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ISOLATION OF LACTOBACILLUS BACTERIA FROM THE STOMACH OF HONEY BEES AND THEIR EFFECT AS A PROBIOTIC IN INCREASING THE NUMBER OF BACTERIA.

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Abstract

This study was conducted in the apiary of the College of Agricultural Engineering Sciences, University of Baghdad, and 15 hive of honey bees, Apisi smellifera, were selected. *Lactobacillus* bacteria were isolated from the stomach of honey bees and diagnosed phenotypically by conducting biochemical tests, then they were added to bee food to see their effect on increasing the numbers of bacteria and colonies. The local isolate was compared with the commercial preparation by adding it to a diet consisting of 10 gm of pollen to a sugar solution at a concentration of 1:1.The results of the phenotypic tests showed that the bacteria had circular colonies, with a convex diameter at the top, and white surfaces.Gram-positive bacteria, and physiological tests proved that the bacterium is anaerobic and unable to grow at temperatures of 5 and 15 C. While it grows at 45 °C, it also grows in a saline medium of 4% and 5%, while it does not grow in a saline medium of 6%.The results of the biochemical examination indicated that the bacteria are negative for catalase, do not consume citrate, do not produce ammonia from arginine, and have the ability to ferment sugars. Finally, results were shown. Counting the numbers of bacterial cells and colonies showed an increase in the number of cells and bacterial colonies isolated from the stomach of bees during feeding periods. The local isolate treatment achieved a concentration of 3% at the highest rate, as it reached at the end of the experiment 197.00 x 105 in2, then the treatment of the commercial preparation at a concentration of 3%. It reached 105 × 162.20 in2, then the local preparation treatment, a concentration of 1%, followed by the treatment of the commercial preparation, a concentration of 1%. This indicates that the concentration of 3% of the local isolate has achieved higher results in increasing the numbers of bacteria cells and colonies.

Keywords: Lactobacillus bacteria, stomach, honey bees, probiotic

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 (Villalba et al., 2020, Zheng et al., (2017) Al-Hujaimi. 2003. This was mentioned in The Holy Quran:

There comes forth from their bellies a drink of various colours, in which is healing for people." Surah An-Nahl 69

Honey bees are exposed to infection with many pathogens, and despite the fact that honey bees live in a high population density and the presence of food, as well as the decrease in immune genes associated with the immune function compared to solitary insects, makes it a target for many pathogens, but honey bees have developed defensive means at the level of the individual and the

group. Among these means are living organisms that inhabit the digestive system, and these organisms work to help bees to carry out their tasks by helping to digest food, remove toxins from particles in the stomach of honey bees, provide protection from pathogens and parasites, provide growth factors and increase immunity (Engelc.Moran. 2013; Al-Ali, 2011,)The presence of microorganisms in the digestive tract of honey bees depends on preference and coexistence, and they are present, including lactic acid bacteria (Lactobacillus (Babandrillus. Vasguezct et al., 2012). Lactobacillus bacteria is one of the largest bacterial groups that were discovered inside one insect. These bacteria add to honey bee workers a high ability to resist microbes and remain in the stomach of bees (Alejandra. Vasguez 2012; (Al-Jourani et al,2004; Al-Hujaimi et al 2012)It was found that pollen is attractive to honey bees to varying degrees and is a major source of proteins in honey bee diet, and that the protein level and quality affect the lifespan and performance of honey bee colonies. Feeding bees on pollen in a sugar solution at a rate of 100 gm for the period from August to September led to an increase in brood by 23% compared to the fed colonies, and feeding bees on pollen before the nectar season led to an increase in the productivity of honey bee colonies compared to unfed colonies (2010 .Schneider.Brods).The beekeeper resorts to feeding the bees with food supplements instead of honey, especially in seasons when natural food is not available or in case of insufficient food in the hive and due to the lack of studies on the use of bacteria isolated from the stomach of honey bees LAB and adding them to bee food and its effect on the activity of Lactobacillus bacteria present in the stomach of bees by calculating the numbers of colonies and bacterial cells.

The study aimed to:

1- Evaluation of the efficiency of Lactobacillus isolated from the stomach of honey bees in affecting the bacteria in the stomach and increasing their number.

Materials and methods

-1Isolation and cultivation of Lactobacillus bacteria from the stomach of honey bees

The process of isolating Lactobacillus bacteria from the stomach of honey bees before and after feeding was conducted in the Girls' Diseases Laboratory , Girls' Prevention Department , College of Agricultural Engineering Sciences. (5) workers were taken from each hive and placed in plastic boxes and transferred to the laboratory for isolation.

-2 Developing isolation on MRS broth

The liquid medium was prepared as mentioned in the previous paragraph from the preparation of the liquid medium, then the stomach was pulled through the stinging machine by forceps in the last abdominal ring inside the isolation room (Hood). The stomach was mashed in a drop of sterile water and mediated by the inoculation needle after passing it over the flame each time to sterilize it before use and then grow the bacteria in MRS broth in test tubes and using pivot pressure walls. The tubes were incubated at a temperature of 37 C for a period of 48 hours in anaerobic conditions.

-3 Purification of bacteria

For the purpose of obtaining pure colonies of LAB bacteria, isolated colonies from honey bee stomachs were planted in dishes containing Aagar MRS with three replicates for each sample.As a smear was taken from the growing colonies in the previous paragraphs from the development of

the bacterial isolate, and it was cultivated in 3 dishes representing 3 replicates, by the method of planning by means of an inoculation needle, and it was incubated at a temperature of 37 C for 48 hours in anaerobic conditions.

Laboratory tests of bacterial isolates

A- Physical tests

The phenotypic traits were recorded on the colonies growing on MRS agar medium, through which they can be identified from these characteristics: color, edges, shape, degree of viscosity and gloss, and diameter.

b. Microscopic tests

A smear was taken from the growing colonies on MRS agar solid culture media by inoculation needle and placed on a glass slide mixed well in a drop of distilled water and passed the slide on the flame to dilute. Methylblue dye was added to the sample and a coverslide was placed. Bacterial colonies were tested using a light microscope (Proway composite) with 1000x magnification.

C- Staining bacteria with a gram stain

A smear was taken from the growing colonies on the solid culture medium MRS agar with the mediation of the inoculation needle and placed on a glass slide a drop of distilled water was added and spread by the inoculation needle and it was dyed with a gram dye, Iodinesolution, Crystalviolet for one minute and Ethanol and Safranin for half a minute after the addition of these four dyes and each Separately, the excess dye was washed off with water, and the bacteria were examined under a microscope (Coeuret et al., 2003.(

Physiological and biochemical tests

A- Growing in aerobic conditions

The tubes containing MRS broth were inoculated with bacteria, and the tubes were incubated at 37 °C for 48 hours under aerobic conditions, and the appearance of growth in those tubes was monitored.

B- Growth in different concentrations of NaCl

Prepare the liquid culture medium MRS broth and add sodium chloride solution at 5%, 4% and 6% to the medium and complete the volume to 100 ml with distilled water. Sterilize the medium at 121 °C. It was pressurized for 15 minutes and distributed in tubes and incubated in the incubator at 37 °C for 48 hours under anaerobic conditions using pivot walls in the Department of Industries / College of Agricultural Engineering Sciences / University of Baghdad. The formation of turbidity in the medium containing sodium chloride was recorded as an indication of the growth of bacteria.

C. growth at different temperatures

The liquid culture medium MRS broth was inoculated in tubes with bacteria and the tubes were incubated at temperatures (45, 15, 5) for 48 hours under anaerobic conditions and monitoring the appearance of turbidity in the medium.

Biochemical tests

A- Catalase tested

MRS agar was prepared and inoculated with bacterial isolates in petri dishes, and the dishes were incubated at 37°C for 48 hours under anaerobic conditions.

A swab of bacterial colonies growing on the solid medium was transferred to a glass slide, and a drop of hydrogen peroxide (Hzoz) was added to it and mixed well. The appearance of bubbles is evidence of a positive test (Andrews, 1997).

2- Choose the consumption of Citrate

Transferring a portion of the growing bacterial colonies onto the solid medium by means of an inoculation needle. It was spread on the medium of consumption of citrates (Simmons citrateagar) prepared by the company () by the planning method in Petri dishes, and the dishes were incubated at a temperature of 37 C for a period of 7 days under anaerobic conditions.The color change from green to blue indicates that the reaction is positive (Mccanae, Harrigan 1976).

Internal feeding of honey bee colonies

it used industrial pollen powder from the local markets of Chinese origin, to which sugar solution was added at a ratio of 1/1 and local bacteria isolated from the stomach of honey bees with two concentrations of 3% and 1%, and commercial bacteria with two concentrations, 1% and 3% in a volume of 100 ml per hive for a period of four months, twice a week, and the treatments were as follows:

1- Pollen + local bacteria at a concentration of 1% + 99 ml of sugar solution
2- Pollen + local bacteria at a concentration of 3% + 97 ml of sugar solution
3- Pollen + commercial bacteria at a concentration of 1% + 99 ml of sugar solution
4- Pollen + commercial bacteria at a concentration of 3% + 97 ml of sugar solution
5- Pollen + 100 ml sugar solution.

Results and discussion

Phenotypic and biochemical tests results

The results of Table (1) showed that there is a great similarity in most of the phenotypic, physiological and biochemical characteristics of LAB bacteria when comparing the local isolate with the specifications of the standard isolate.

Table (1) Biochemical tests of the local isolate from the stomach of honey bees compared with the standard isolate according to Williams and Willkins (1994)

standard isolation	isolation traits	Biochemical tests
−	−	A- Catalase tests
−	−	B- citrate consumption test
−	−	C- Production of ammonia from arginine
−	−	The reduction of nitrates
−	−	The fermentation of sugars
+	+	1- Glucose
+	+	2 fructose
+	+	3 screws
−	−	4- Arabinose
−	−	5- Xylose

Calculate the true number of bacterial cells and colonies

Calculate the actual number of bacterial cells

The results of Table (1), which includes the calculation of the number of bacterial cells isolated from the stomach of honey bees, are shown as it was found that the treatment of pollen grains with sugar solution added to the local isolate at a concentration of 3%, the average number of bacterial cells was 197.00 × 105 compared to other treatments, and the lowest rate of bacterial cells was in the comparison treatment, which amounted to 98.73 × 105. We conclude from this that the treatment with pollen and sugar solution added to the bacteria of the local isolate caused an increase in the number of bacterial cells and the progression of the time period with feeding, that is, adding bacteria as a probiotic to the food leads to an increase in the numbers of bacterial cells .Where these bacteria cells give an effective role to honey bees in protection against pathogens, and increasing these numbers is considered a treatment for honey bee colonies (Vaquez.Olofsson (2008).As it was noted from the table, the longer the time period, the higher the number of bacterial cells. Pollen plants with bacteria recorded 3% locally in June, the highest number of bacterial cells. It amounted to 263.33 x 10^5 and less than the control treatment, where the number of bacterial cells reached 118.33 x 10^5.The results of the specialist analysis also showed that there were high significant differences from the local isolation coefficient of 3% and other treatments, and significant differences in the treatments and control.(Audiso.2018.fancitiotti) 2017. An increase in the productivity of honey bee cells increased by feeding on the enriched sugar solution. In addition, the incidence of two diseases, Nosema and Varroa, decreased.

Table / Number of LAB bacterial cells in the digestive system of honeybee workers at different treatments during successive time periods after treatment

Bacterial cells (cell) 1 ml x 10 ^{5}						
average	**Month**(6)	(5) **Month**	**Month**(4)	(3) **Month**	**Month**(2)	treatments
167.60	**201.67**	**186.67**	**180.33**	**168.33**	**101.00**	The preparation is commercial 1%
162.20	**221.33**	**199.00**	**180.00**	**127.33**	**83.33**	The preparation is commercial 3%
140.03	**206.03**	**189.03**	**130.03**	**105.03**	**70.00**	local isolation 1%
197.00	**263.33**	**237.33**	**209.33**	**190.33**	**84.67**	local isolation 3%
98.73	**118.33**	**125.33**	**86.67**	**86.67**	**76.67**	control
15.30	**34.21**					lsd 5%
	202.14	**187.47**	**157.27**	**135.54**	**83.13**	average
	15.30					**lsd5%**

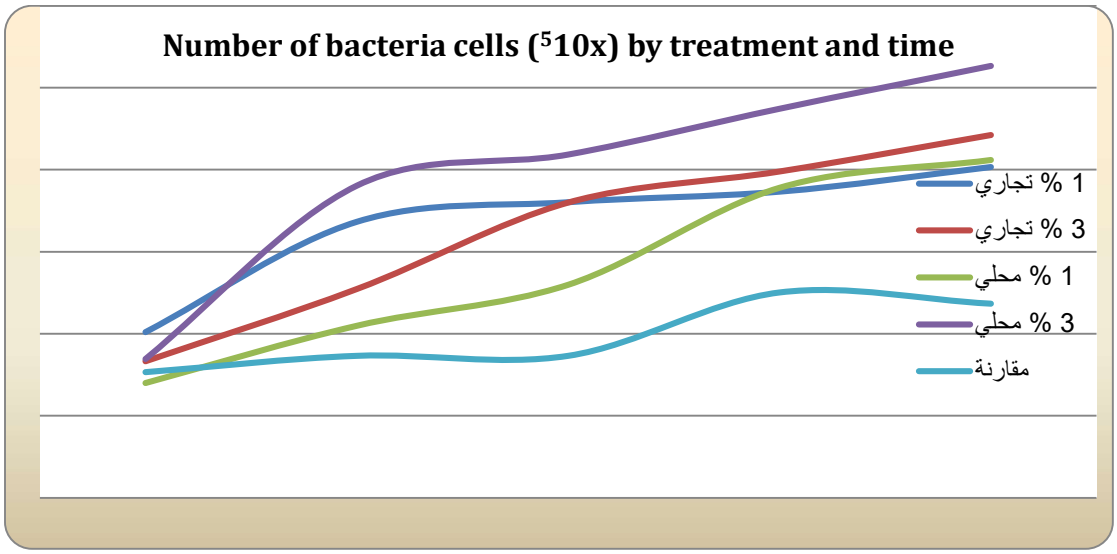

Figure (1) Preparation of bacteria cells for the treatments during the time periods

Calculate the true number of bacterial colonies

The results in table below (Table 3) are based on calculating the numbers of bacterial colonies isolated from the stomach of honey bees and for all treatments before and after feeding. It excelled the rest of the other treatments as it reached its peak in the month of June. The number of bacterial colonies was 263.3, followed by 221.3% commercial, 1% local, and then 1% commercial control shows that the number of bacterial colonies increases with the progression of the feeding period. We conclude from this that the numbers of bacteria colonies increase as the time period progresses with feeding, as well as the addition of local and commercial isolates to honey bee food, as adding bacteria to bee food leads to an increase in the number of colonies .As the processed microorganisms added to honey bee food have an important role in increasing these numbers of bacterial colonies.This is consistent with Al-Azzawi, (2021), where he showed that feeding with sugar solution increased bacterial colonies with increasing time periods.

Table (3) / Number of colonies and numbers of LAB bacteria stomach of honeybee workers of different treatments during successive periods of time before and after feeding

Number of colonies during the study months						treatments
average	June	May	April	March	February	
1676	**201.67**	**186.67**	**180.33**	**168.33**	**101.00**	preparation is commercial 1%
170.1	**221.33**	**199.00**	**178.33**	**168.33**	**83.33**	preparation is commercial 3%
135.1	**202.33**	**185.00**	**125.67**	**92.33**	**70.00**	local isolation 1%

197.0	**263.33**	**237.33**	**209.33**	**190.33**	**84.67**	local isolation 3%
98.7	**118.33**	**125.33**	**86.67**	**86.67**	**76.67**	Control
24.18**	**30.46****	**61.9****	**37.06****	**38.23****	**18.13***	preparation is commercial 1%

References

Al-Ali, Abdul-Baqi Muhammad, 2011, Book of Beekeeping, Plant Protection Department / College of Agriculture - University of Baghdad, pg. 434.

Al-Jourani, Reda Sakkab, Kamila Ward Shaher, Alaa Sharif Abbas, 2004, Evaluating the Efficiency of Some Means for Controlling American Foul Brood Disease (AFB) on Honeybees, Iraqi Journal of Agricultural Sciences, 35 (4): 100-95

Al-Hujaimi, Kamila Ward Shaher, Taher, Intisar Jabbar, 2012, Pollution of honey bees with lead and cadmium, Iraqi Journal of Agricultural Sciences. 43(15): 68-63

The Holy Quran

Al-Hujaimi, Kamila Ward Shaher, Hussain Khudair Al-Tai, 2008. Training needs of beekeepers and their problems in Iraq. Iraqi Research Journal of Agriculture) Vol. 13, No. 2, pp. 173-0187

Al-Azzawi, Hussein Ali Khaled (2021). Isolation and identification of Lactobacillus bacteria from the stomachs of Apis mellifera honey bees and the effect of food on their activity. Master Thesis. College of Agricultural Engineering Sciences. Baghdad University. 40 pages

Audisio, M. C. and Bente 2-Ahrendts, M. R. 2011. Lactobacillus Johnsonii CRL 1647, isolated from APS: mellifera L.bec-gut, exhibited a beneficial effect on honey bee Colonies. Bene f Microbes 2:29-34.

Babendreier D; Joller D; Romeis); Blglerf, and Widmer f. 2007. Bacterial community structuresin honey bee intestines and their response to two insecticidal proteins. FEMS Microbiol Ecol:59: 600-10.

Brodschneider, R. and Crailsheim, K. 2010. Frits, Nutrition and health in honey bees. Apidologie, (3): 278- 29/٠٠٧

coeuret, V.; Dubernet, S.; Bernar deans Mag Guegun, M. and vernouxj, P. 2003. Isolation, Characterization and identification of lactobacillus focusing mainly on cheese and other dairy products. La , 83: 29-306

Engel, P. and Moran, N.A. 2013. The gut microbiota ofinsects diversity in structure and function. FEMS Microbiology Reviews, 37(5): 699-735.

Flint, H.J; Scott, K. P; Louis P, and Duncan, S.H. 2012. The role of the gut microbiotain microbiota in nutrition and health. Nature Reviews. Gastroenterology and He Patology, 9(16): 577-589

Harrigan, w. f. and Mc Cance, M. E. 1976. Laboratory method in food and dairy microbiology Academic Press, London, UK. Page. 452.

olivera, R.P.D.S.; Perego, P.; Deoliveria, M. N. and Converti, A. 2011. Effect of inulin as prebiotics and symi biotic interaction between probiotics to improve fermented milk firmness. j. food. Engineering., 107:40 -46.

9- Olofsson, T. Cand vasquez, A. 2002. Detection and identification of a novel lactic acid. bacterial flora with in the honey stomach of the honeybee Apis mellifera. Currmicrobiol. 59:356-363.

10- WiIIiams, S. T. and WiIIkins, M. 1994.Bergys manuaI of determinative bacterioIogy., U. S. A. 832.

11- Vasquez, A., Forsgren, E., Fries,I., Paxton, R. J., FIaberg, E. and OIofsson, T. C. 2012. Symbionts as major moduIators of insect heaIth:Iactic acid bacteria and honey bees. PLoS One, 7:e33188.