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ISOLATION AND IDENTIFICATION OF BACTERIA GENUS *LACTOBACILLUS* AND COUNTING THEIR NUMBERS FROM THE HONEY BEE *APIS MELLIFERA* STOMACHS

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ABSTRACT

This study was conducted in the College of Agricultural Engineering - the University of Baghdad, the apiary of the protection department and laboratory of the Department of Food Sciences on *Apis mellifera* hybrid honeybee colonies during the period from October 2019 to February 2020. The main study aim was isolating the bacteria genus Lactobacillus from the stomachs of honeybees and identifying them by performing morphological and microscopic examinations as well as conducting physiological and biochemical tests. The results of morphological examinations indicated that the colony is oval-shaped, shiny, with a milky white color, and its diameter does not exceed 1 mm. The microscopic examination results showed that the bacteria were gram-positive, not motile, and not forming spores. Whereas the physiological examinations proved that, the bacteria are anaerobic, inability to grow at temperatures of 5 and 15 ºC, while they grow at a temperature of 45 ºC and grow in saline media by 4% and 5%, while they do not grow in a saline media 6%. Finally, the biochemical test results showed that the bacteria are catalase-negative, do not consume citrate, do not reduce nitrates, do not produce ammonia from arginine, and have the ability to ferment certain sugars.

Keywords : Genus Lactobacillus, Honey Bee, *Apis mellifera*

Introduction

The honey bee insect is of high value and importance all over the world for its great importance to humans and ecosystems. This insect is of great importance in the pollination of many economically important crops as well as wild plants and others (Van Engelsdrop and Meixner, 2010). There is a close connection between the honey bee insect and the microorganisms that live inside its intestine, as these microorganisms, including lactic acid bacteria (LAB), digest food as well as remove toxins in harmful or toxic particles and provide essential nutrients to honey bees (Bourtzis and Miller, 2003). However, there are very important and useful microbes that are found in the intestines of honeybees, and these microbes are found in pollen and honey. Thus, gut microbial communities can affect pollinators in different ways, starting from feeding to defense against diseases (Koch and Schmid-Hempel, 2011 and Kwong *et al*., 2014).The microorganisms present in the insect gut are affected by many factors, including nutrition, physiology, and immune function. Therefore, these microbes are of great importance to honeybees (Eckburg *et al*., 2005 and Backhed *et al*., 2005). Intestinal microbes are important to honey bee health (Hamdi *et al*., 2011 and Vasquez *et al*., 2012), for example, Lactobacillus bacteria can increase honeybee immunity, and they protect them from pathogenic bacteria (Guarner and Malagelada, 2003 and Evans and Armstrong, 2006). The intestinal germ that is found in the intestines of honey bees, consists of 9 basic bacterial species and is present in adult bees (Cox-foster *et al*., 2007 and Martinson *et al*., 2012). However, the composition of the gut microbial community of honeybees is dynamic rather than static, as the intestinal microbes provide different advantages to honey bees at different ages (Kwong and Moran, 2016). In addition, intestinal germs are transferred from adult bees to newly emerging bees from the six eyes through feeding and secretion within the colony (Koch *et al*., 2013). Due to the lack of studies on Lactobacillus bacteria in Apis mellifera hybrid honey bees in Iraq. The study aimed to isolate the bacteria from the stomachs of honey bees and to detect them by performing physiological and biochemical tests to identify them and estimate the number of colonies.

Materials and Methods

Creating colonies

15 boxes were selected from the local bee race, as these boxes were identical and equal in terms of strength and bee population and their queens were fertilized from spring 2019, where the number of frames covered with bees is about 3 frames. Then, all work was carried out on the bees until the experiment was conducted on the cells on 1/10/2019 in the apiary of the College of Agricultural Engineering Sciences - University of Baghdad.

Isolation and growth of Lactobacillus bacteria from honey bee stomachs.

The process of isolating Lactobacillus bacteria, or the lactic acid bacteria (LAB), was performed from the stomach of honey bees in the laboratory of the Department of Food Sciences / College of Agricultural Sciences - University of Baghdad. As 5 workers were taken from each cell and placed in plastic boxes for the purpose of preservation, and then

transferred to the laboratory for the isolation process, where the stomach was withdrawn through the stinging machine in the last abdominal ring of the worker by forceps. Then, they were placed in a small amount of distilled water and crushed well by the loop for growing on the MRS Broth medium (Tajabadi *et al*., 2011).

- **Preparing the culture media**

Preparation of MRS broth medium

The culture media MRS broth was prepared according to the recommendations of the producing company (Oxoid / England) by dissolving 52 g in one liter of distilled water. Further, a magnetic stirrer was used to dissolve it and then distribute it in 10 ml plastic tubes, after that the medium was sterilized by autoclave at 121 ° C for a period of 15 minutes, as this medium was used in growing the bacterial cells. The solid medium MRS agar was also prepared in the same manner mentioned above, with the addition of 18 g of the agar for counting the colonies of bacteria growing on it through the colony counter.

- **Cultivation of isolates on MRS medium**

Subsequently, isolating the bacteria from the pollen and honey, they are cultured on a sterile liquid MRS medium, where a smear of both pollen and honey is taken from the loop on the inside of the isolation room (Hood). Besides, they transferred to the tubes containing the liquid medium after good sterilization on the flame, and each time. After that, the isolates are transferred to the incubator at a temperature of 37 ° C for a period of 48 hours in anaerobic conditions, where the PH of the liquid medium is measured before transferring the isolates, after which the bacterial isolates are cultured.

- **Study the morphological characteristics of colonies growing on MRS medium**

1. Morphological examination

The colonies growing surface on the MRS agar plate were selected with morphological characteristics, where the morphological examination was carried out in order to identify their morphological characteristics in terms of color, shape, edges, shining, diameter, degree of viscosity, etc.

- **Microscopic examination**

Part of the growing colonies (single and contiguous) were stained on MRS agar medium with methyl blue dye after a smear of bacteria was taken from the plate by the loop. Then, a drop of distilled water was placed on a clean and sterile glass slide, and the bacteria were mixed with distilled water on the slide by the loop and after that, it was passed on the flame three times to dry it. Furthermore, it was stained with methyl blue dye and the bacteria was examined under the microscope with a magnification power of 1000, and then their numbers were counted. Finally, the number of bacterial cells was counted by moving the slide under the microscope to the right and randomly left ten times, and then the bacterial cells are counted.

- **Staining of bacteria with a gram stain**

A portion of the growing colonies on MRS agar medium (single and contiguous) was stained using gram stain, which is four stains, Crystal violet, Iodine solution for one minute, and Ethanol 95%, Safranin for half a minute. The colonies were transferred by the loop onto a clean and sterile glass slide and the four stain were added to them separately, and the excess stain was washed with water, after which the bacteria were examined under a microscope (Coeuret *et al*., 2003).

- **Examination of cells using the hanging drop method**

The tubes containing the MRS broth medium were inoculated after sterilization and incubation at 37 ° C for 48 hours and in anaerobic conditions. Further, a drop of the bacteria growing in the center of the sterile slide cover was taken by the inoculation needle after continuous sterilization, a small amount of distilled water was placed on the four corners of the slide cover. The cover was placed on a flat surface and then a concave slide was placed on it, taking into account that the drop of the bacterial culture was at the center of the concave area with careful pressure on the slide for the purpose of sticking with the surface tension method. Finally, the slide was flipped with the cap on its face after making sure that the culture was hanged in concavity, the slide was placed under the microscope, taking into account the highlighting of the hanging drop, as well as observing the movement of the microorganisms.

- **Physiological and biochemical examinations**

1. Physiological examinations

- **Growing in aerobic conditions**

The tubes containing the MRS broth medium were inoculated with bacteria that had been isolated from the stomach of honey bees and understudy inside the isolation room, the tubes were incubated at 37 °C for 48 hours in aerobic conditions, with follow-up the bacterial growths in these tubes.

- **Growth at different temperatures in the liquid medium MRS broth**

After the tubes containing the sterile MRS broth culture medium were prepared. These tubes were inoculated with the bacterial cultures during the study and incubated at temperatures 5, 15, and 45 °C for 48 hours in anaerobic conditions with following up the appearance of sediment or turbidity (Andrews, 1997).

- **Growth in different concentrations of NaCl**

The MRS broth medium was prepared with 80% of distilled water, then different concentrations of NaCl were added, with a ratio of 4%, 5%, and 6%, after which the volume was completed to 100 ml. Then, they were distributed in test tubes, the tubes were sterilized with the autoclave at 121 °C for a period of 15 minutes, the tubes containing the saline media were inoculated with bacterial isolates and incubated at 37 °C for 48 hours and in anaerobic conditions. The readings were recorded by the appearance of sediment or turbidity in the medium containing salt for each concentration.

2. Biochemical examinations

- **Catalase test**

After the MRS agar medium was prepared, the bacterial isolates were cultured during the study on this medium separately at a temperature of 37 °C for a period of 48 hours and in anaerobic conditions. After that, a smear of the growing bacterial colonies by the loop was transferred to a clean and sterile glass slide, and then a drop of hydrogen peroxide solution was added to the slide containing the bacterial isolate and was mixed well, the appearance of gas bubbles as evidence of the positive test (Andrews, 1997).

- **Ammonia production test from arginine**

The tubes containing the prepared medium for the formation of ammonia from arginine were inoculated with bacterial isolates, which at the age of 24 hours were then incubated at 37 °C for 48 hours and in anaerobic conditions. Then, 1 ml of the inoculated medium was taken and placed in a sterile test tube and 1 ml of Nessler's reagent was added to it. The constancy of the orange color and its non-change to red color is evidence of the inability of isolates to produce ammonia from arginine (Harrigan and McCance, 1976).

- **Nitrate reduction test**

The tubes containing the medium for nitrate reduction called sterile Nitrate peptone, where this medium was prepared by dissolving 0.2 of KNO_3 potassium nitrate in 100 ml of water were inoculated with bacterial isolates and both separately, after which the tubes were incubated at 37 °C for 48 hours. Then, two solutions were prepared: solution A, which was prepared by dissolving 8 g of Sulphonic acid in a liter of 5 N acetic acid, in addition to solution B, that was prepared by dissolving 5 g of α-naphthyl amine in a liter of 5 N acetic acid. Further, 1 ml of solution A and 1 ml of solution B was added to each test tube containing the bacterial isolates. The non-coloration of the tubes indicates the negative reaction, this means, the inability of the isolates to reduce nitrate to nitrite (Andrews, 1997).

- **Citrate reduction test**

Bacterial isolates of 24 hours age were inoculated on citrate consumption medium called Simmons citrate agar, which was supplied from Difco Company by the loop and by the streaking method on the plate. Subsequently, the plates were incubated at a temperature of 37 °C for 7 days, and in anaerobic conditions, the medium color was changed from green to blue indicates a positive reaction (Harrigan & McCance, 1976).

- **Fermentation of sugars test**

The tubes containing the medium for fermentation of sugars were inoculated with the activated bacterial isolates at 24 hours of age with a rate of 1%. These tubes were also incubated at a temperature of 37 °C for a period of (3-5) days and under anaerobic conditions, after which the Chlorophenol red indicator was added at a concentration of 2%. Each sugar was prepared separately after controlling the pH of up to 6.2, during the medium preparing, where the xylose sugar was sterilized by filtration using Millipore filters that its holes diameter of 0.22 µm before adding it to the sterile medium. The readings were recorded by changing the color of the medium inside the tubes from purple to yellow, and this was due to a decrease in the pH and this indicated the positive reaction (Cowan, 1974).

- **Estimating or counting the total number of bacteria**

The total number of bacteria present in the stomachs of honeybees was counted by taking 1 ml of the medium containing the isolates. Then, a serial decimal dilution was created between the tubes, after which a smear is taken from the tubes containing the isolates by the loop and placed on a sterile slide containing a drop of distilled water. The slide was passed over the flame for a period of three times for the purpose of drying, after which a methyl blue dye was added to the slide, the dye was left for a minute, after that, it was washed with water and then placed under a microscope, after which the numbers of bacteria were counted. As for the pour plate method mentioned by (Dave and Shah, 1996), it was conducted using MRS agar, the plates were incubated at a temperature of 37 °C for 48 hours and in anaerobic conditions. After the incubation period ended, the number of bacterial colonies growing in the plates was counted using a colony counter, where the bacterial number was counted by multiplying by the reciprocal of the decimal dilution as reported in APHA (1978).

Results and Discussion

- **Isolation of Lactobacillus**

Lactobacillus bacteria were isolated from the stomachs of the honeybee, *Apis mellifera* from all the experimental treatments, where the identification was based on the morphological characteristics, staining the bacterial cells, and the physiological and biochemical examinations.

- **Morphological and microscopic characteristics of Lactobacillus**

The results in Table 1 showed both the morphological characteristics, microscopic examinations, and the hanged drop tests of the Lactobacillus, and compared them to the standard isolation of bacteria. Morphological characteristics of bacteria were characterized in the form of smooth circular colonies and whitish surfaces on the MRS Agar medium. As for the microscopic examinations of the bacteria, the results showed that the bacteria are gram-positive, and they are long or short bacilli. As well as, they are in the form of chains or single and not formed from spores, while the motility test using the hanging drop showed that the bacteria are not motile.

Table 1 : The morphological and microscopic examinations of the local isolate from the stomach of honey bees compared with the standard isolation according to (Bergy's manual, 1994)

Examination type	The isolation characteristics	Standard isolation
Morphological characteristics		
A- Colony		
Shape	Convex from the top	Convex (like a dome)
Color	Milky white	Milky white
Shining	Shiny	Shiny
Edge	Smooth	Smooth
Diameter	1 mm	1 mm
B- Cells		
Shape	Single or double lanceolate cocci	Single or double lanceolate cocci
Spores	Not forming spores	Not forming spores
Motility	Not motile	Not motile
Gram stain	Gve +	Gve +

- **Physiological and biological examinations**

The results in Table 2 showed both the physiological and biochemical examinations of the local isolate from the stomachs of honeybees for identifying the Lactobacillus. Physiological examinations showed that there are few growths of bacterial isolates in aerobic conditions, and these bacteria were grown at different temperatures, which are 5 °C. Moreover, the results showed the inability of these bacteria to grow at a temperature of 5 °C, as well as their inability to grow at a temperature of 15 °C and their ability to grow at a temperature of 45 °C; this is consistent with what was mentioned by (Holt *et al*., 1986). Whereas, the growth in the different saline concentrations, which are 4%, 5%, and 6%, the results of Table 2 showed the ability of this bacteria to grow at 4% salt concentration, as well as its ability to grow at 5% saline concentration. As for the concentration of 6%, these bacteria showed their inability to grow in this saline concentration.

Table 2 : Physiological and biochemical examinations of local isolation from honey bee stomachs compared to standard isolation according to (Bergy's manual, 1994).

Examination type	Local isolation	Standard isolation
2- Physiological examinations		
A- Growth in aerobic conditions	+/-	-
B- Growth in temperatures		
5	-	-
15	-	-
45	-	-
C-Growth in different concentrations of NaCl		
%4	+	+
%5	+	+
%6	-	+
Anaerobic growth	+	+
3- Biochemical tests		
A- The catalase test	-	-
B- Producing ammonia from arginine	-	-
C- Reduction of nitrates	-	-
T- consumption of citrate	-	-
D- Fermentation of sugars		
1- Glucose	+	+
2- Fructose	+	+
3- Sucrose	+	+
4- Raffinose	+	+
Arabinose	-	-
Xylose	-	-

However, these biochemical tests were carried out as the last stage of the selection process to ensure that they belong to the genus Lactobacillus, as the results of Table 2 showed that these isolates belong to the genus Lactobacillus based on the standard isolation. This bacteria is catalase-negative due to its inability to produce the enzyme peroxidase, which is evidenced by the emergence of gas bubbles. The results also showed the inability of these bacteria to release ammonia from arginine, as well as their inability to reduce nitrates, due to the lack of enzyme Nitrate reductase, which works to convert nitrates into ammonia, which is consistent with (De Vos *et al*., 2009) findings. These results also showed its inability to consume citrate, its ability to ferment certain sugars such as Glucose, Fructose, Sucrose, and Raffinose, and its inability to ferment other sugars such as Arabinose and Xylose, and this is consistent with (Holt *et al*., 1986

- **Counting the total number of bacteria**

The results of Table 3, which includes counting the real number of colonies and bacterial cells after they were isolated from the stomach of honeybees, showed that box No. (14) recorded the highest number of bacterial colonies, as it reached (101,000) colonies, while both boxes No. (3) and (8) recorded the lowest number of bacterial colonies, as it reached (92,000) colonies for each, respectively. As for the count of bacterial cells, box (9) recorded the highest rate of bacterial cells, as it reached (74) cells/ml, while the lowest number of bacterial cells was recorded in both boxes No. (3) and (7) reached (60) cells/ml each. It can be concluded from this that the numbers of both colonies and bacterial cells depend mainly on the strength and activity of honey bee colonies, as it also depends on the type of nutrition and the availability of pastures well around the apiary site.

Table 3 : Counting the number of colonies and bacterial cells isolated from the stomachs of honey bees

Box number	Bacterial colonies number	Bacterial cells number
1	99000	65
2	96000	61
3	92000	60
4	97000	68
5	95000	63
6	100000	64
7	99000	60
8	92000	66
9	93000	74
10	97000	63
11	91000	73
12	97000	62
13	93000	69
14	101000	72
15	98000	65

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