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EFFECT OF LACTIC ACID AND PROBIOTICS AS GROWTH PROMOTERS IN HONEYBEE'S NUTRITION

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Abstract

The study was aimed at determining the effect of four different concentrations of lactic acid 0.002, 0.004, 0.006, and 0.008% on the survival rate of *Lactobacillus acidophilus* and *Bacillus subtilis* and the effect of their mixture as growth promoters in honeybee's nutrition. The best result of the survival rate of bacteria was 102.56 ± 3.21 % for the concentration 0.004%. Honeybee workers' feed consumption in the cages under study ranged from 2.17 ± 0.7 to 6.67 ± 1.53 cm^3 during 17 hours. The statistical analysis of the mortality rate for workers in the cages showed no significant differences in any concentration. As for pH values of intestinal honeybee workers exhibited the highest result for the concentration 0.002% with pH 5.18 ± 0.1. It can be concluded from this study that, the mixture of Lactic acid with *L. acidophilus* and *B. subtilis* can be used as a growth promoter in bee's nutrition that causes a decrease in the intestinal pH of honeybee workers thus inhibiting the growth of pathogenic microbes.

***Keywords*:** lactic acid, probiotic, honeybee's nutrition, consumption rate, mortality rate.

1. Introduction

Honeybees (*Apis mellifera*) are the most economically valuable pollinator for more than 100 important crops worldwide in addition to contributing about 9.5% to the total of agricultural economics [1]. Keeping them up at good health during the whole season to be able to collect an adequate supply of nectar and pollen additionally requires enough nutrition during periods when nutrients are limited, or at the time when weather conditions are not suitable for the honeybees to search for nectar and pollen [2]. Despite the high benefits of pollen and nectar substitutes, these substitutes don't contain beneficial bacteria that can enrich feed additives with lactic acid. Using lactic acid positively affects live forms and makes the gastrointestinal tract of the bees not suitable for the growth of pathogenic bacteria which live in the intestine and increases the immunity of bees [3, 4]. Improvement of the pollen substitutes related to honeybee nutrition is necessary; furthermore, pollen substitutes are a suitable option for the reduction of natural sources of feed for bees. Feeding honeybees with sugar syrup containing acidifying substances is aimed to decrease the intestinal pH to inhibit the pathogenic microbes and subsequently improve the yield of such colonies [5]. Furthermore, acidifying substances such as lactic acid promotes the growth of probiotic bacteria and keep living places favourable to them [3, 6, 7]. Probiotics are microorganisms that improve digestion in organisms such as humans and insects. They aid to stabilize the local microflora balance; at the same time, it works to strengthen the immune barrier of the intestines [8]. This research aimed to evaluate the effect of sugar syrup supplemented with lactic acid and probiotic mixture on the consumption rate of sugar syrup, pH of the intestinal content, and mortality rate of honeybees in the cages.

2. Materials and Methods:

2.1. Preparation of probiotic suspension

L. acidophilus and *B. subtilis* strains were isolated, identified, purified, and were kindly supplied by the Regional Center for Food and Feed (RCFF), Agricultural Research Center, Ministry of Agriculture, Egypt. The mixture of *L. acidophilus* and *B. subtilis* (1: 1) was prepared according to [9] as follows:

The bacterial cultures were inoculated in 2 separate sterilized flasks each containing 200 ml of autoclaved MRS broth which then were incubated at 37 ºC for 24 hrs. The bacterial count was adjusted to give 10^7 cfu/ml of *L. acidophilus* and 10^7cfu/ml of *B. subtilis*

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[9]. The bacterial suspensions were counted according to [10]. The harvested cells from 1 ml of each bacterial suspension were washed in Phosphate-buffered saline (PBS) according to [7] to prevent the matrix effect on its count and viability, and the obtained pellet was re-suspended in 1 ml of sugar syrup and was kept at 4-8 ºC to be used during the experiment.

2.2. Estimation of the effect of different lactic acid concentrations on the count of probiotics *in vitro*

Nine sugar syrup tubes (1kg sucrose sugar: 1L water) were supplemented with the prepared bacterial suspension to have 10^7 cfu/ml for each type of bacteria. Only 8 tubes were supplemented with different concentrations of Lactic acid 0.002, 0.004, 0.006, 0.008, 0.02, 0.04, 0.06, and 0.08% while the last one with no added acid was used as a negative control treatment. All tubes were kept at room temperature for 72 hrs. during which sub-samples were withdrawn at 0, 2, 4, 6, 8, 24, 48, and 72 hrs. intervals. The count of *L. acidophilus* and *B. subtilis* was performed to estimate the effect of the different acid concentrations on the count of the probiotic bacteria according to [10].

2.3. Cage experiment

Honeybee's workers under study were divided into 5 equal groups with 3 replicates for each. Each wooden cage contained 40 individuals whose mean weight ranged from 2.8-3 g. The first group was supplied with 30 cm^3 of sugar syrup only as of the control group while the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} groups were supplied with the mixture of 10^7 cfu/ml probiotics together with Lactic acid in concentrations of 0.002, 0.004, 0.006 and 0.008%, respectively. The consumption rate of sugar syrup, pH of honeybee workers' gut content and their mortality rate were estimated and recorded during 17 hrs.

2.4. pH measurement:

The values of pH were measured after the cage experiment ended using the Orion 420A pH meter. Fresh guts extracted from 5 dead bees from each cage were homogenated in 5 ml of distilled water.

2.5. The survival rate

Survival rate (%) = (log cfu N_t / log cfu N_0) ×100. N_0 and N_t represent the surviving count at zero time and after 2, 4, 6, 8, 24, 48 and 72 hours respectively [11].

2.6. Statistical analysis

Data were analysed using General Linear Model (GLM) and using the SAS 9.4 TS Software (2013). Means are compared using Duncan's Multiple Range Test. The means differences are significant at P-value ($P < 0.05$).

3. Results

Data obtained in Table 1 demonstrated the survival rate of both *L. acidophilus* and *B. subtilis* after 72 hrs. of exposure to lactic acid in concentrations of 0.002, 0.004, 0.006, 0.008, 0.02, 0.04, 0.06, and 0.08%. The concentrations ranging from 0.002% to 0.008% showed the highest survival rate ranging from 102.56 ± 3.21 to 92.21 ± 7.16%. It was observed that the concentrations of 0.02% to 0.08% showed the least survival rate ranging from 73.43 ± 15.85 to 56.59 ± 14.5 %. The results of all concentrations at the same time elucidated that the highest survival rate of the count of probiotic bacteria was 91.98 ± 1.65 % after 2 hrs. and the lowest survival rate was 71 ± 2.34 % after 72 hrs. of exposure to sugar syrup supplemented with lactic acid.

Table 1: Means of different concentrations of lactic acid on the mixture of *L. acidophilus* and *B. subtilis* compared with the control within different exposure periods *in vitro*:

Survival rate (%)			
Concentrations %	**Means ± SD**	**Times**	**Means ± SD**
Control	93.44^{b} ± 7.62	**2 h**	91.98^{b} ± 1.65
0.002	98.84^{ab} ± 3.29	**4 h**	86.09^{cd} ± 1.65
0.004	102.56^{a} ± 3.21	**6 h**	80.70^{d} ± 1.65
0.006	92.21^{b} ± 7.16	**8 h**	72.91^{e} ± 1.65
0.008	92.46^{b} ± 5.80	**24 h**	77.08^{ab} ± 2.34
0.02	73.43^{c} ± 15.85	**48 h**	81.46^{a} ± 2.34
0.04	65.87^{d} ± 17.65	**72 h**	71.87^{bc} ± 2.34
0.06	57.83^{e} ± 14.00		
0.08	56.59^{e} ± 14.5		

Data are expressed as mean ± SD (standard deviation). P values were < 0.05. Means with the same letter are not significantly different.

Data in Table 2 established that the concentrations ranging from 0.002% to 0.008% showed the lowest acidity ranging from 4.54 ± 0.16 to 3.96 ± 0.19 %. It was observed that the concentrations of 0.02% to 0.08% showed the highest acidity ranged from 3.38 ± 0.02 to 3.05 ± 0.03. There are no significant differences in pH values of all concentrations at the same time of exposure to lactic acid and the probiotic mixture.

Data in Table 3 showed the consumption rate of the supplemented sugar syrup with lactic acid concentrations added to the mixture of *L. acidophilus* and *B. subtilis*. The results revealed that there are no significant differences between the concentrations of lactic acid 0.002, 0.004, 0.006, 0.008% if compared to the control. Normally, there are significant differences in consumption rate during 17 hrs. of exposure to the different concentrations of lactic acid and the mixture of the probiotics in the cages. The results ranged from 2.26 ± 2.46 to 2.29 ± 2.08 cm^3.

Table 3 also showed that the concentration of 0.006% has the lowest mortality rate with 1.14 ± 0.91 during the

experiment and the highest mortality rate was 2.90 ± 0.94 for the concentration 0.008%. We can also notice that there are significant differences in the mortality rate of honeybee workers between the concentrations 0.002, 0.004, and 0.006 % compared with the control. Furthermore, the results showed that there are no significant differences between the control and the fourth concentration 0.008 %.

One of the important indicators of good digestion with the acidic feeding to honeybee workers is the pH of the intestinal content of honeybee workers under study in the wooden cages. The data obtained in Fig.1 showed the pH values of intestinal content of honeybee workers before and after feeding with sugar syrup only as of the control treatment. The results showed that there are significant differences between the concentrations of 0.002, 0.004, 0.006, and 0.008% compared with the control. The lowest pH for intestinal content was recorded at a concentration of 0.002%, with a value of 5.18 ± 0.1.

Table 2: Means of different concentrations of lactic acid on the pH of supplemented sugar syrup with *L. acidophilus* and *B. subtilis* compared with the control within different exposure periods *in vitro*:

The pH of supplemented sugar syrup			
Concentrations %	**Means ± SD**	**Times**	**Means ± SD**
Control	$6.81^{a} \pm 0.00$	2 h	$4.19^{a} \pm 1.11$
0.002	$4.47^{c} \pm 0.13$	4 h	$4.15^{a} \pm 1.11$
0.004	$4.54^{b} \pm 0.16$	6 h	$4.15^{a} \pm 1.10$
0.006	$4.03^{d} \pm 0.15$	8 h	$4.16^{a} \pm 1.11$
0.008	$3.96^{e} \pm 0.19$	24 h	$4.16^{a} \pm 1.10$
0.02	$3.38^{f} \pm 0.02$	48 h	$3.99^{a} \pm 1.23$
0.04	$3.35^{f} \pm 0.06$	72 h	$3.92^{a} \pm 1.19$
0.06	$3.13^{g} \pm 0.02$		
0.08	$3.05^{h} \pm 0.03$		

Data are expressed as mean ± SD (standard deviation). P values were < 0.05. Means with the same letter are not significantly different.

Table 3: Means of consumption and mortality rate of honeybee workers after exposure to the supplemented sugar syrup with lactic acid concentrations added to the mixture of *L. acidophilus* and *B. subtilis* compared with the control in the cages:

mixture of probiotics compared with the control in the cages.

Consumption rate (cm^3)		**Mortality rate (worker bee/cage)**	
Concentrations%	**Means ± SD**	**Concentrations%**	**Means ± SD**
Control	$2.24^{a} \pm 2.30$	**Control**	$2.00^{a} \pm 1.92$
0.002	$2.26^{a} \pm 2.46$	**0.002**	$1.24^{b} \pm 1.22$
0.004	$2.64^{a} \pm 2.49$	**0.004**	$1.57^{b} \pm 1.78$
0.006	$2.48^{a} \pm 2.52$	**0.006**	$1.14^{b} \pm 0.91$
0.008	$2.29^{a} \pm 2.08$	**0.008**	$2.90^{a} \pm 0.94$

Data are expressed as mean ± SD (standard deviation).
P values were < 0.05. Means with the same letter are not significantly different.

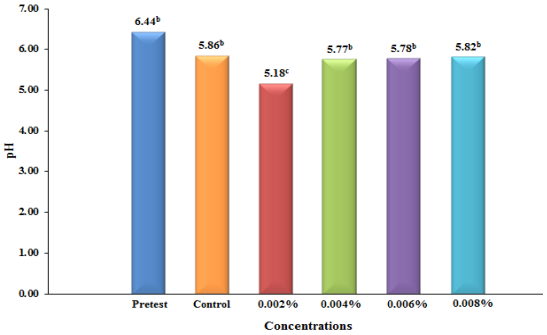

Fig. 1: pH of the intestinal content of honeybee workers before and after exposure to the supplemented sugar syrup with lactic acid concentrations added to the

4. Discussion

This study is considered to be the first one in Egypt. Recently, probiotics and lactic acid have become of particular importance in nutrition and disease prevention in beekeeping. As for, the probiotic bacteria reach the intestines of the bees with the sugar syrup and help to stabilize the balance of local bacteria and strengthen the immune barrier of the honeybee. Microorganisms selected as commercial probiotics are highly resistant and have a great ability to survive, even in unsuitable environments, and can increase in honeybee intestine and may exclude natural symbiotic microflora. As for, using the mixture of the microorganisms such as *L. acidophilus* and *B. subtilis* that a previous study proved the positive effect of this mixture on the strength of honeybee colonies [12].

On the other hand, feeding honey bees with sugar syrup containing acidifying substances which represents lactic acid is aimed to decrease the intestinal pH to inhibit the growth of the pathogenic microorganisms and improve the health of the colony [5, 13]. Mishukovskaya *et al*., 2020 proved that feeding honey bees with organic acids leads to an increase in the number of adult bees and increased the survival of colonies [12].

The main purpose of the *in vitro* experiment was to estimate the survival rate of the probiotic bacteria and to make sure that they reached the bee's intestines with sugar syrup, so they could perform their role perfectly. The results showed that the highest survival rate of probiotic bacteria was 102.56 ± 3.21% that was recorded for the 0.004% concentration of lactic acid. The survival rate reached more than 100% compared with the control because the acidifying substances encourage the growth of probiotic bacteria and maintain a favorable environment for them. This concentration achieved the pH value of sugar syrup 4.45 and these results were consistent with the results of studies that demonstrated that feeding bees with acidified sugar syrup with a pH of 4 showed good brood production and improvement of the intestinal lumen [5, 11, 13, and 16].

Feed consumption of the supplemented sugar syrup did not differ between the bees in the different experimental groups compared with the control group. The present study confirmed the findings of another study approved that no significant differences between treatment groups that received sugar syrup containing lactic acid or probiotic products [14]. During *in vivo* tests, it was found that the groups that received sugar syrup supplemented with lactic acid at concentrations 0.002, 0.004, and 0.006% added to the mixture of the probiotics showed that the mortality decreased compared with the control group but significantly increased in the groups that received the high concentration with 0.008% of lactic acid which is consistent with the data of the study proved that probiotic products decreased the number of dead bees about 25% [12]. Finally, it is clear our results that the concentration of 0.002% has an excellent pH value of the intestinal content of honeybee workers with 5.18. That's due to achieving a suitable medium for the probiotic bacteria and expected to reduce the total number of pathogenic spores. This explanation confirmed the previous study's findings that reported that the expected pH value inhibits the germs in the bee gut with a value < 5.2 [15].

5. Conclusions

At the end of this research, we can summarize that our primary concern is maintaining honey bee health by preserving their digestive tract through providing external substances such as probiotics and lactic acid in their nutrition, which works to reduce the pH of the intestine not suitable for the growth of the pathogenic bacteria that cause serious risks to the honey bees such as European foulbrood and American foulbrood. The sugar syrup supplemented with lactic acid concentrations 0.002% and 0.004% added to the mixture of *L. acidophilus* and *B. subtilis* could be considered an excellent growth promoter that increases bees' immunity.

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