 mL of sterile 0.01 *M* phosphate buffer saline (PBS, Sigma, USA) in a test tube and heat-shocked in a water bath at 80 °C for 10 min, to kill vegetative bacterial cells of other microorganisms, homogenized by vortex and series of dilutions were prepared for isolation. The MYPGP (Mueller-Hinton broth, Yeast extract, Potassium-phosphate, Glucose, and Pyruvate, Oxiod, UK) medium (Dingman and Stahly, 1983) amended with nalidixic acid 10 μg mL^{-1} was used for the isolation of *P. larvae*. Then, incubated anaerobically at 37 ± 0.5 °C and examined daily for 2–7 days (De Graaf et al., 2006).

2.3. Identification of the selected isolates

Microbiological and biochemical characterization was used, according to Hoyo et al. (2001). Along with these, an artificial infection procedure in the apiary was carried out, according to Yost, (2014). The standard strain of *P. larvae*, identified by 16S rRNA gene sequence analysis, was used as a standard strain for the identification procedures. Total DNA was extracted from fresh bacterial cells (overnight culture) according to Nonthapa and Chanchao's method (2015). Thermo Fisher Scientific Inc. synthesized the primers utilized in this research per the method outlined by Tama et al., (2003). The PCR primers employed in this study were derived from a region of the 16SrRNA gene specific to *P. larvae*. A 452 bp PCR amplicon, which was exclusive to *P. larvae*, was amplified by the primers. Upon comparing all known DNA sequences in the accessible databases, the primers demonstrated homology exclusively to *P. larvae* (Table 1).

2.4. Bacteriophages

To find and describe environmental bacteriophage, we obtained the samples from three diverse sources: dead bees, the soil underneath the hives, and wastewater. The selection of bacteriophages was determined by the vulnerability of the identified P. larvae isolates to lysis and the standard strain. Phage presence was ascertained by developing plaques on soft agar overlays containing *P. larvae* (Yost, 2014). Pure cultures of isolates were prepared on MYPGP agar plates, 24 h following incubation, then 10 μL of the filtered isolated bacteriophage was added to each bacterial culture plate and incubated at 37 °C ± 0.5 for 24 h later on; each isolated bacteriophage was tested against all examined bacteria. The studied bacteria were dispersed at a concentration of 10^7 CFU mL^{-1} in sterile distilled water. Plates of basal medium were then coated with a 0.5 mL mixture containing 2 mL of melted semi-solid agar MYPGP medium. After placing 20 μL of phage suspension on the agar over the layers, these plates were incubated at 37 °C ± 0.5 for one night. Positive reactions were denoted by clear convergent lysis, turbid confluent lysis, or individual plaques; extremely indistinct zones denoted negative reactions. (Ghorbani-Nezami et al., 2015).

2.5. Apiary experiment

This experiment was carried out to examine the ability of the isolated bacteriophages to control the isolated bacteria that can infect honeybees

Table 1
The sequences of forward and reverse primers (Chemurot et al., (2016):

Primer name	Sequences (5′ to 3′)	Target	Product size (bp)
AF1-F	GCTCTGTTGCCAAGGAAGAA	*P. larvae*	452
AF2-R	AGGCGGAATGCTTACTGTGT		

(*Apis mellifera* L.) and cause AFB disease under apiary conditions. Each identified bacterial isolate was grown on MYPGP broth medium under shaking at 125 rpm at 37 °C ± 0.5 for 72 h for bacterial culture preparation. Alongside, each bacterium was mixed with its specific After cultivating the phage on MYPGP broth medium using a rotary agitator (Millipore, Bedford, MA, USA) at 125 rpm for 15 min, the sample was centrifuged at 10,000 xg for 15 min to eliminate any bacterial cells or detritus. The resulting supernatant was filtered through a 0.22 μm membrane. The filtrate was diluted ten-fold dilutions with sterilized water (10^1 plague forming units (PFU) to prepare high titer suspensions of phages Tanaka et al. (1990). As well as, Oxytetracycline (55 mg g^{-1} powder) was obtained from Al-Nasr Company for Medicinals and Antibiotics, Abu-Za'bal, Kalyobiya, Egypt to use for antibiotic treatment at a concentration of 1g L^{-1} sugar syrup/colony (Plavša et al., 2011).

2.5.1. Honeybee colonies infection

As previously mentioned, a liquid culture of each identified bacterial isolate plus the standard bacterial strain containing 10^8 mL^{-1} CFU was prepared (Arai et al., 2012). Liquid cultures of each specific phage were prepared and amended with 3 % cornflour and 5 % sucrose (Yost, 2014).

Honeybee combs were sprayed with bacterial culture and phages individually or in the mixture at a rate of (25 ml/comb) following (Balogh, Russo, & Russo, 2003). A randomized complete block design (RCBD) comprised of 28 treatments and three replicates was utilized to conduct this study. The following treatments were divided into five groups:

Group (1): Control (treated with a recommended dose of oxytetracycline) (4 treatments)

Group (2): treated with each bacterial strain (4 treatments)

Group (3): treated with each bacterial strain mixed with its specific phage (4 treatments)

Group (4): treated with each bacterial strain mixed with each of the other three phages separately (12 treatments)

Group (5): treated with each bacterial strain mixed with phages mixture (4 treatments)

The number of infected larvae and the infection reduction rate (%) were calculated according to methods described by De Graaf et al. (2013).

2.5.2. Histopathological studies

Larvae from all treatments were collected after 72 h and rinsed for several seconds with 70 % ethanol for light and Scanning Electron Microscope examinations (SEM, JEOL, UK). For routine histology by light microscopy, the method described by Takamatsu, Sato, and Yoshiyama (2016) was used to observe the differentiation in honeybee's mid-gut between all treatments. For Scanning Electron Microscopy (SEM), the method described by Kanbar and Engels (2002) was used to examine the differentiation in larvae morphology between all treatments.

2.6. Statistical analysis

The quantitative data were statistically based on standard deviation (SD), which was calculated as a mean of three replicates. Version 23 of the SPSS statistical program (SPSS Inc., USA) was used for all statistical computations, and Microsoft Excel 2019 was used to create the graphs.

3. Results

3.1. Collection of American foulbrood diseased brood combs

AFB symptoms survey from diseased combs, collected from 8 apiaries of six locations, is shown in Figure S1. Thirty-eight honeybee colonies from eight apiaries developed AFB symptoms distinguishable with the sealed broods. The odor of the colonies was strong, particularly in the late retrogressive cases; the sealed brood was discolored as very dark, contrary to the standard color; the brood cells capping were sunken inside the cells and punctured at more than one side; the color of the dead brood was light brown or coffee brown to dark and sometimes black; the brood which recently dead was soft and made a sticky or a ropy; much of these dead broods were dried and make black scales lie flat on the lower side of cells and adhered tightly to the cells walls Fig. 1.

3.1.1. Holst milk test

AFB can be diagnosed in the field by visual inspection; however, this diagnosis must be confirmed by the Holst milk test. This test effectively differentiates *P. larvae* and *M. pluton,* the causative agents of AFB and EFB, respectively. The most conspicuous clinical manifestation of AFB remains the pulpy or dark brown larval remnants that resemble adhesive. However, it is inconclusive: taken from the exhibited colonies were examined for the Holst milk test, where 68 % (26 out of 38) of the infected larvae gave a positive reaction. The milk curdled in less than 40 sec. On the glass slide, 32 % (12 out of 38) of the samples gave an adverse reaction.

3.1.2. Isolation and identification of P. larvae

Thritin isolates from AFB samples were isolated on MYPGP medium coded as Hb1, 2, 3, 4, …13, only 5 isolates, Hb2, Hb3, Hb4, Hb5, and Hb10 gave typical reactions with *P. larvae* (Table 2). The bacterial colonies had irregular edges and were flat and light grey under the light microscope.

3.1.3. 3.1.3. Artificial infection procedure in the apiary

The traditionally identified *P. larvae* isolates were subjected to an artificial experiment in the apiary to confirm their ability to cause the same clinical symptoms on honeybee combs. The larvae, fed separately with each of the five bacterial inoculums of *P. larvae*, exhibited most of the clinical symptoms of AFB disease relative to control, which developed into pre-pupae.

3.2. Comparison between standard strain and isolates of P. larvae by 16SrRNA

3.2.1. Detection of bacterial 16SrRNA gene

The results presented in Fig. 2 indicated that the amplifications with the AF1 and AF2 primers were positive in all strains; also, the AF_1 plus AF_2 primers detected *P. larvae* DNA in the three strains. A search for sequence similarity was conducted utilizing the Blast server of the National Center for Biotechnology Information. The PCR product had the same length (452–540 base pairs) as the RNA gene of P. larvae located among the primers. A sequence alignment between the result of PCR and the 16SrRNA gene sequence of *P. larvae* revealed that the two sequences were identical. Results illustrated by (Supplementary Figure S2) showed the sequence of this 452-bp PCR amplicon.

3.3. Bacteriophages

The identified *P. larvae* strains Hb2, Hb3, and Hb10 that can cause the clinical symptoms on honeybee combs, in addition to the standard strain *P. larvae*, were subjected to their specific phage isolation. Three sources for isolation were: i) dead bees, ii) soil under the hives, and iii) wastewater. Data in Table 3 indicated that lysogenic phages of *P. larvae* strains were found in all used sources. The highest lytic phages with (+++) were 50 % from dead bees and 50 % from soil under the hives, according to the ability of the yielded phages to lyse *P. larvae* strains (Supplementary Figure S3).

3.4. Apiary experiment

3.4.1. Infection reduction rate (%) in the apiary

Honeybee brood combs sprayed with the inoculum of each bacterial strain (control) gave the highest number of infected larvae compared to other treatments, where the number of infected larvae ranged from 10 to

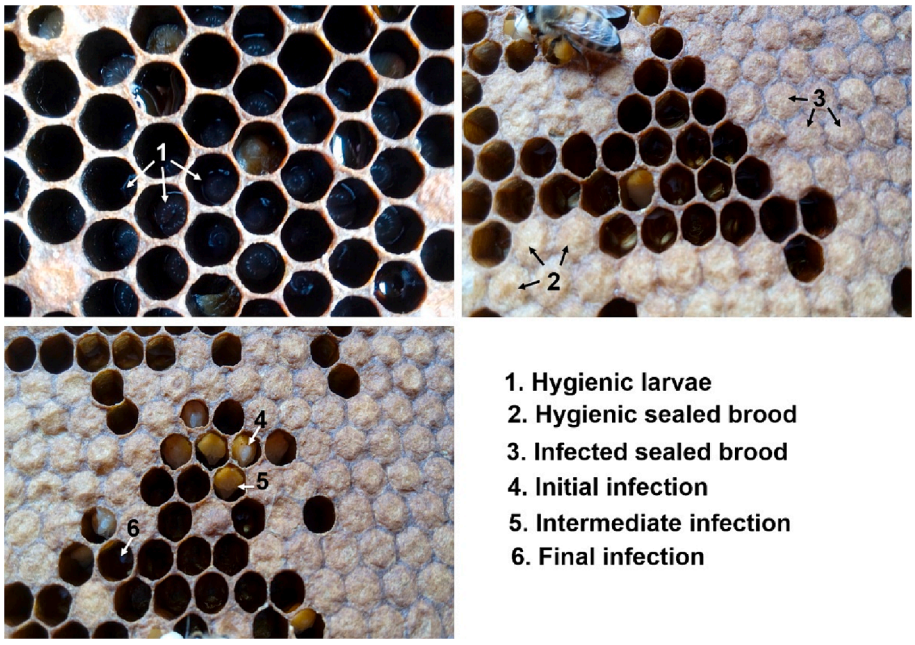

Fig. 1. Symptoms of AFB in honeybee larvae: honeybee colony disorder.

Table 2
Morphological and biochemical characteristics used for identification of the selected isolates.

Tests	Bacterial isolates													
	SS.	**Hb1**	**Hb2**	**Hb3**	**Hb4**	**Hb5**	**Hb6**	**Hb7**	**Hb8**	**Hb9**	**Hb10**	**Hb11**	**Hb12**	**Hb13**
	Morphological characteristics													
Colony color	grey	creamy	grey	grey	grey	grey	white	creamy	white	white	grey	white	white	creamy
Colony diameter (mm)	4.0	3.6	4.0	4.1	4.0	4.0	3.8	3.8	3.9	4.0	4.0	3.9	3.8	3.9
Spore staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gram staining	G+	G+	G+	G+	G+	G+	G+	G+	G+	G+	G+	G+	G+	G+
Motility	+	−	+	+	+	+	−	−	−	−	+	−	−	−
	Biochemical characteristics													
Growth velocity	very slowly	slowly	very slowly	very slowly	very slowly	very slowly	slowly	slowly	slowly	slowly	very slowly	slowly	slowly	slowly
Pigments production	−		−	−	−	−	−	−	−	−	−	−	−	−
Growth on N-agar medium	−	+	−	−	−	−	+	+	+	+	−	+	+	+
Catalase production	−	+	−	−	−	−	+	+	+	+	−	+	+	+
Nitrate reduction	+	+	+	+	+	+	−	−	−	−	+	−	−	+
V. P. test	−	−	−	−	−	−	+	+	+	−	−	−	+	−
Indole production	−	−	−	−	−	−	−	+	+	−	−	−	+	+

SS: Standard strain Hb: Honeybee

18 larvae/comb, as presented in Table 4.

On the other hand, brood combs treated with antibiotics gave a lower number of infected larvae than combs treated with only bacterial strains. The same treatment is given a higher number of infected larvae when compared with honeybee combs sprayed with each phage separately or in a phage mixture. The infection rate was reduced to approximately 94.6 to 100 % through the use of a phage mixture, as opposed to 20 to 85.7 % when each phage was administered individually or 78.6 to 88.9 % when antibiotic treatment was implemented.

3.4.2. Fluctuations in the midgut histologically of honeybee larvae

3.4.2.1. Light microscopy. The anatomical segment demonstrates the midgut of the control larvae, according to Fig. 3a, which shows that the basement membrane encircles the midgut epithelium. They exhibit ovoid, prominent nuclei that are situated near the center. The mid-gut in 2 days of infection revealed definite changes in Fig. 3b, where epithelial cells separated from the basement membrane in numerous locations. They are interspersed with small goblet cells with spherical nuclei and diminished granular cytoplasm. In striated (brush) borders, columnar cells extend microvilli from their free extremities to form the epithelium.

Two separate muscle fibers lined the intestinal wall: longitudinal fibers along the exterior and circular fibers along the interior. Connective tissue nearly fills the cavities between the various layers of the intestinal wall, and the fat bodies are normal.

Additionally, Fig. 3c clearly shows the effect of phage treatment on the larvae, especially on the mid-gut tissues, where the effect is more pronounced and extensive within 2 days of the treatments. Some cells resumed their original morphology, and the basement membrane encircled the epithelium. They exhibit ovoid, prominent nuclei that are

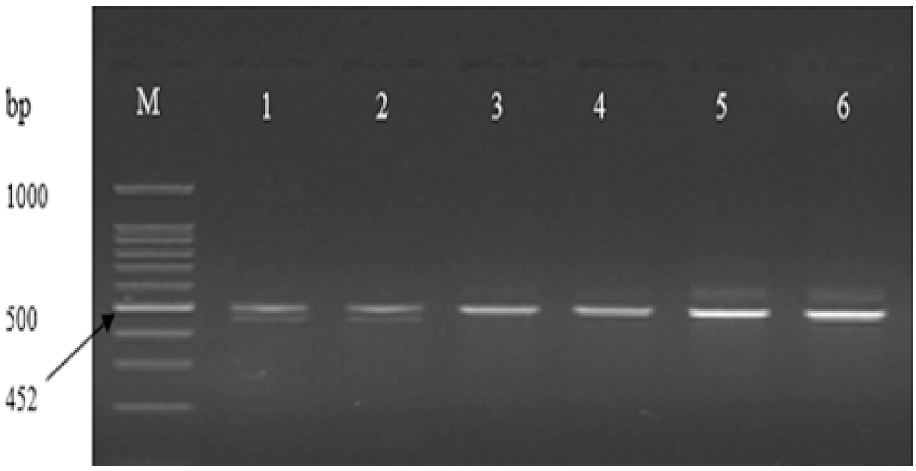

Fig. 2. Gel electrophoresis of PCR products of *P. larvae* amplified with 16SrRNA gene primer pairs AF1 plus AF2. Lanes: M, molecular size marker (100-bp ladder); 1,2(Hb2); 3,4(Hb3); 5,6(Hb10). Hb 2, 3, and 10 are *P* larve isolates.

Table 3
Source of isolated lytic phages.

Phage source / *P. larvae* isolates	Dead bees	Soil under the hives	Wastewater
Hb2	+++	++	+
Hb3	++	+++	++
Hb10	+++	+	++
SS.	++	+++	++

(+): degree of lysis SS: Standard strain
4. Infection reduction rate (%) in honeybee colonies under different treatments.

situated near the center. They are interspersed with small goblet cells with spherical nuclei and diminished granular cytoplasm. In reality, the striated (brush border) on the epithelial was composed of microvilli protruding from the free extremities of columnar cells.

On the contrary, the impact of antibiotic therapy on the mid-gut sections of larvae was elucidated in Fig. 3d. The basement membrane encompassing the mid-gut epithelium is striated and contains ovoid, visible nuclei. Small goblet cells with reduced granular cytoplasm and spherical nuclei appeared scattered between them.

3.4.2.2. Examination of the a. Mellifera larvae Scanning electron microscopy surface. The SEM examination of treated larvae was observed in Fig. 4 and Supplementary Fig. S4I, II, and III. The shape of the hygenic larvae was normal Fig. 4A compared to the infected larvae, which became abnormal, sticky, and disjointed after 2 days of infection with each *P. larvae* Fig. 4B. Meanwhile, the larval shape in antibiotic treatment was nearly similar to the hygienic one Fig. 4C. Meanwhile, in phage mixture treatment, the shape of the examined workers was reasonable, and their magnitude was better than that of the larvae from antibiotic treatment or untreated larvae (Fig. 4D).

4. Discussion

American Foulbrood (AFB), caused by the bacterium *Paenibacillus larvae*, is a highly contagious disease of honey bees that is notifiable in many countries. Upon detection, infected hives are typically destroyed to prevent the spread of the disease (Truong et al., 2023). This strict approach is due to the extreme contagiousness of AFB spores and the subsequent high risk of transmission to surrounding apiaries, including those managed by beekeepers and wild pollinator populations. The wide spread of the American foulbrood causative agent, as suggested by (Daisley et al., 2020; Matović et al., 2023), that *P. larvae* may exist as a pathobiont in the native microbiota of adult worker bees, from where it is passively and constitutively transmitted throughout the hive to fresh brood cells. All the symptoms matched descriptions of AFB disease by Genersch, (2010a) and Yost et al., (2016).

Additionally, the spread of the disease through all locations was observed. This may be attributed to the fact that *P. larvae* generate spores, which adult bees unintentionally transport back to their hives (Daisley et al., 2023). Although adults remain unaltered by the infection, they serve as vectors for transmitting susceptible larvae. Spores, on the whole, exhibit resistance to heat and antibiotics and can endure for decades, posing a significant challenge to eradicating the illness (Yost et al., 2016). After a single larva's infection and subsequent demise, most spores may be generated and discharged within the hive. Thirty-six hours after hatching, honeybee larvae are at their highest risk of contracting *P. larvae*. As the disease consumes the larvae of a hive, fewer bees mature into adults; consequently, the colony cannot support its population and ultimately fails.

Genersch, (2010a) reported that the causative bacterium of AFB disease produces a high level of proteolytic enzymes in skim milk during the sporulation stage. Hence, the milk proteins precipitate, and then the mixtures become clear. The positive samples contain *P. larvae,* the

Table 4
Infection reduction rate (%) in honeybee colonies under different treatments

P. larvae isolates	No. of IL from each strain separately	Oxytetracycline		Bacteriophages									
				Hb2-phage		Hb3-phage		Hb10-phage		S.S.-phage		Phages Mixture	
		No. IL	IRR (%)	No. IL	IRR (%)	No. IL	IRR (%)	No. IL	IRR (%)	No. IL	IRR (%)	No. IL	IRR (%)
(Hb2)	10	2	80.0b	1	90.0a	3	70.0c	2	80.0c	4	60.0d	1	90.0b
(Hb3)	12	2	83.3b	3	75.0c	2	83.3a	3	75.0d	4	66.7c	0	100a
(Hb10)	18	2	88.9a	3	83.3b	4	77.8b	0	100a	3	83.3b	0	100a
(SS.)	14	3	78.6c	2	85.7b	3	78.6b	2	85.7b	1	92.9a	0	100a

No. IL: No. of infected larvae/comb, IRR: Infection reduction ratio (%), (*): mean phage SS: standard strain, lowercase letters in columns indicate significant differences ($p<0.05$).

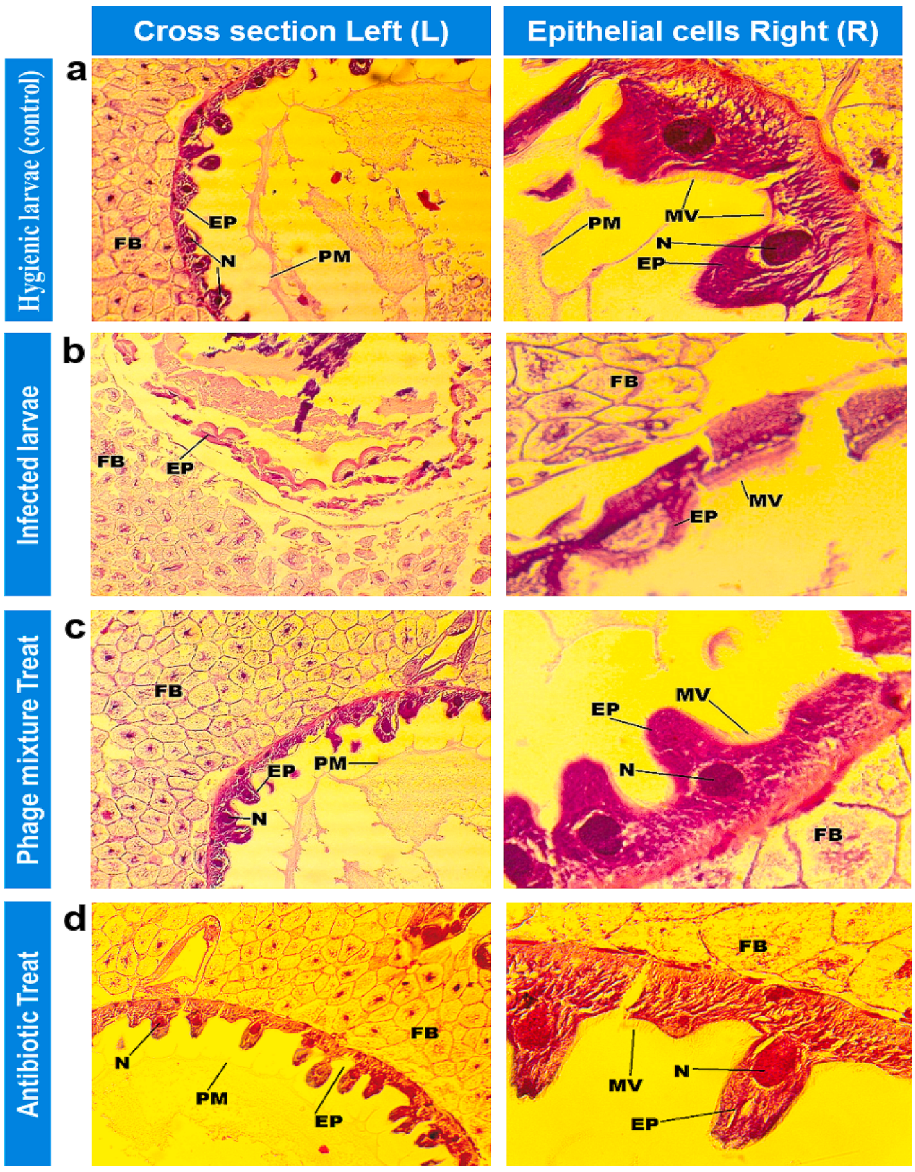

Fig. 3. From Left to Right: Cross section in *A. mellifera* larval midgut (10 x), Epithelial cells in *A. mellifera* larval midgut (100 x) (Right). **PM,** peritrophic membrane – **EP,** epithelial cells – **N,** nuclei – **MV,** Microvilli – **FB,** Fat bodies.

causative bacterium of AFB disease, but *M. pluton,* the EFB causative bacterium, does not have this capability (Takamatsu, 2023).

The single colony measures about 4 mm. in diameter, is motile, and belongs to *P. larvae,* according to Yost, (2014). Additionally, *P. larvae* colonies were very slow on the MYPGP medium, non-pigmented, and did not grow on the N-agar medium. Also, they showed an adverse reaction with catalase, VP, and indole tests, yet they showed a positive one in the nitrate reduction test. Garritty et al., (2004) confirmed that the catalase and nitrate reduction tests were used for the biochemical diagnosis of *P. larvae*.

Results showed that 60 % of the tested bacterial isolates Hb2, Hb3, and Hb10 gave the typical clinical symptoms of AFB, whereas 40 % could not cause these symptoms. This may be because although the remains of infected larvae have the clinical symptom of AFB, it is inconclusive, as reported by Erban et al., (2017).

The conclusive identification of *P. larvae* strains, the genus-specific 16SrDNA gene analysis was carried out using two PCR primers for the specific 16SrDNA gene of *P. larvae* (GenBank accession No. X60619) and the expected 973-bp PCR amplicon unique to *P. larvae* was amplified. The obtained nucleotide sequence of the 16SrRNA PCR product aligned with that of the *P. larvae* 16SrRNA gene sequence. These results were in harmony with those obtained by Erban et al., (2017) who proved that the molecular identification of the bacterial isolates was accomplished via 16SrRNA analysis. Amplification of the PCR amplicon was observed exclusively with the *P. larvae* strains that exhibited the desired phenotype. No PCR products were detected with the remaining nine *Bacillus* strains.

Additionally, the size and sequence of the resulting PCR product matched that of the region found on typical *P. larvae*. 16SrRNA gene between the two primers. Similarly, Govan et al., (1999) identified a solitary amplicon generated by particular primers; conversely, Dobbelaere et al., (2001) discovered four amplicons generated by the primers designed for the 16SrDNA gene.

Phages that can lyse *P. larvae* strains are highly abundant in nature and could be utilized as a potential treatment for AFB. Suttle and Fuhrman (2010) had the same result. Yost, (2014) also indicated that most isolated phages are obtained from soil under and around beehives. The soil around beehives is easily available and was a reliable source of lytic phage that lysed all or nearly all of the *P. larvae* strains tested. Besides, we characterized the isolated phages by plaque morphology

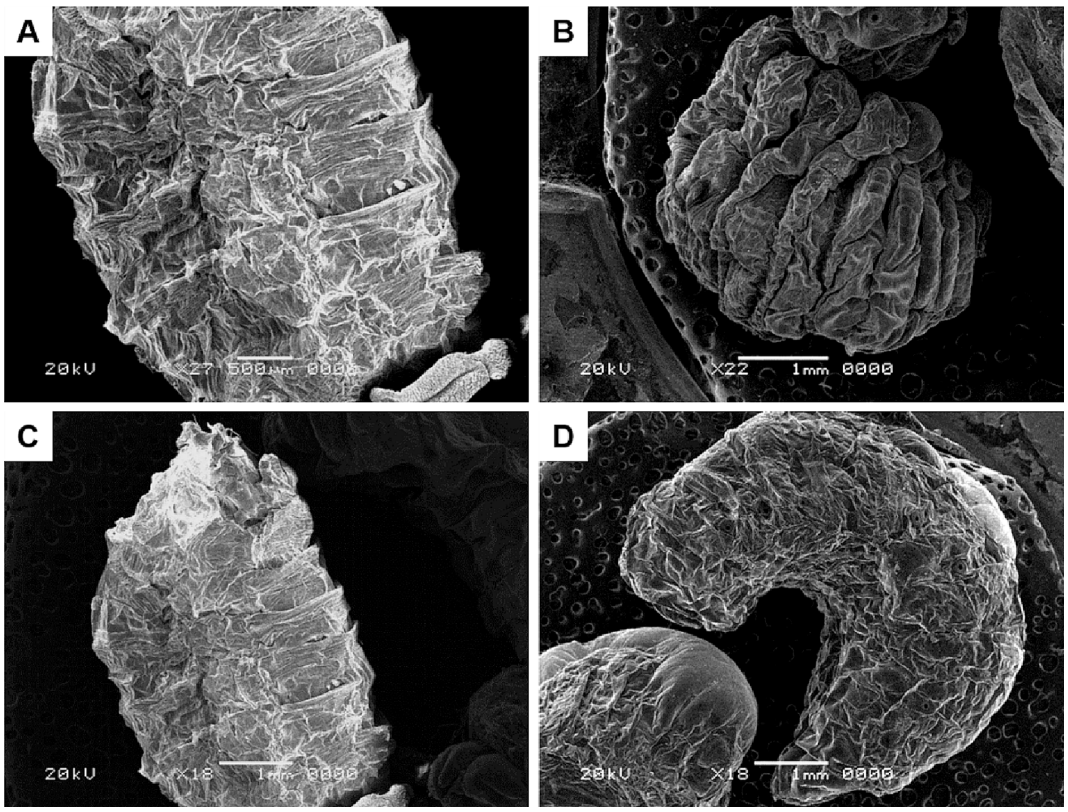

Fig. 4. Scanning electron microscopy of *A. mellifera* larvae, A: Hygeinc larvae, B: Infected larvae, C: Antibiotic –treated Larvae, D: Phage-treated larvae.

and host range on various bacterial species. The host range was useful in choosing an active phage for potential AFB treatment. Likewise, Ghorbani-Nezami et al. (2015) isolated phages based on the vulnerability of *P. larvae* to lysis. Phosphate presence was ascertained by developing plaques on soft agar overlays containing *P. larvae*. In their study, Yost et al., (2016) isolated lysogenic phages of P. larvae from the following environmental sources and locations: bees (18.89 %), the soil beneath beehives (15.9 %), hive samples (excluding beehive soil), water sources, cosmetics, and plant material (10.71 %).

This variation in infection rate may be due to the virulence of each strain depending upon strain and genotype (Genersch et al., 2005; Alpay Karaoğlu et al., 2023), and the force of the bee community or *P. larvae* spores can be infective. In this respect, Genersch (2010a) reported that a meager mortality rate occurs when 300 spores are administered as an initial dose to infect larvae. A comparison between brood combs sprayed with each *P. larvae* strain and those sprayed with each *P. larvae* strain combined with its specific phage indicated that phage decreased the number of infected larvae by 93 %. No infected larvae were observed when honeybee colonies were treated with *P. larvae* Hb10 mixed with its specific phage. Also, it was clear that Hb2-phage decreased the infection to 90 % when mixed with *P. larvae* Hb2 compared to 80 % using antibiotics, as illustrated in Table 4. Yost, (2014) reported that administering phage increases larval survival compared with infected larvae.

Moreover, the infection percentage was decreased by 90 to 100 % when the phage mixture combined with each *P. larvae* strain was applied. This result could be attributed to the necessity of employing a phage combination capable of efficiently lysing the widest variety of *P. larvae* strains; these results were confirmed by those obtained by Murray, Aronstein, and de Leon (2007), and Loganathan et al., (2024) who reported that antibiotics are insufficient to combat the disease due to increasing resistance among *P. larvae* strains. Honeybee products have been found to contain oxytetracycline residuals, which are frequently employed in the treatment of AFB (Tian et al., 2012). Additionally, they demonstrated that eight antibiotic-resistant genes to tetracycline, which has historically been employed to treat AFB, were prevalent in honeybee intestinal microbiota. Antibiotics are not viable treatments for AFB due to their ability to disrupt the systematic microbial community of the honeybee gastrointestinal microorganisms and the rise in resistance among bacterial strains.

Gregorc and Bowen (2000) reported that 20 % of honeybee larval columnar cells from the Control treatment were positive. Additionally, certain cells and their thickness were disrupted in mid-gut larvae, and their cytoplasmic contents were discharged into the interstitial space that separated epithelial and peritrophic membranes. The mid-gut epithelial cells subsequently lost their columnar structure and became more deformed, while the peritrophic membrane remained intact in many regions, and the fat bodies were destroyed. Furthermore, the mid-gut epithelial cells exhibited the presence of dispersed vacuoles, the basement membrane became apparent, and certain areas of the epithelium cells were obliterated (Kok et al., 2023; Ye et al., 2023).

In this respect, Gregorc and Bowen (2000) reported that in antibiotic-treated larvae, the midgut epithelium is renewed from a population of regenerative stem cells situated in the basal layer of the gut. The new cells generated from these are carried upwards through the epithelial sheet until they reach the exposed surface, where they die and are sloughed off into the lumen (Kok et al., 2023).

This phenomenon could be attributed to the capacity of *P. larvae* to secrete a diverse array of secondary metabolites and chitin-degrading enzymes. These enzymes facilitate the degradation of the peritrophic matrix, permit penetration of the midgut epithelium, invade the hemocoel, and ultimately decompose the larva into a ropy mass (Daisley et al., 2020, 2023).

5. Conclusion

P. larvae strains were utilized to generate phages with exceptional lytic specificity. These phages were readily isolated from nature and functioned as an alternative biological approach to antibiotics, which have traditionally been employed to control AFB. A mixture of phages gave a 100 % reduction in infection rate compared with antibiotics or

each specific phage. In larvae from all treatments, some cells recovered to the regular shape, the basement membrane encircled the mid-gut epithelium, and the cells contained oval, prominent nuclei positioned nearly centrally. The microvilli of columnar cells protruded from their unbound extremities, revealing the intestine cross-section showed healthy longitudinal and circular muscles; all these were observed in phage treatments. Moreover, the SEM results showed that the shape of honeybee workers in phage, treatments separated or mixed, the shape of the examined workers was ordinary, and their magnitude was better than the larvae from antibiotic treatment or untreated larvae. Phage had the potential to solve the problem of AFB in honeybees since they were easy techniques to apply, relatively cost less, and are handy products for beekeepers to use as far as preparation in the lab is concerned.

CRediT authorship contribution statement

Rasha M. El-Meihy: Resources, Methodology, Data curation. **Eman O. Hassan:** Writing – review & editing, Software, Methodology, Formal analysis. **Soha A. Alamoudi:** Resources, Methodology, Funding acquisition, Writing – review & editing. **Sally Negm:** Validation, Formal analysis, Methodology. **Nawal Al-Hoshani:** Methodology, Data curation. **Mariam S. Al-Ghamdi:** Resources, Methodology. **Elhosseny E. Nowar:** Writing – original draft, Resources, Methodology.

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Appendix A. Supplementary material

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