Isolate of Bacteria and Fungi Associated with Dead Honey Bee (*Apis mellifera*) In Certain Egyptian Governorates and *in Vitro* Treating Some of Related Bacteria by Nano Chitosan_ Propolis

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***Abstract*—** Apiaries of sixteen localities in Egypt (New valley, Minya, Gharbiya, Demyat, Sharqyia, Ismailia, Mansoura, Cairo, Suez, North Sinai, Banisweif, Qalubeiah, Giza, Behera, Alexandria and Alaqsour) were investigated for diseases. Thirty adult workers were collected from the hives represent every locality and analyzed for microbial presence. Bees dead in many cases by the infection with microorganisms; fungi observed in New valley, Mansoura, Cairo and North Sinai, while bacteria observed in the all localities except Alaqsur, because bacteria significantly increased fastly and spread inner and between honeybee colonies through the bee operations. Three fungal species (*Rhizopus* spp., *Penicillium* spp., and *Saccharomyces cervisia*) found in bodies and guts of dead bees, but three species of bacteria (*Bacillus* spp., *Escherichia coli* and *Pseudomonas* spp.) were in most localities. Propolis loaded on nano chitosan laboratory proved its efficiency at very low concentrations against the isolated *Escherichia coli* bacterium more than *Bacillus* spp.

***Keywords*—** Chitosan, Microbs, Nano-propolis, REMA

I. Introduction

Honey Bees Are A Subset Of Bees In The Genus *Apis*, Primarily Distinguished By The Production And Storage Of Honey And The Construction Of Perennial, Colonial Nests Out Of Wax. Honey Bees Are The Only Extant Members Of The Tribe *Apini*, All In The Genus *Apis*. Currently, There Are Only Seven Recognized Species Of Honey Bee With A Total Of 44 Subspecies, Though Historically, Anywhere From Six To Eleven Species Have Been Recognized. Honey Bees Represent Only A Small Fraction Of The Approximately 20,000 Known Species Of Bees. Some Other Types Of Related Bees Produce And Store Honey, But Only Members Of The Genus *Apis* Are True Honey Bees. There Is A Report That The Microorganisms Associated With Honeybees, *Apis Mellifera*, And Their Food Include Bacteria (Gram-Variable Pleomorphic Bacteria, *Bacillus* Sp., And *Enterobacteriaceae*), Molds (Primarily *Aspergilli* And *Penicillia*), And Yeasts **[1]**. Emerging Adult Bees Acquire Intestinal Micro Flora By Food Exchange With Other Bees In The Colony And Through Consumption Of Pollen. Biochemical Contributions Of Microorganisms To Honeybees; The Role Of Microorganisms In The Conversion, Enhancement, And Preservation Of Pollen Stored As Bee Bread In Comb Cells; And The Production Of Antimycotic Substances By Molds And *Bacillus* Sp. From Honeybee Colonies That Are Resistant To The Fungal Disease; Chalkbrood, Are Well Known According To **[2]** And **[3]** Respect To The Association Of *Bacillus* Sp., With Bees Including Honeybees, Stingless Bees, And Solitary Bees From Tropical And Temperate Zones That Appears To Have Evolved In Which Female Bees Inoculate Food Sources With These Bacteria Whose Chemical Products Contribute To The Elaboration And/Or Protection From Spoilage Of Food That Is Stored In The Nest.

Microbes Associated With Honey Bee May Range From Pathogenic To Non-Pathogenic Forms. Among These Pathogens, Bacteria Of The Genera *Escherichia Coli*, *Flavobacterium*, *Klebsiella*, *Proteus*, And *Pseudomonas* Are Commonly Found. The Fungal Genera Mostly Include Are *Penicillium* Sp., *Aspergillus* Sp., And Sometimes Frequently *Torulopsis* Sp., Most Of These Fungi May Be Not Be Harmful To Honey Bee But Certain Fungi That Colonized Insect Gut And May Kill Them Altogether. These Insect Fungi Exist As Commensals Deriving Nutrition From Gut Contents Without Causing Harm To The Host, As Ectoparasites Getting Nutrition From Cuticular Waxes, Or As True Insect Pathogenic Fungi Obtaining Nutrients From Within The Insect. After Death, The Fungus Reverts To A Filamentous Form And Typically Digests The Remaining Internal Organs, Leaving Only The Chitin/Protein Exoskeleton. Such Fungi That Colonized Insects And May Cause Death Are Commonly Known As Entomopathogenic Fungi. These Fungi Comprise A Heterogeneous Group Of Cover 100 Genera With Approximately 750 Species, Reported From Different Insects And Living In Diverse Habitats Including Fresh Water, Soil Surfaces And Aerospace, Many Of Which Offer Greater

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Potential In Pest Management. Fungi Have Worldwide Distribution And Grow In A World Range Of Habitats, Including Extreme Environment Such As Deserts Or Areas With High Salt Concentration Or Ionizing Radiation, As Well As In Deep Sea Sediments **[4]** And **[5]**.

This Study Aimed Two Targets; The First Is Isolate And Identify The Microorganisms (Bacteria And Fungi) Which Associated With Or Responsible About The Death Of Adult Honeybee Workers. The Second Aim Was Examination The Antibacterial Activity Of Propolis In Shape Nano-Particles Against The Isolated Bacteria, *Bacillus* Spp., *Escherichia Coli*, Using Resazurin Microtiter Assay (Rema) For Determination The Mic.

II. Material & Methods

Honeybees samples (30 adult workers / sample) were collected from sixteen governorates in Egypt (New valley, Minya, Gharbiya, Demyat, Sharqyia, Ismailia, Mansoura, Cairo, Suez, North Sinai, Banisweif, Qalubeiah, Giza, Behera, Alexandria and Alaqsour) during years 2021-2022, then all experiments were performed at the Specific Microbiological Lab of Beekeeping Department & Nanotechnology Unit of Animal Health Institute under the suitable sterilize conditions.

A. Preparation of samples and used media

Any sample of the 16 localities was divided to two parts;
1st part used for the fungal isolation, whereas the dead bees were rapidly (15 seconds) passed through path of 70% ethanol due to surface sterilization using wire net and spread over sterilize tissue paper until the drying, then wings and legs of bees were removed, hence these bee bodies were directly cultured on plates of potato dextrose agar (PDA) medium then the plates were incubated at both 25 ± 1°C and 30 ± 1 ^{0} C for one week. Percentage of fungi recovered was calculated after 3 days of incubation, whereas the observation continued each day after inoculation. Fungal colonies growing on the plates were transferred to freshly prepared PDA slants. Pure cultures of fungi were stored at 4 ^{0} C at refrigerator for farther study. To identify the isolated fungi, method of **[6]** was conducted.
The 2nd part of sample (dead bees) used for bacterial isolation from the bodies and guts of bees using differential and selective media according **[7]**, as primarily, the sample (30 dead adult bee workers) per any location were dipped into distilled water for 5 minutes, dipped in 70% alcohol for 3 minutes, then dipped in sodium hypochlorite solution for 1 minute, lastly dipped into sterile water for 1 minute then all honeybees were kept into sterilize tissue paper for oven dried at 37 ^{0} C for 6 hours overnight. Then the bodies and guts of bees were grounded, homogenized, diluted in sterile normal saline 0.9% and the infusion was cultured for bacterial growth by using sterile swab sticks streaked on the media plates. The nutrient agar was used to identify the isolated bacteria **[8]**; also, Columbia sheep blood agar selective medium was used to confirm *Paenibacillus larvae*, which causes American foulbrood **[9]**.

B. Identification of isolated microbs

Generally, all the cultures were evaluated as bacteria or fungi by the visual examination to morphology of the growths **[7]** and **[6]**. Respect to isolated fungi, the identification was done by morphological and microscopic characterization, whereas the colonies growing out the plates were subculture into freshly prepared potato dextrose agar slant, and the mycellium of each isolate was taken in a clean glass slide and stained with lactophenol cotton blue. The mounted specimen was teased gently with a help of sterile needle and observed under binocular Olympus model microscope at 40X and 100X. Practical Mycology, Manual for medically important fungi– A guide to identification by **[10]**.

Respect to isolated bacteria, the identification was done by the following tests which showed in Table I. according to **[11]** and **[12]**.

TABLE I.
THE OUTCOME OF PERFORMED TESTS WHICH INDICATES TO PRESENCE OR ABSENCE OF *PAENIBACILLUS LARVAE* BACTERIUM IN SAMPLE

Test / Result	Presence	Absence
Gram stain	+ve	-ve
Holst milk	+ve	-ve
Catalase activity	-ve	+ve
Starch hydrolysis	-ve	+ve
Casein hydrolysis	+ve	-ve
Gelatin liquefaction	+ve	-ve
Nitrate reduction	+ve	-ve
Voges proskauer	-ve	+ve
Growth on blood agar	+ve	-ve
Growth on nutrient agar	-ve	+ve

C. Determination of antibacterial activity by minimal inhibition concentration MIC using Resazurin microtiter assay (REMA)

REMA method was carried out as described by **[13]**, whereas Resazurin was prepared at 0.015% by dissolving of 0.015 g, vortexed and filter sterilized (0.22 lm filters) and stored at 4℃ for a maximum 2 weeks after preparation. 100 µl Mueller Hinton broth (MHB) were dispensed in each well of the 96-well plate. 100 µl of stock solution of each concentration of propolis loaded on Nano chitosan loaded (serial 12 dilutions start from 100%), and their couple combinations in ratio 1:1 were added and serial two-fold dilutions were done in columns on the plate with resulted concentrations ranging from 100 to 0.781 mg/ml for each extract. One hundred µl of each bacterial inoculum of concentration 1-3 × 105 colonies for unit (CFU)/ ml was added in each well. The plates were incubated at 37 ^{0} C for 24 hrs. After incubation, 30 µl of resazurin solution was added to each well and the plates were re-incubated for 1-2 hrs. The change in color from blue to pink indicated that reduction of

resazurin and to presence of the bacterial growth. The MIC was defined as the lowest concentration of the drug that prevented this change in color.

III. Results & Discussion

The microbial examination of the bodies and guts of bees showed the presence of three fungal species (*Rhizopus* spp., *Penicillium* spp., and *Saccharomyces cervisia*), and presence of three species communities of bacteria in most of ecological localities, such as *Bacillus* spp., *Escherichia coli* and *Pseudomonas* spp.

Three species of fungi; *Rhizopus* spp., *Penicillium* spp., and *Saccharomyces cervisia* were isolated and identified from the intestines of each samples (30 adult workers of honey bees).

In Table II, the results of the microbial examination to the bodies and guts of bees indicated to the presence of three species of fungi (*Rhizopus* spp. , *Penicillium* spp., and *Saccharomyces cervisia*) (Figs. 3 and 4) in little number of governorates, whereas they found in six localities; New valley, Mansoura, Cairo and North Sinai, Giza and Alexandria areas, While the results showed to the large presence of three bacterial species community in most ecological localities, these bacteria were *Bacillus* spp., *Escherichia coli* and *Pseudomonas* spp. (Figs. 5 and 6), and found in the all localities except Alaqsour due to the species of bacteria significantly increased fastly and spread of colonies of bees through the bee operations.

Generally, Table II shows the organisms isolated and the frequency of occurrence. The microbes associated with bees included both bacterial and fungal genera. The intestine of bees has been found to contain 1% yeast, 27% Gram +ve bacteria including *Bacillus*, *Bacteridium*, *Streptococcus* and *Clostridium* sp., 70% Gram -ve or Gram variable bacteria including *Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus*, and *Pseudomonas*. Secondary sources of microbial contamination in honey are human, equipment, containers, wind, dust *etc*. Yeasts have been recovered from equipment in honey houses; contaminated equipment can introduce yeast into clean honey. Possible of the routes transmission into extracted honey would include air (in the house or while the honey was packed), and food handlers (from skin infections, sneezing or *faecalis* contamination). Most of these microorganisms may remain associated as normal contaminant and may not lead to death. Certain fungi commonly called as entomopathogenic fungi colonized insect guts and may cause severe infection. Insect pathogenic fungi are unique in being able to infect across the insect cuticle the first barrier to infection. Germinating conidia produce extracellular lipases, chitinases, and proteases to initiate cuticle invasion. Once inside the host, the fungus develops as an insect’s immune system, modify the insect’s behavior, or act as post-mortem antibiotics against competing microorganisms. After death, the fungus reverts to a filamentous form and typically digests the remaining internal organs, leaving only the chitin/protein exoskeleton. Such fungi may cause death to variety of insects. Therefore it is speculated that the dead of honey may be due to such fungi.

During the study periods fungi of the genera *Penicillium*, *Rhizopus*, *Saccharomyces*, were isolated from surface sterilized fragments of bees (Figs. 1 and 2). Although all these fungi are not reported as entomopathogens, fungi like *Verticillium*, *Aspergillus*, *Fusarium* and *Paecilomyces* have been known to be entomopathogenic. Several of these genera are principally or exclusively associated with a single family, genus or a few species of insect pests. According to **[14]** that a number of publications discuss insect mycosis caused by *Entomopthora*, *Beauveria*, *Metarhizium* and *Aspergillus*. Among the entomopathogenic fungi, *Metarhizium* sp. and *Verticillium lecaniii* (Zimm) have wide host range.

TABLE II.

INFECTED AND NON INFECTED LOCALITIES WITH FUNGI OR BACTERIA ASSOCIATED WITH DEAD HONEYBEE FROM THE 16 CHOSEN LOCALITIES AND THE MOST OF MICROBS FOUND

Locality / Microbe	New valley	Minya	Gharbiya	Demyat	Sharqyia	Ismailia	Mansoura	Cairo	Suez	North Sinai	Banisweif	Qalubeiah	Giza	Behera	Alexandria	Alaqsour
Rhizopus spp.	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Penicillium spp.	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-
Bacillus spp.	-	-	-	+	-	-	+	+	-	+	-	-	+	-	-	-
Escherichia coli	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+	-
Pseudomonas spp.	+	+	+	-	+	+	+	+	+	+	-	-	+	+	-	-
Saccharomyces cervisia	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-

+ **and** - ; The microbe was present or absent in sample of dead honeybee

Honey bees are known to be infected seriously by fungi such as *Aspergillus flavus*, *Pericystis apis* and *Mucor hiemalis* **[15]**. In this present study of several species of *Aspergillus* were isolated. Therefore it may be assumed that the cause of honey bee might be due to infection of several *Aspergillus* species. Some of the fungal genera such as *Geotrichum*, *Epiccocum* and *Chaetomium* may not cause insect dead but these fungi are mostly found as normal contaminant according to **[16]** and **[17]**. The infective unit in most of the entomopathogenic fungi is a conidium or spores which when land on a susceptible host, put forth germ tubes or infection pegs from appressoria **[1]**. These structures secrete a complex of cuticle degrading enzymes viz., chitinases, proteases and lipases, which are capable of hydrolyzing corresponding cuticular constituent's viz., chitin, protein and lipids. There are many threats to bee survival , including the risk of diseases caused by microorganisms such as viruses, fungi, protozoa and bacteria which are all known to cause infections in bees, sometimes leading to collapse of colonies and causing serious threats to the bee-keeping industry **[18]**. Bees have two distinct life forms, brood (egg, larva and pupal stages) which develops within the hive and adult. Most diseases are specific to just one of these life stages, while the list of diseases is quite long, only a few are of serious concern to apiculturists **[19]**.

American foulbrood (AFB) is an infectious and highly contagious disease caused by a gram +ve spore-forming bacterium, *Paenibacillus larvae*, (Figs. 7 and 8).

AFB has been known to affect honeybees for hundreds of years.

AFB was found around the entire world, though different genotypes predominate in different areas and it was considered by many to be the worst disease of honeybee, the results in Table III showed this bacterium was observed in the apiaries' samples of four localities, which were Gharbiya, Banisweif, Giza and Alexandria governorates.

The diseases caused by the spore-forming bacterium, *Paenibacillus larvae*, was first described in 1769. AFB is probably the most virulent diseases of honeybee brood and is capable of causing the collapse of bee colonies.

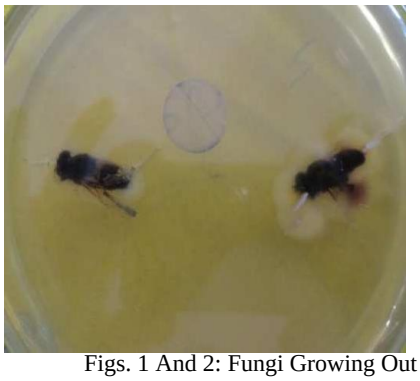
The readings attached in Table IV reflect the test reactions shown in Fig. (9), whereas it showing the effect of Nano-particles of chitosan loaded with propolis by 12 concentrations against growths of two strains pursuant both of isolated bacteria; *Escherichia coli* and *Paenibacillus larvae*, comparison with the standard strain for each one, and the negative control. The effect or minimal inhibition concentration (MIC) was evaluated using Resazurin Microtiter Assay (REMA), this effect of the treatment explained by not discoloration or change in varying degrees from blue to pink, which indicates that resazurin didn't reduced, because of success of Nano chitosan propolis for inhibition of the bacterium growth. According to **[13]**, the MIC was defined as the lowest concentration of the treatment somewhat preserved the presence of blue.

TABLE III.

INFECTED AND NON INFECTED SAMPLE WITH PAENIBACILLUS LARVAE BACTERIUM; THE AGENT OF AMERICAN FOULBROOD (AFB) DISEASE FROM THE 16 CHOSEN LOCALITIES

Locality / Microbe	New valley	Minya	Gharbiya	Demyat	Sharqyia	Ismailia	Mansoura	Cairo	Suez	North Sinai	Banisweif	Qalubeiah	Giza	Behera	Alexandria	Alaqsour
Paenibacillus larvae	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-

+ **and** - ; The microbe was present or absent in sample of dead honeybee



Figs. 1 And 2: Fungi Growing Out Of Surface Sterilized Honey Bee Workers On Pda Medium

Figs. 3 And 4: Cultural Morphology Of Some Fungi Isolated On PDA Medium

Fig. 5: BACTERIAL Growth On Nutrient Agar

Fig. 6: Pure Bacterium Isolated On MYPGP

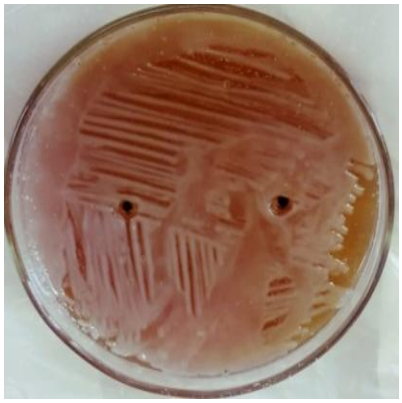


Fig. 7: Bacterial Growth On Blood Agar

Fig. 8: Pure P. Larvae Bacterium Isolated On Sheep Blood Agar Medium

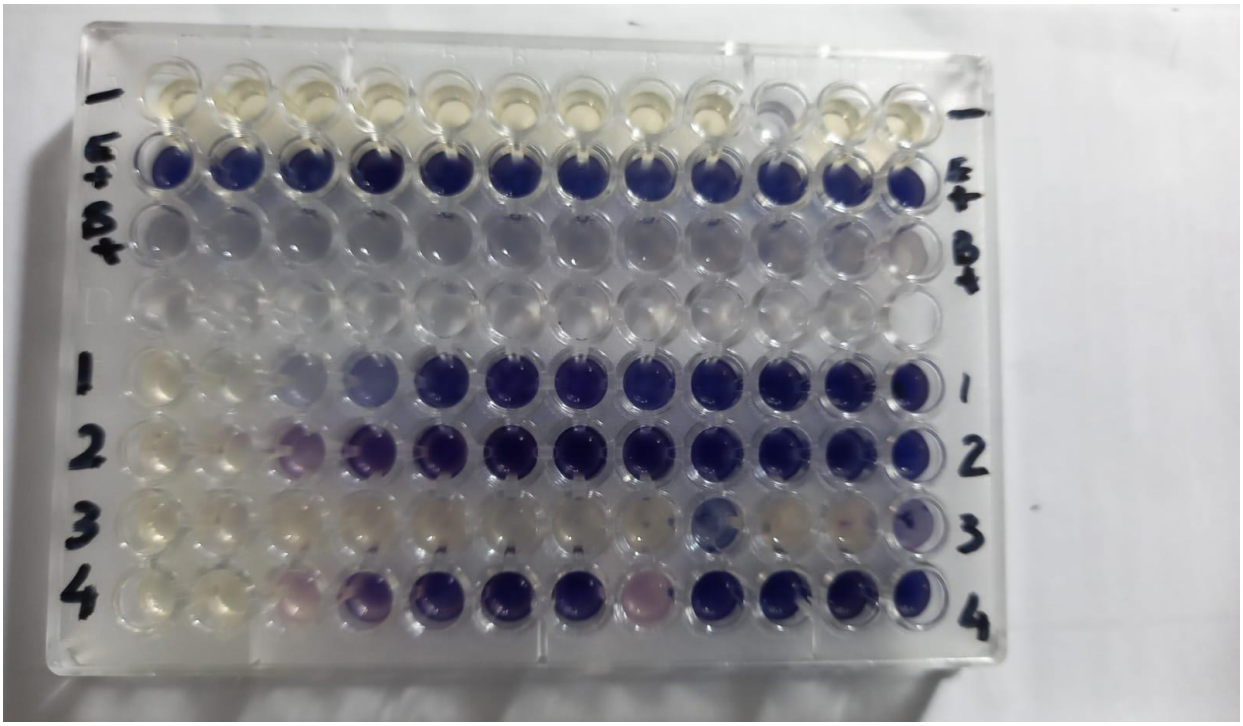


Fig. 9 : Differences Of Change For The Medium Color From Blue To Pink By Growth Of E. Coli Or P. L. Strains Under Effect Of Nano Chitosan + Propolis (Rema Method)

TABLE IV.

MIC OF NANO CHITOSAN – PROPOLIS AGAINST GROWTH OF E. COLI AND P. L. MEASUREMENT BY USING REMA METHOD

Nano chitosan & propolis Conc. % / Bacterial Strain	**0.0488**	**0.0977**	**0.195**	**0.391**	**0. 781**	**1.563**	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
-Ve control	-	-	-	-	-	-	-	-	-	-	-	-
E. coli (+ve control)	X	X	X	X	X	X	X	X	X	X	X	X
P. l. (+ve control)	√	√	√	√	√	√	√	√	√	√	√	√
E. coli (isolated strain 1)	√	√	~MIC	~	X	X	X	X	X	X	X	X
E. coli (isolated strain 2)	√	√	~MIC	~	~	X	X	X	X	X	X	X
P. l. (isolated strain 1)	√	√	√	√	√	√	√	√	~MIC	~	~	~
P. l. (isolated strain 2)	√	√	~MIC	~	~	~	~	~	X	X	X	X

MIC; minimal inhibition concentration, ***E. coli****; Escherichia coli* bacterium, ***P. l.****; Paenibacillus larvae* bacterium, **REMA**; resazurin microtiter assay method, **-** ; None inoculate, **X**; Non growth, √; Growth, ~; Growth traces (Haze)

IV. Conclusion

In the present study fungi associated with honey bee was studied. Naturally dead honey bees were collected from in and around the locality (16 localities) and fungi were isolated from surface sterilized fragments. Dead of bees usually occur due to infection of microorganisms. The microbes associated with dead bees included both bacterial and fungal genera. The intestine of bees has been found to contain gram +ve bacteria including *Bacillus* spp., *Escherichia coli* (*E. coli*), and *Saccharomyces* fungus. The fungi were found little than bacteria over the 16 localities were studied in Egypt, and it is noticeable that Alaqsour district was found empty or free from any disease. Also, it was noticeable that the dangerous bacterium, *Paenibacillus larvae* (*P. l.*) which cause the foulbrood disease in honeybee didn't largely found in the mentioned governorates, only in four samples from 16 governorates they were found, and the bacterium, *E. coli* was found in honeybee sample by or due to drinking travels of honeybee to the ponds and drains of polluted water. Nano chitosan loaded with propolis has succeed in vitro for inhibition isolates of *E. coli* and *P. l.* bacteria but it was best against *E. coli* bacterium at very low concentration than *P. l.* bacterium. Secondary sources of microbial contamination in honey are human, equipment, containers, wind, dust etc.

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