Isolation and identification of Lactobacillus bacteria in the stomach of honey bees and recording a type of bacteria

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Abstract

This study was conducted in the apiaries of the College of Agricultural Engineering Sciences / University of Baghdad, and 15 packages of *Apis mellifera* honey bees were selected. As Lactobacillus bacteria were isolated from the stomach of honey bees, where samples were taken from the bees at the rate of (20) bees for each treatment , the stomach of the bees was withdrawn, Then, it was mashed and inoculated on MRS agar solid culture medium in plates, and the plates were incubated in the incubator at 37°C for 48 hours. The results of the molecular diagnosis by PCR technique showed that the proportions of matching the nucleotide sequence of a part of the 16S ribosomal RNA gene of the bacterium *Apilactobacillus kunkeei i*solated from honey bees in Baghdad province, which were multiplied using the primer kit (16 SrRNA) and its equivalent sequences retrieved from the gene bank. The phylogenetic tree, which was drawn by the (Tamura) program of the type of macrophages, shows the genetic relationship that was built from the nucleotide sequences of part of the genetic region 16 SrRNA ribosomal gene belonging to the bacterium Apilactobacillus kunkeei isolated from honey bees in Baghdad Governorate (marked with the symbol ○). They were duplicated using primers set (16 srRNA) and their equivalent sequences retrieved from the GenBank.

Introduction

Apis mellifera bees are social insects that live in groups that work as one unit to preserve their species and contribute to the improvement of the ecosystem in the medium in which they live. The importance of bees of all kinds lies in their ability to pollinate many crops and vegetables. Their outputs (honey, propolis wax, royal jelly) are important in human food and medicine (11,12 ,4)

From their guts, a liquid with many different hues that is therapeutic for humans emerges. 69th Surah An-Nahl, Despite living in a high population density, having access to food, and having fewer immune genes associated with immune function than solitary insects, honey bees are susceptible to infection from a variety of pathogens. However, honey bees have evolved both individual and societal defense mechanisms to combat these threats. Living organisms that reside in the digestive system are one of these methods. These organisms aid in the digestion of food, the removal of toxins from particles in honey bees' stomachs, protection from pathogens and parasites, provision of growth factors, and improvement of immunity. (1,5) and LAB bacteria are among the largest bacterial groups that were discovered inside one insect, adding to honey bee workers a high ability to resist microbes (10). There were many types of Lactobacillus bacteria, where 45 species of bacteria were isolated and identified from bees, bee products and flowers, and Lactobacillus was the most common genus, as it formed, 950% of the bacteria found in honey, 74.6% in pollen, 83.9% in bee bread, 93.3% in royal jelly, and 30.3% in the whole alimentary canal (2,3) , and the. Kunkeei type is the most common. (9) showed that the use of LAB (*Apislactobacillus Kunkeei*) in glucose solution over 5 months reduced mummification of larvae in chalkbrood disease by more than 80%. These data highlight that giving probiotic lactic bacteria. In the diet of honey bees it can be a valid strategy for biological control of diseases.

This study aimed:

Isolation and Identification of lactic acid bacteria Lactobacillus in honey bee workers by PCR technique.

Materials and methods

Partial diagnosis by PCR technique

Sample preparation

For each treatment, (20) bees collected samples from the bees. The bee stomach was removed, crushed, and inoculated in plates on MRS agar. 48 hours were spent incubating the plates in the incubator at 37 °C.

DNA extraction

1- Colonies growing on MRS agar were washed by adding distilled water 100 µl for the purpose of obtaining bacteria. In tubes with covers, the germs were scraped off and put. The tubes underwent a 5-minute centrifugal treatment in a centrifuge. A precipitate was ignored while a filter was developed. . he filtrate was added to the precipitate 750 µl of DNA EiuTion Buffer and mixed well.

2- Add 50-100 mg (wet weight) of the bacteria that had previously been suspended in 200 microliters of water or isotion solution (PBS).or approximately 200 mg of tissue into a ZR Bashing Baed Tm lysis tube (0.1 mm and 0.5 mm) and 750 µL of lysis solution was added to tube 2.

3- The tube was installed in a granules beater attached to a 2 ml tube holder, then the beater was operated at maximum speed for 5 minutes.

4- The centrifugation process was coundected on the tube containing the decomposition medium ZR Bashing Baed Tm in the centrifuge at a speed of 1000 cycles for one minute.

5- Transfer about 400 microliters of the supernatant to the Zymo Spin TM IV spin filter Corang ToP in the collection tube and centrifuge at 7000 rpm for one minute.

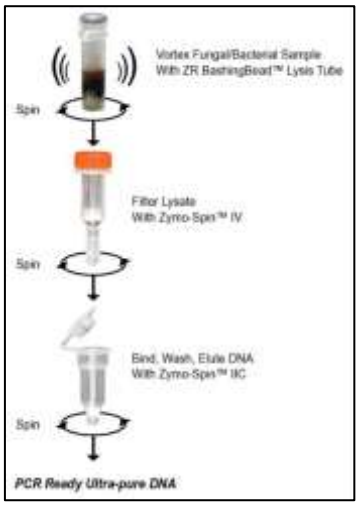
6- 1200 microliters of bacterial DNA binding buffer solution was added to a filter in the collection tube, step (5).

7- 800 microliters of the mixture in step (6) was transferred to an IIC column3 Zymo Spin TM in the collection tube, and the centrifugation process was carried out at 10,000 cycles for one minute.

8- Discard the supernatant from the collection tube and repeat step (7)

9- Add 200 microliters of DNA wash Buffer solution to the IIC Zymo Spin TM column.
In the new collection tube, it was centrifuged at 10,000 revolutions for one minute.

10- The IIC Zymo Spin TM column was transferred to a clean centrifuge tube with a capacity of (1.5) ml, and 100 microliters of DNA buffer solution was added directly to the column (Column matrix) and a centrifugation process was performed at a speed of 10,000 cycles for 30 minutes to extract DNA.



. PCR reaction

1. Primer

For the purpose of conducting a chain amplification reaction (PCR), the primer that targets the specific sequence of the 16srRNA gene was used. It is a general primer produced by Integrated DNA Technologies Company USA to detect types of bacteria, and it consists of the sequences listed in Table (1)

Table 1: primers targeting the specific sequence of the 16srRNA gene

Primer	Sequence	Tm (Cº)	GC (%)	Product size
Forward	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250 Base
Reverse	5'- GGTTACCTTGTTACGACTT- 3’	49.4	42.1	Pair

Dissolve the lyophilized primers in deionized water to reach a final concentration of 100 picomol / microliter as a standard solution and keep this standard solution at -20 °C, and to prepare 10 picomole / microliter to make the primers suspension 10 µL of standard solution was taken in 90 µL of deionized water to reach a final volume of 100 µL, and the assay was done by IDT (Integrated DNA Technologies company, Canada).

2. Prepare the Monoplex PCR master mix

The PCR mixture was prepared using the Maxime PCR PreMix kit (i-Taq) 20µlrxn (Cat. No. 25025) which was prepared by (Korean Intron) and according to the company's instructions.

The reaction mixture was prepared in a final volume of 25 microliters by mixing the ingredients listed in Table (2,3) and for each sample.Then, the contents of the reaction mixture were mixed for several seconds by means of a Vortix mixer, then the tubes were transferred to a PCR Thermocycler to conduct PCR thermocycler conditions.

Table (2) Components of the Maxime PCR PreMix kit (i-Taq)

Components	concentration
Taq PCR PreMix	**5µl**
Forward primer	**1 µl**
Reverse primer	**1 µl**
DNA	**1.5µl**
Distill water	**16.5 µl**
final size	**25µl**

Table (3): Components of the Monoplex PCR master mix

Material	concentration
i-Taq DNA Polymerase	**5U/ µl**
DNTPs	**2.5Mm**
Reaction buffer (10X)	**1X**
Gel loading buffer	**1X**

3. Thermal cycles program for DNA amplification

The polymerase enzyme reaction was coundected using a thermocyclur in PCR purification using the 16srRNA gene and according to the thermal conditions listed in the following table:

Table (4): Conditions used in the PCR thermometer

No.	Phase	Tm (C^o)	Time	No. of cycle
1	Initial Denaturation	95C^o	5 min.	1 cycle
2	Denaturation -2	95C^o	45sec	35 cycle
3	Annealing	58C^o	45sec	
4	Extension-1	72C^o	45sec	
5	Extension -2	72C^o	7 min.	1 cycle

Electrophoresis of DNA on an agarose gel

Electrophoresis was carried out on a 1.5% agarose gel to identify the DNA fragments after extraction in the presence of standard DNA and to characterize the size of the DNA fragments resulting from the PCR technique.

Preparation of agarose **gels**

1.5% was prepared according to the method of Sambrook et al., 1989 by dissolving 1.5 g of agarose in 100 ml of TBE Buffer solution.Heat the jelly to the point of boiling, then leave to cool to a temperature of 45-50°C. According to the gel in the gel sheet after installing a comb to make holes or drill to put the DNA. Calculate the gel in a gentle way to avoid the formation of bubbles and leave for 30 minutes to harden. Softly lift the comb out of the gel

Fix the paper in the electrophoresis basin for a horizontal shape. Fill the basin with TBE Buffer solution so that it covers the surface of the gel.

Then, operate the relay using a current of 7 V/cm2 for 1-2 hours.

After the end of the migration process, the gel containing the PCR results was examined using the Vlightsource.U ultraviolet light at 360 nm wavelength to determine the product with the measurement unit.

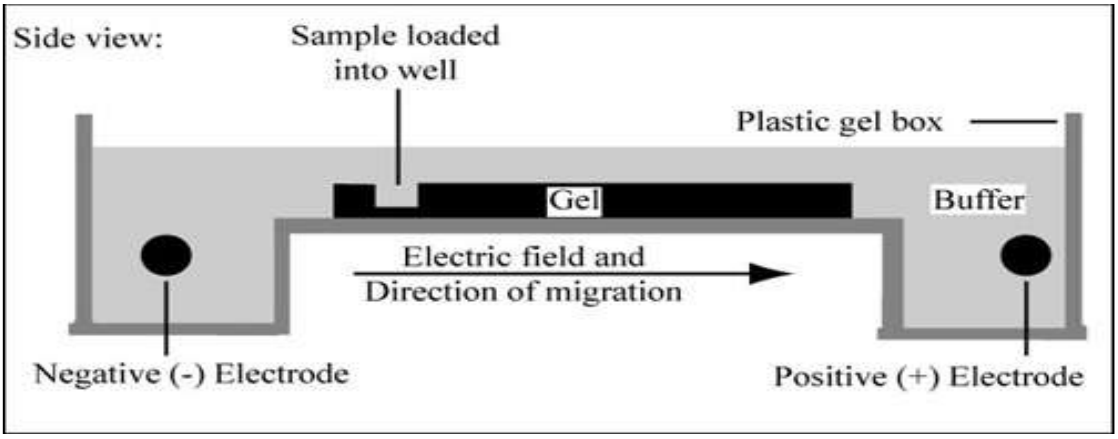


Fig (1) the electrophoresis system

Results and discussion

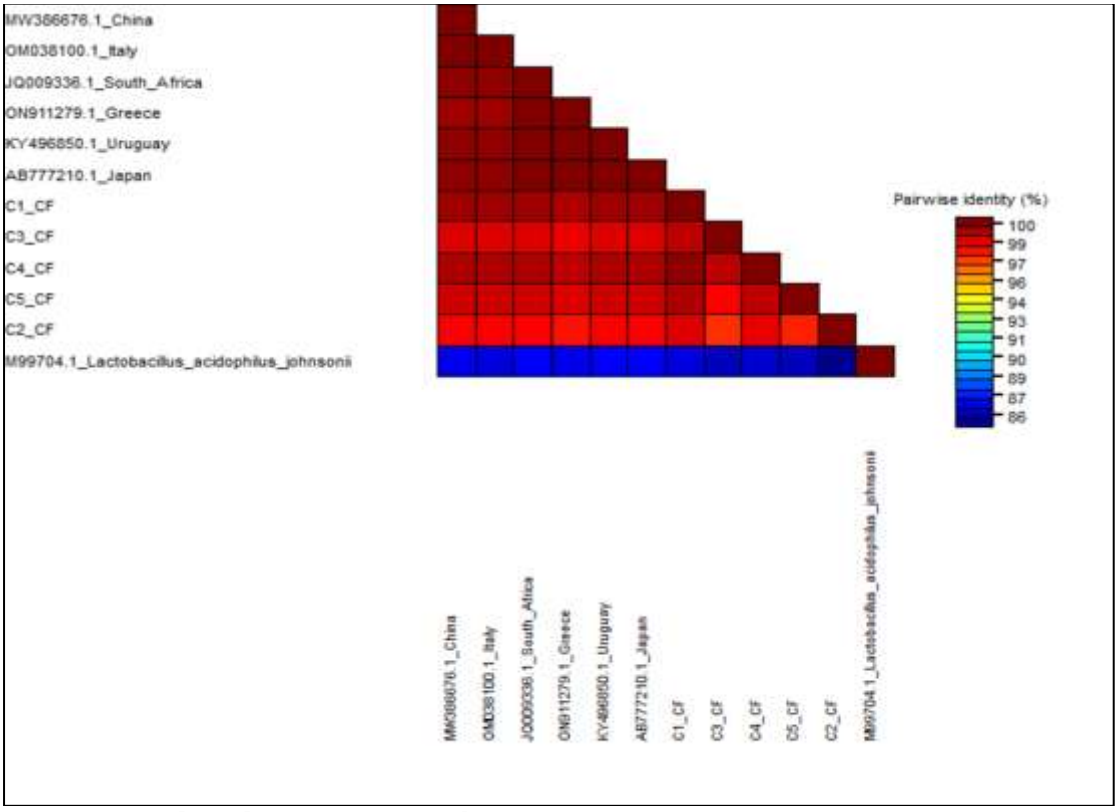


Fig (2) : Nucleotide sequence matching ratios .

Percentages matching the nucleotide sequence of part of the 16S ribosomal RNA gene region of the bacterium Apilactobacillus kunkeei isolated from honey bees in Baghdad province, which were multiplexed using the primer set (16 SrRNA) and its equivalent sequences retrieved from the Genbank. The nucleotide sequence of the 16S ribosomal RNA gene of Lactobacillus acidophilus johnsonii was used as an outgroup comparison. Results were analyzed using Muhire software (SDTv1.2 et al. 2014)

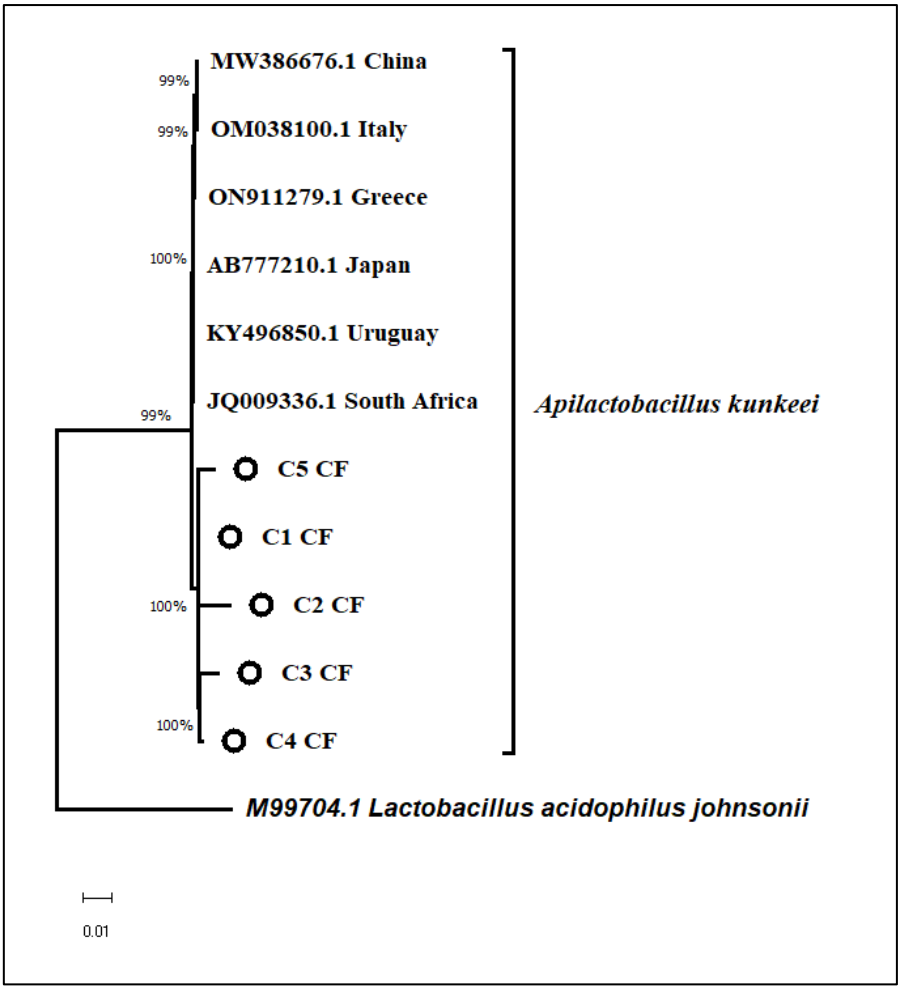


Fig (3) . *Apilactobacillus kunkeei*

The phylogenetic tree of the type connective tissue adjacent to it shows the genetic relationship. It was built from the nucleotide sequences of a part of the 16S ribosomal RNA gene region belonging to the bacterium *Apilactobacillus kunkeei* isolated from honey bees in Baghdad provaince (marked with the symbol ○), which were replicated using the primer set (16 SrRNA) and its counterparts. of its equivalent sequences retrieved from the GenBank. The nucleotide sequence of the 16S ribosomal RNA gene of Lactobacillus acidophilus johnsonii was used as an outgroup comparison. This tree was drawn using the MEGA11 program (8).

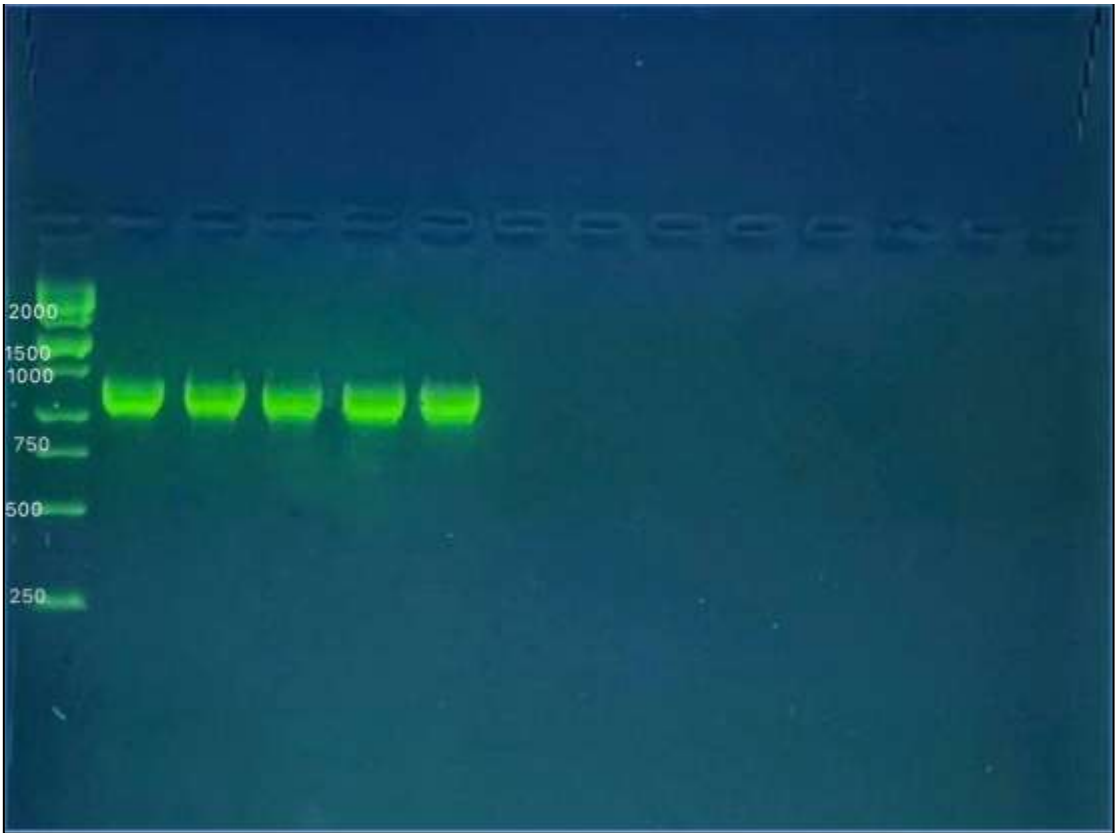


Figure (4)of the DNA duplicate product using crystal chain reaction (PCR) isolated from the stomach of the honey bee *Apis mellifera*

The results of the SKONSC were analyzed by the Korean company using the (BLAST) program available at the National Center for Biotechnology Information (NCBI) and included using the program BioID). (Tamura et al., 2011).

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