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Field Application of Honeybees' Endogenous Lactic Acid Bacteria for the Control of American Foulbrood Disease

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ABSTRACT

The purpose of this study is to investigate a new applicable and promising treatment for American Foulbrood (AFB) disease in honeybee colonies. AFB is a very serious honeybee disease caused by the spore-forming bacteria *Paenibacillus larvae larvae* (*P. l. larvae*). Five honeybee endogenous lactic acid bacteria (LAB), isolated from honeybee workers' guts, were previously identified and tested for their inhibitory effects on *P. l. larva in vitro.* Artificial infection was accompanied by the administration of a mixture of the five LAB strains, two belong to *Lactobacillus plantarum*, two different strains of *Lactobacillus kunkeei*, one strain of *Lactobacillus sp.*. It was observed that the honeybee endogenous LABs inhibited *P. l. larvae* in an *in vivo* system. LAB mixture added to the larval food in honeybee colonies significantly reduced the number of infected larvae ($P \simeq 0.000$, $p < 0.001$). Confidence intervals analysis showed no significant difference from adding LAB to the food on first or second-day post infection and throughout the feeding period. *In vivo* studies demonstrated that LAB microbiota in *Apis mellifera* inhibits the bacterial brood pathogen *P. l. larvae*. The results pointed to new avenues for the prophylactic or therapeutic treatment of honeybee diseases.

INTRODUCTION

Apiculture has a great economic impact; therefore, many countries consider that the health status of honeybees is of utmost importance. The honeybee *Apis mellifera* plays an important role in pollination and therefore in agriculture processes. Also, they are the main source for many products like honey, wax, propolis, royal jelly, pollens and venom which have been widely used in food and medicine.

Recently, Lactic acid bacteria (LAB) which are recognized as beneficial microbes in healthy humans, animals, and insects (Hammes and Hertel, 2006; Vásquez *et al*., 2012), were also found within honeybee guts (Olofsson and Va´squez 2008). LABs are known for their production of lactic acid during their metabolism (Klaenhammer *et al.*, 2002).

They also produce many substances of antimicrobial properties such as antimicrobial peptides, organic acids and hydrogen peroxide (De Vuyst and Vandamme, 1994). LABs are commensal bacteria that share in immunomodulation and interference with pathogens (Mitsuoka, 1992); therefore, they maintain the healthy microbiota and subsequently honeybee health. LABs also protect honeybee products from microorganisms and protect the foraging bees (Vásquez *et al*., 2012).

AFB is a very dangerous honeybee disease caused by the spore-forming bacteria *P. l. larvae*. Recently, it was reported that LAB microbiota inhibits *P. l. larvae* spores within the honeybee crop (Forsgren *et al*., 2010). Other experiments have shown that LAB also decreases broods infected with a similar disease, the European Foulbrood (EFB) (Vásquez *et al*., 2012). All these experiments lack field application, where they proved AFB and EFB inhibition by LAB *in vitro*.

The present study aimed to investigate if the newly identified LABs from honeybee gut inhibit the growth of *P. l. larvae* and influence AFB disease development in infected honeybee colonies.

MATERIALS AND METHODS

Maintenance of Honeybee Colonies:

Twenty colonies of healthy hybrid Carniolian honeybees were maintained in the apiary yard of the Honeybee Research Department, Plant Protection Research Institute, Agriculture Research Centre, Ministry of Agriculture, Dokki, Egypt. The experiment was carried out during summer 2017.

***P. l.* Larvae Preparation for Colonies' Artificial Infection:**

Previously identified *P. l. larvae* with KAT-PCR, designed by Allipi *et al*. 2002, was cultivated on J-agar (5.0 gm tryptone,15.0 gm yeast extract, 3.0 gm K_2HPO_4, 2.0 gm glucose and 20.0 gm agar and adjusted to 1000 ml by distilled water; pH 7.3 - 7.5) according to **(Gordon *et al.*, 1973).** Cultured bacteria were incubated at 35 ◦C and 5% CO2 for 10–14 days to form spores. 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.

LABs Used and Their Preparation for Treatments:

1. LAB Strains:

Five endogenous honeybee LABs previously isolated, identified and tested for their inhibitory effects on *P. l. larvae*. They gave the most effective probiotic inhibitory results, of all isolated endogenous LABs, against *P. l. larvae* spores in vitro and were selected for field treatment. These strains were:

Two strains belong to *Lactobacillus plantarum* (GenBank Accession numbers: MK780211 and MK780215), two different strains of *Lactobacillus kunkeei* (Accession numbers: MK780216 and MK780218) and one strain of *Lactobacillus sp*. (Accession number: MK780212).

2. LABs Preparation for Treatments:

Each LAB strain was cultured anaerobically (using a gas pack system and a gas pack kit, oxoid) in MRS broth (oxoid) with 0.5% L-cysteine (pH 6.2) and incubated at 37 °C for 24 h. LAB mixture of about $5x10^4$ bacteria/ml was prepared freshly for each experimental dose.

2. Field Experiment:

A.Treatment of Colonies:

To get larvae of the same age, each colony was caged for 4 hours to a single comb, using queen excluder, in the center of the brood nest. A total of 20 honeybee colonies were prepared for the experiment. They were divided into four groups, with five replicates for each one:

Group 1 (-ve control): untreated honeybee colonies. The colonies of this group were fed only on 20% sugar solution during the whole experimental practices. They were kept isolated from treated colonies.

Group 2 (+ve control): colonies were infected once on the first day of the experiment, with *P. l. larvae* spores (approximately 5 x 10^4 spores/ml) dissolved in 20% sugar solution. The colonies were sprayed with 100 ml of the solution.

Group 3: colonies were supplied, on the first day of the experiment, with *P. l. larvae* spores (approximately 5 x 10^4 spores/ml) mixed with the 5 types of LABs (approximately 5 x 10^4 bacteria/ml) which were all dissolved in 20% sugar solution. About 100 ml of solution is added by spraying. Treatment with only LAB mixture was continued for the next four days with the same dose and ended on the fifth day of the experiment. A Revitalization dose of LAB mixture was given once a week, for two weeks, from the last day of LABs treatment.

Group 4: colonies were supplied, on the first day of the experiment, with *P. l. larvae* spores (approximately 5 x 10^4 spores/ml). Treatment with the 5 types of LAB mixture (approximately 5 x 10^4 bacteria/ml) dissolved in 20% sugar solution (100 ml) began from the second day of the experiment and continued for the next 4 days and on the sixth day of the experiment. A revitalization dose of LAB mixture was given once a week for two weeks from the last day of LABs treatment.

Colonies were distributed in a completely randomized design. The experimental procedures for inoculation were conducted as described by Alippi *et al.* (1999) and Forsgren *et al.* (2010).

B. Monitoring Larval Mortality:

The number of larvae in an area of about 20 inches was recorded before treatment (at zero time) and seven days' post-treatment with capped cells.

C. Data Analysis:

Statistical analysis was used to evaluate the influence of LAB, and the timing of treatment on *P. l. larvae* spores by assessing the proportion of infected larvae. The numbers of dead and survived last instar and sealed larvae, seven days post-treatment, in relative to the numbers of initial larvae marked at zero time prior to infection, were transformed using SPSS (version 20) for Person's Chi-square test analysis. Chi-square test was performed to compare percentages of dead and survived larvae. Person's Chi-square test, especially suited for large samples, was run to estimate the infection among treatments in all three groups. Results from all three groups were combined for that purpose (Spivak and Reuter, 2001).

Confidence intervals of the difference between the percentages of the dead larvae in the treated groups (G3 & G4) corresponding to the untreated group (G2: +ve control), were conducted separately, also between the two treated groups (G3: treatment on day 1 & G4: treatment on day 2), for indication of how reliable the samples' proportions. Confidence intervals were calculated manually by the following equation:

$$(\hat{p}_1 - \hat{p}_2) - z_{\alpha 2}\sqrt{\frac{\hat{p}_1\hat{q}_:}{n_1} + \frac{\hat{p}_2\hat{q}_2}{n_2}} < p_1 - p_2 < (\hat{p}_1 - \hat{p}_2) + z_{\alpha 2}\sqrt{\frac{\hat{p}_1\hat{q}_1}{n_1} + \frac{\hat{p}_2\hat{q}_2}{n_2}}$$

n_1: population 1
P_1: Phat1 (population 1, positive response)
q_1: Qhat1 (population 1, negative response)
n_2: population 2
P_2: Phat2 (population 2, positive response)
q_2: Qhat2 (population 2, negative response)
$\alpha/2 = 0.025$
$z_{\alpha/2} = 1.96$

The investigators desired level of confidence most commonly 95%, but any level between 0-100% can be selected.

D. Checking Colonies and Culturing of the Probiotic LAB and the Pathogen *P. l. larvae* from Honeybees:

On the fifth day of treatment, Last instar larvae were collected, from groups 1, 3 & 4, and cultured on both MRS agar (larval guts only) and J-agar (whole larvae) for detection of LAB and *P. l. larvae* respectively. For

group 2 (+ve control), larvae were cultured on J-agar only.

3. Field Efficacy:

Colonies were observed for two months to record any AFB disease relapsing results. Colonies were scored as to their degree of disease like Alippi *et al.* (2005). Larvae with AFB clinical symptoms were measured in the same way in all the experimental colonies. Measurement of clinical signs of AFB were estimated according to a seven levels scale, where level 0 is non-detectable AFB symptoms; level 1, between 1 and 10 larvae with clinical signs of AFB; level 2, between 11 and 30 larvae with clinical signs of AFB; level 3, between 31 and 99 larvae with clinical signs of AFB; level 4, more than 100 larvae with clinical signs of AFB; level 5, Queen superseding due to AFB and level 6, colony death, respectively.

RESULTS

Monitoring Larval Mortality:

Numbers of survived and dead larvae in each group, from day one before infection (zero time) and Seventh-day post infection and treatment with the probiotic LAB mixture, were recorded in table1.

Table 1: Mean numbers of survived and dead honeybee larvae and capped cells in the four groups of the experiment at zero time (before infection) and Seventh-day post infection.

Groups	Mean number of larvae at Zero time	Mean number of larvae at 7th day	
		Survival	Dead
***G1 (-ve)**	2250	2447	103
***G2 (+ve)**	2350	375	1975
***G3 (P)**	2475	1825	650
***G4 (PA)**	2300	1660	640

*G1: -ve control, G2: +ve control, G3: treatment on the first-day post infection and G4: treatment on second-day post infection.

Crosstabulation for expected total counts, of dead and survived brood larvae and sealed larvae, on 7th-day post-experiment was listed in the table (2). The counts of each group, infected and treated with LAB, were represented on a bar chart (Figure 1). Percentages of survived larvae of the treated Groups 3 and 4 were 73.7 % and 72.2 %, respectively, while the infected group 2 showed 16 % survival with a great loss in brood of about 84 % (Table 3). The stratified bar chart (Figure 2) illustrates the percentages of survived and dead larval brood after treatment with LAB.

Overall, the results collected from field experiments, in relation to the infected group (+ve control), the result of the Person's chi-squared test is 2.064E3. The LAB treated colonies showed significant differences in respect compared to the AFB-inoculated controls $P \simeq 0.000$ ($p < 0.001$) (table 4).

Table 2: Survive * Treatment Crosstabulation of the expected count of dead and survived brood larvae on seventh-day post-experiment.

Mean larvae:		Treatment			Total
		*G2(+ve)	*G3(P)	*G4(PA)	
Dead	Count	1975	650	640	3265
	Expected Count	1076.9	1134.2	1054.0	3265.0
Survive	Count	375	1825	1660	3860
	Expected Count	1273.1	1340.8	1246.0	3860.0
Total	Count	2350	2475	2300	7125
	Expected Count	2350.0	2475.0	2300.0	7125.0

*G2: +ve control, G3(P): treatment on the first-day post infection and G4(PA): treatment on second-day post infection.

Table 3: Survive * Treatment Crosstabulation of percent within the treatment of the dead and survived brood larvae on seventh-day post-experiment.

Mean larvae:		Treatment			Total
		G2(+ve)	G3(P)	G4(PA)	
Dead	Count	1975	650	640	3265
	% within Treatment	84.0%	26.3%	27.8%	45.8%
Survive	Count	375	1825	1660	3860
	% within Treatment	16.0%	73.7%	72.2%	54.2%
Total	Count	2350	2475	2300	7125
	% within Treatment	100.0%	100.0%	100.0%	100.0%

*G2: +ve control, G3(P): treatment on the first-day post infection and G4(PA): treatment on second-day post infection.

Table 4: Person's Chi-Squared Test

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.064E3[a]	2	0.000
N of Valid Cases	7125		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 1053.96.

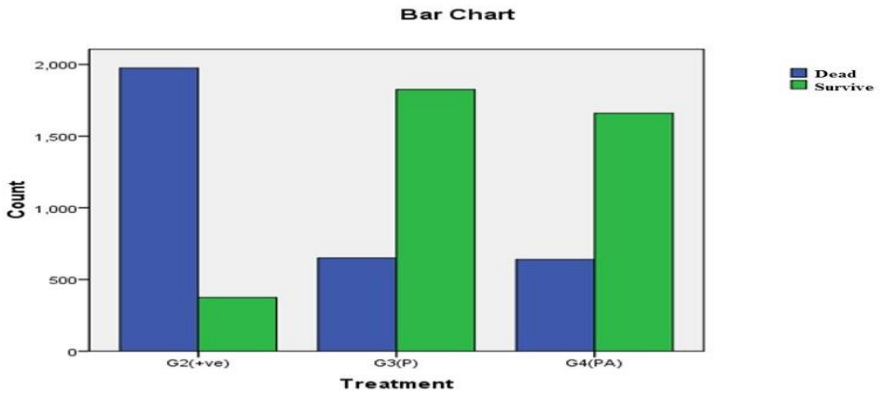

Fig. 1. A bar chart represents the counts of survived and dead larval brood post-treatment with LAB (groups of G2: +ve control, G3(P): treatment on the first-day post infection and G4(PA): treatment on second-day post infection).

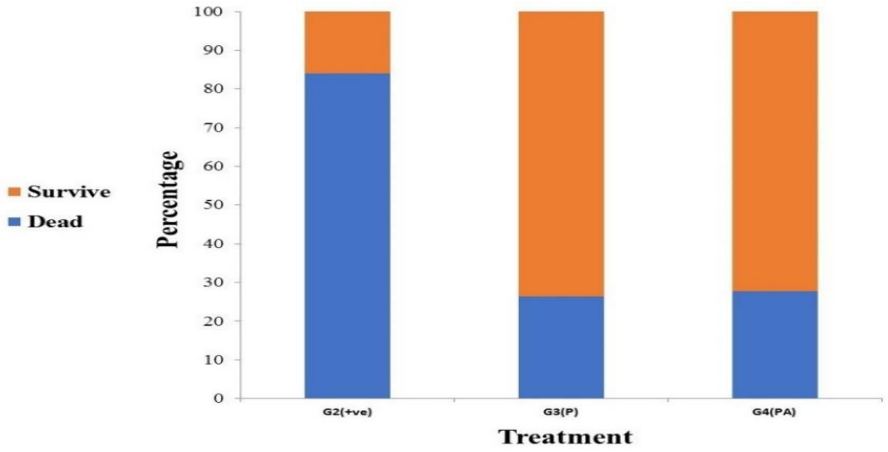

Fig. 2. A stratified bar chart represents the percentages of survived and dead larval brood post-treatment with LAB (groups of G2: +ve control, G3(P): treatment on the first-day post infection and G4(PA): treatment on second-day post infection).

Confidence intervals of the difference between the percentages of the dead larvae within different groups were as follows:

Between groups (G2 and G3): 55.4% < P_2-P_3 < 59.9%

Between groups (G2 and G4): 53.8% < P_2-P_4 < 58.6%

Between groups (G3 and G4): -4.02% <P_3-P_4 <1.02%

Confidence Levels were high between treated and both untreated groups between G2 and G3 ranged between 55.4% and 59.9%. However, G2 and G4 fall between 53.8% and 58.6%. It means that should the experiment be repeated or surveyed over and over again, about 57% of the time, the results will match our results get from a population (in other words, our statistics would be sound) and this is a reliable result.

Confidence intervals analysis on the effect of the time LAB was added to the colony showed a little negligible difference in the proportion of dead larvae in both treated groups. The effect of adding LAB in combination with *P. larvae* at the same time of spore administration was not significantly different from adding LAB to the food second-day post infection and throughout the feeding period, zero involved in the confidence interval between treated groups; G3 and G4 (-4.02% <P_3-P_4 <1.02%).

Checking Colonies and Culturing of the Probiotic LAB and the Pathogen *P. l. larvae* from Honeybees' Larvae:

A Check was done for the four different groups of colonies on the fifth day of treatment and different observations were recorded as follows:

In the first group, untreated honeybee colonies, (-ve control): the numbers of the brood larvae on the fifth day were relatively the same as at zero time, the brood and brood cells were healthy, colony affairs, queen and honeybee workers' behavior were all normal. Collected honeybee larvae were cultured on J-agar and dissected larval guts on MRS agar for the detection of both LAB and *P. l. larvae*, respectively. Both cultures gave negative results and completely barren of both bacteria.

In the second group, treated honeybee colonies with AFB, (+ve control): a great decrease in brood numbers was observed and the development of clear AFB clinical symptoms in all treated colonies was recorded. Symptoms included: 1. Rotten or bad smell of the brood. 2. Irregular or scattered brood patterns; there were many cells that appear empty. 3. Sick and partially decaying larvae changed their colors from the normal glistening white to off-white or light brown. Infected larvae were stretched out on the wall of their cells and when a stick is inserted into the suspected cell and then withdrawn; the infected larva sticks tenaciously, and its contents are drawn out into a long thread or rope (+ve ropy test**).** A change in workers' behavior was observed, they became very aggressive and once the cover of the colony was opened, in spite of smoking which keeps workers calm, they attack in a very aggressive manner. The high growth of *P. l. larvae* was detected when partially decayed honeybee larvae were cultured on J-agar.

In the third group, treatment of AFB by LAB started on the first day of infection: a relatively little loss in brood larvae was recorded, but any of AFB clinical signs were not detected in both colonies. The brood, queen and honeybee workers' behavior were all normal. LABs were detected in all larval guts cultured on MRS agar, but *P. l. larvae* disappeared from all larval cultures on J-agar.

In the fourth group, treatment of AFB by LAB started on the second day of infection, colony check was on the sixth day of the experiment: Few brood larvae were also lost in this group and clinical signs of AFB were not detected. Colony affairs and brood were all in order and undamaged. LABs were detected in all larval guts cultured on MRS agar, also *P. l. larvae* only appeared in a small amount from only one larval culture on J-agar.

Field Efficacy:

Two months post-experiment; the colonies were checked once again for recording any AFB diagnostic signs. For groups 1, 3 and 4; they were all plotted in level 0 with non-detectable AFB symptoms, while in group 2 (+ve control) one colony was plotted in level 5, it was nearly free of brood with a superseding queen and the other colonies represented level 6 which indicated for complete death of the colony.

DISCUSSION

This study aimed to investigate a new promising, applicable and effective way for the treatment of American Foulbrood (AFB) disease in honeybee colonies. Field results strongly suggest that the probiotic lactic acid bacteria (LAB) linked to the honeybee stomach have a strong inhibitory effect on *P. l. larvae* spores affecting the honeybee pathology in general and for AFB tolerance in particular. Recently, Stephan *et al.* (2019) in their field studies, found that honeybee LABs inhibit the multiplication of *P. larvae* vegetative cells, but that spore germination appeared to be unaffected and that it decreased the mortality of infected larvae**.** However**,** our results suggest that honeybee LABs have an acceptable inhibitory effect on vegetative and spore forms of *P. l. larvae* in honeybee colonies. This contradiction between the two studies may be due to different field environmental conditions such as the high temperature in Egypt during

spring and summer which may reach between 30 to 45 °C (preferable temperature to LAB growth). Also, there are three different strains belong to two newly isolated and identified LAB species from bee guts that were used in our field studies. These two species (*Lactobacillus plantarum* and *Lactobacillus sp*) gave us very promising inhibitory effects in vitro and field studies. Stephan *et al.* (2019) used SymBeeotic™, a proprietary mixture of honeybee-LAB species, they treated the colonies twice with LABs, one week before and one week after inoculation by *P. l. larvae*, in each time 3 equal doses for only 3 days. Here we used freshly prepared and counted viable LABs; At first 5 equal doses were given for 5 days post-infection and repeated twice as one dose for another two weeks post-treatment.

The overall effect from adding the LAB mixture to the larval food in honeybee colonies was a significantly reduced number of infected larvae when pooled data from all experiments were analyzed ($P \simeq 0.000$, $P < 0.001$). Confidence intervals analysis on the effect of the time LAB was added to the colony showed a little negligible difference in the proportion of dead larvae in both treated groups. These small differences may be due to differences in honeybees hygienic behavior by which bees clean out dead or diseased larvae from their cells (Spivak & Gilliam, 1998 a,b).

Hygienic behavior helps to remove brood pathogens from the colony, and therefore, it is considered as a part of the immune response of honeybees (Woodrow and Holst, 1942; Chen *et al*., 2000; Wilson-Rich *et al*., 2009).

Our results demonstrated that LAB niches were constructed in larval guts by adding LAB mixtures to their food, in contrary these niches were very weak or completely absent from untreated healthy larvae. These promising satisfactory results prove that LAB can synergist the immune system of honeybee larvae and will keep both larval health in particular and honeybee colonies in general safe.

Checking colonies for clinical symptoms were carried out by visual inspection of the brood combs; the most common method for the detection of AFB (Shimanuki, 1997). The clinical symptoms of AFB are typical and clear in artificially infected untreated colonies (+ve control), with the brown, viscous larval remains forming a ropy thread when drawn out with a matchstick. It has an unpleasant odor that can be noticeable. The worker bees became very aggressive and once the cover of the colony was opened, workers attack in a very aggressive manner. Checking those colonies was very difficult.

It is well-known from previous field observations that some colonies show no clinical symptoms despite a high spore concentration (Hansen and Brødsgaard, 1999). So far, these differences have been explained by differences in host tolerance and hygienic behavior of honeybees (Woodrow, 1942; Woodrow and Holst, 1942; Hansen and Brødsgaard, 1999). Checking of colonies results were illustrated by a followed culturing of larvae on J-agar media which proved our field observations and absence of the causative AFB pathogenic bacteria, *P. l. larvae* from treated colonies, in spite of its heavy appearance in cultures from infected colonies. Further field proceedings for verification of field efficacy of our treatment; showed that after 2 months' post-treatment with LAB, colonies retained their health situation and colony affairs were all absolutely alright. On the other hand, infected untreated colonies were completely destroyed and died.

This work boosts an urge to develop an alternative treatment strategy, which recommends natural symbiotic lactic acid bacteria as an alternative for artificial antibiotics for the treatment of many honeybee diseases.

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ARABIC SUMMARY

التطبيق الحقلي لبكتيريا حمض اللاكتيك ذاتية النمو الملازمه لنحل العسل لمكافحة مرض تعفن الحضنة الأمريكي.

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[2] قسم بحوث النحل، معهد بحوث وقاية النباتات، مركز البحوث الزراعية، وزارة الزراعه، الدقي، الجيزه، مصر.

الغرض من هذه الدراسة هو إيجاد علاج جديد يساعد في القضاء علي مرض تعفن الحضنة الأمريكي في مستعمرات نحل العسل. تعفن الحضنة الأمريكي هو من أمراض نحل العسل البكتيرية وهو مرض خطير للغاية و تسببه بكتيريا *البانيباسيللس لارفي لارفي* . تم إعداد خمسة سلالات من بكتيريا حمض اللاكتيك، المستخرجه من معي نحل العسل، والتي تعرف بتأثيرها المثبط لكونها من المضادات الحيوية الأولية وذلك لإختبار قدرتها المثبطة في القضاء علي البكتيريا المسببه لمرض تعفن الحضنة الأمريكي. تمت التجربة الحقلية أولا بعمل عدوى اصطناعية مصحوبة بالعلاج بخليط من سلالات بكتيريا حمض اللاكتيك الخمس،وكانت سلالات البكتيريا المعالجه تنتمي الي أنواع: سلالتان ينتميان إلى *لاكتوباسيللس بلانتارم* ، وأخريتان من *لاكتوباسيللس كوينكياي* ، وسلالة واحدة من نوع مختلف من أنواع ا *لاكتوباسيللس*. لوحظ أن بكتيريا حمض اللاكتيك المستخرجه من نحل العسل لها القدرة علي تثبيط نمو بكتيريا *البانيباسيللس لارفي لارفي* والتقليل من ظهور ألأعراض المصاحبة للمرض في أفراد الحضنة. فبإضافة خليط من البكتيريا المعالجة إلى طعام اليرقات في مستعمرات نحل العسل تراجع بشكل كبير عدد اليرقات المصابة. وعند تحليل البيانات المجمعة من جميع التجارب كانت النتيجه ذات مغزي إعتباري ($P \simeq 0.000$، $P<0.001$). لم يُظهر تحليل فترات الثقة أن لتأثير الوقت الذي تمت فيه إضافة البكتيريا العلاجية إلى المستعمرة فرقًا كبيرًا عند إضافة العلاج إلى الطعام في اليوم الأول أو الثاني من العدوي. أظهرت الدراسة أن بكتيريا حمض اللاكتيك المصاحبة لمعي نحل العسل *أبيس ميللفرا* تقلل من ظهور ألأعراض المرضية للمرض. والنتائج المبدئية للدراسة أثبتت إمكانية إستخدامها كعلاج فعال لمرض تعفن الحضنة البكتيري. مما يعطى مؤشرات جديدة فى طرق العلاج والبرامج الوقائية لامراض نحل العسل.