Effect of Vitamin C on some biological and immunological aspects of the honeybee, *Apis mellifera* L.

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Abstract

The study evaluated the effect of adding different concentrations of vitamin C (2,4,8,16) mg on certain life aspects of honeybee workers, including consumption and survival rates. Additionally, it impacts immune indicators, such as encapsulation and melanization. The laboratory experiment results indicate that bees preferred adding vitamin C at a concentration of 2% to the nutritional feed in terms of consumption and survival rate. However, the concentration of 16% showed significantly lower consumption rates, but no differences were observed in the survival rate. The consumption rates were recorded as 108.81 and 9.48 mg/bee/day, respectively, while the survival rates were 98.57 and 93.72 %, respectively on concentrations 2and 16 mg . The bees' immune system was tested as a response to the regulation of protein and fat in the diet and vitamin C concentrations. The results showed a positive correlation between the encapsulation index and melanization activity, fat-to-protein ratio, and vitamin C concentrations. The maximum percentage of encapsulation index was (63.63 and 64.54) % in P: F and 8mg VC, respectively. Omit the minimum rate (54.07) % in 16mg VC.
Keywords: honey bees, immunity, vitamin C, biological, survival.

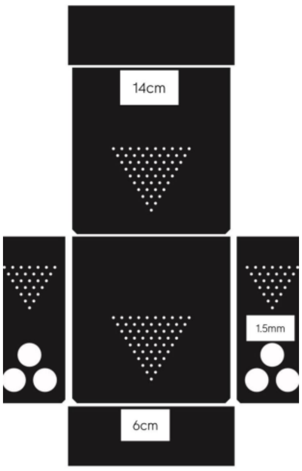
Introduction

The effect of vitamin C on carbohydrate metabolism in healthy bees [1].Honeybee nutrition is complex and unique, differing from other animals in several aspects. Honeybee colonies have a hierarchical system and a well-organized division of labor. Three levels of nutrition exist: colony-level, queen-level, and larval level [2]. Although vitamins are necessary for all organisms, there is limited information available regarding the specific vitamin requirements of honeybees. The presence of these vitamins has been demonstrated, and they are likely obtained from floral sources and the diet consumed by the bees [3]. Despite being necessary for all creatures, little is known about the vitamins that honeybees need. These vitamins are abundant in pollen, typically high in water-soluble vitamins, and deficient in fat-soluble ones [4]. The seven B-complex vitamins—thiamine, riboflavin, pyridoxine, pantothenic acid, niacin, folic acid, and biotin—which are necessary for most insects are typically found in the pollen. Pollen contains inositol, ascorbic acid, and other water-soluble vitamins, an excellent source of these micronutrients [5].The immune system is the most reactive, and its activity can be changed through a group of stressors, as it is evident that the colony's collapse has multifactorial causes and is frequently associated with the emergence of pathogens and parasites [6]. Encapsulation is a cellular immune response used against pathogens that are too large to be phagocytosed; this response is commonly employed by dipteran and lepidopteran larvae in response to infection with the eggs of the parasitoid [7]. Melanization in insects refers to the production and deposition of melanin, a complex biochemistry process that plays a crucial role in immune responses in mosquito vectors[8]. The research aimed to study the effect of adding vitamin C concentration to artificial bee feed on some biological and immune indicators.

Materia and Methods

Bee-rearing laboratory cages

The following requirements were taken into consideration when designing the cages: Advanced acrylic material, which is not foldable and does not break easily, was developed into a cube with a length and width of (14 cm), an upper and lower base, and sides that are (6 cm) in diameter. The feeding holes are 1.5 mm in size, fitting for the Eppendorf tube. The number of feeding tubes was six , and each tube had a capacity of 2 ml. and the cage also had very tiny ventilation holes on both sides (Figure 1).



A

B

Figure (1): A. Basic parts of the cage. B Experimental cage.

Table (1): Concentrations of vitamin C paste used for laboratory immunity experiments treatments

Paste treatment	Components
Vitamin C	(2، 4 ،8 ،16) mg + 20 % casein+10% lecithin + 80 gm of sugar
control +	20 gm of Bee-Pro® + 80 gm of sugar

control -	80 gm of sugar

To summarize the steps involved in the experiments:

1. Preparation of paste: Powdered sugar (with honey 10 %) and enough water were mixed to create a cohesive paste.
2. Packaging the paste: The paste is placed in nylon bags and wrapped in tin foil. The bags are designed to fit inside the bee cages.
3. Presenting the paste to bees: A sufficient quantity of paste is placed in small nylon bags and inserted into the bee cages. A small hole is made in the bags to allow the bees access the paste .
4. Monitoring paste consumption: The weight of the paste is measured before it is presented to the bees. After 24 hours, the weight is measured again to calculate the amount of paste consumed by the bees for 14 days.
5. Recording bee mortality: The number of dead bees is counted daily to track the mortality rate for 14 days.
6. Each treatment had five replicates with 30 bees\replicate. Rearing boxes were incubated at 33C and 66%RH with 24h dark. 9. Input two of Eppendorf tube and filled with water.

Immunology Experiment

To study the health importance of the diets used in this study, an immunological survey of encapsulation and melanization was set up as follows:
1. feeding bees for 14 days 2. Sampling bees in 7 and 14-day-old. **3** immobilize bees in the freezer for 10 min. **4.** A thread with a diameter of 0.08 is cut into small pieces, about 0.5 cm each. These thread pieces are inserted between the third and fourth abdominal segments **5.** Returning bees to the rearing boxes for 24 hours**. 6.** Freezing and dissection bees after 24 hours to get nylon threads out. **7.** Microscopic examination: The extracted thread is transferred under a microscope for photography. This step is conducted to measure immunity-related factors such as encapsulation and melanization.

Figure (2): Nylon filament used in the immune experiment.

Statistical Analysis

Daily consumption data were analyzed using fit general linear model ANOVA using Minitab ®19 (Minitab, LCC, USA). Comparisons were made using the LSD test with 95% confidence with a significance at p =0.05. The impact of diets on survival was analyzed using a GraphPad Prism V.9. [9]

Correlation Analysis

Correlation is a statistical method used to describe the nature and strength of the relationship between two variables [10]. The correlation coefficient is denoted by the letter r and can take the following values:

- r = 0 indicates no relationship between the two variables.
- r = -1 indicates a perfect negative (inverse) relationship between the two variables.
- r = +1 indicates a perfect positive relationship between the two variables.

Results and Discussion

Effect of vitamin C on bee consumption and Survival

According to the statistical analysis of nurse bees' consumption of the four different concentrations of vitamin C over 14 days, there were significant differences in the average consumption ($F_{5,18}$ = 690.32, P = 0.000, Fig 3). Regarding the consumption of the test paste in the concentrations, we can observe that the highest consumption rate reached 108.819 mg/bee/day of 2mg vitamin C. On the other hand, the lowest consumption rate was 9.48 mg/bee/day at 16 mg of vitamin C. The results also showed that the preference for the remaining concentrations (4 and 8 mg) reached 56.36 and 19.44 mg/bee/day respectively. While the consumption rate in the control treatments (ctr+ , Bee-Pro®) and ctr- P:f) was 72.40 and 87.74 mg/bee/day respectively.

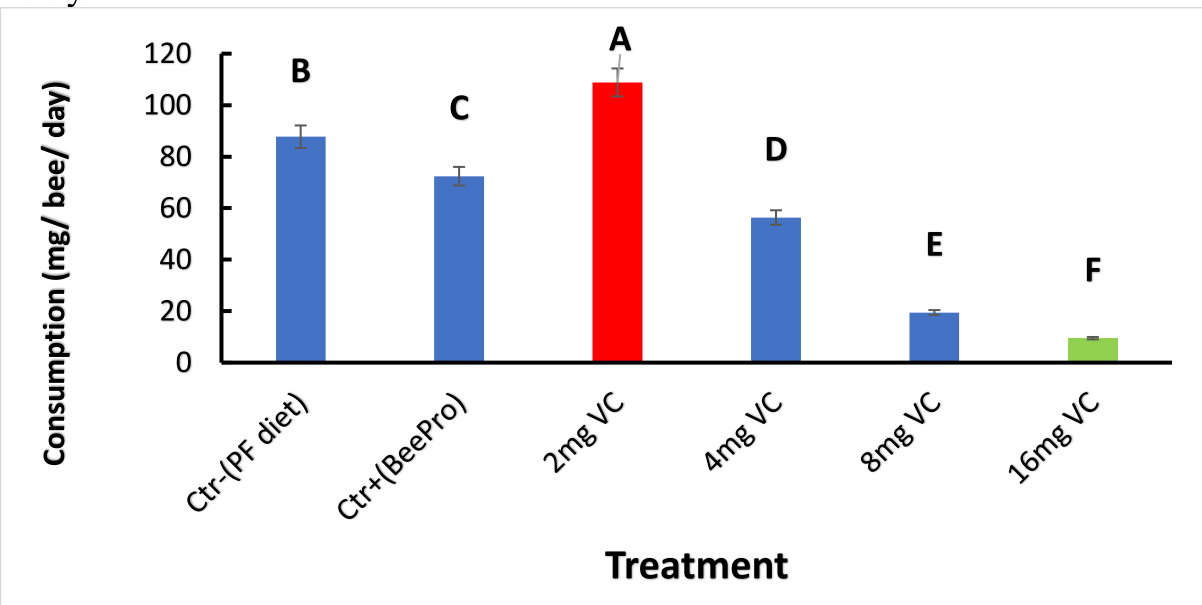


Figure (3): Average diet consumption over 14 days by bees in the cage for different concentrations of vitamin C. means labeled with the same letter do not differ significantly according to Fisher's LSD. N =5 cohorts per treatment.30 bee/ cohort.

The daily consumption for all treatments differed significantly ($F_{(6,161)}$ =1.25, P= 0.284, Fig 4). The bees increased their consumption gradually to reach the peak at day 14 which was 74.3 mg.bee^{-1}.day 1, whereas the consumption rates on days 12 and 10 did not differ significantly (65.08 and 63.77 mg.bee^{-1}.day $^{-1}$ respectively). The consumption on days 8,6 and 4 did not vary considerably, reaching 61.15,58.72, and 53.95 mg.bee^{-1}.day^{-1} respectively. Finally the consumption minimum rate on day 2 was 44.45 mg/bee/day

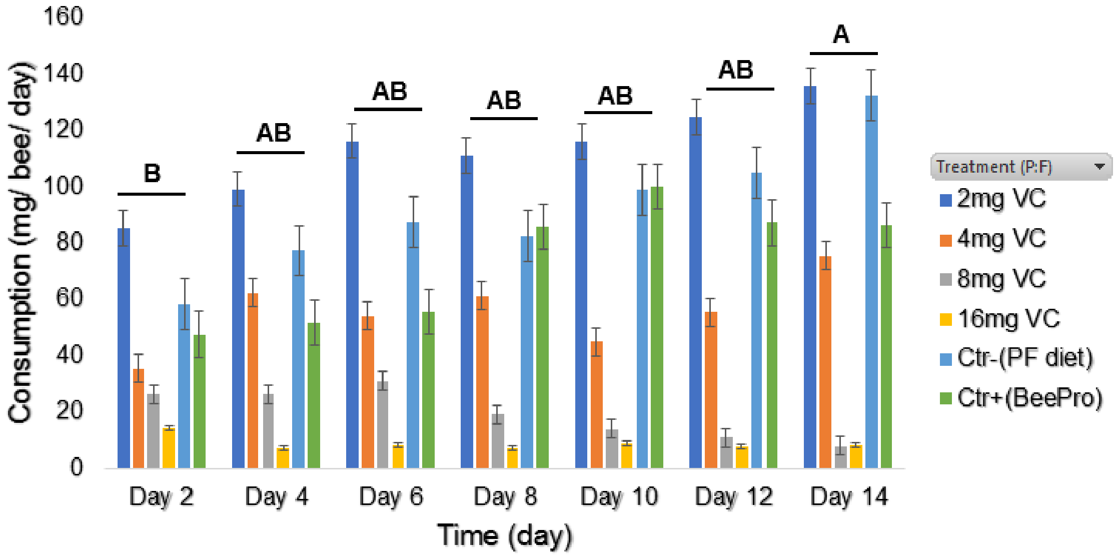


Figure (4): Daily consumption of honeybee nurses for different concentrations of vitamin C . Means labeled with the same letter do not differ significantly according to Fisher's LSD test at P= 0.05. N 5 cohorts per treatment.

The current results of bee survival showed that there was a significant effect ($F_{5.,18}$= 161.01, P = 0.000, Fig 5), with the highest nurse bee survival rate, which was 98.57 and 96.116 % when feeding bees on concentrations 2 and 4 mg of vitamin C respectively and the lowest bee survival rate was 94.24.% and 93.92% when providing on concentration solution 8 and 16 mg of vitamin c respectively while it reached 98.12 and 97.50 % in ctr- and ctr+ respectively.

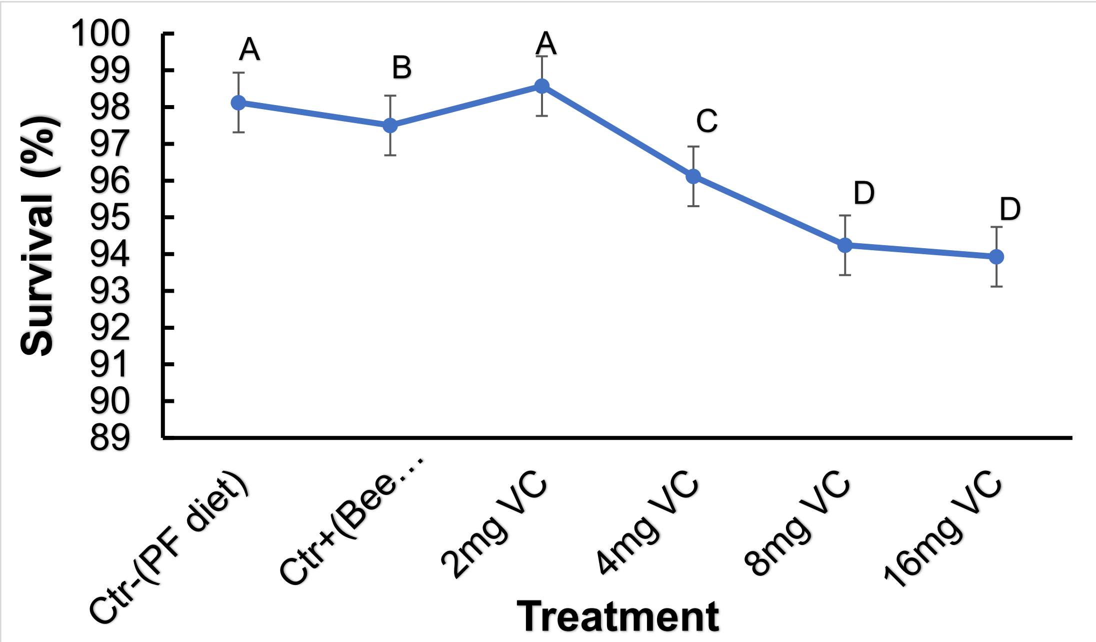


Figure (5): Cumulative survival rates of nurse honeybees *Apis mellifera* after 14 days of feeding on different concentrations of vitamin C in the laboratory. Points with the same latter do not differ significantly at $P \leq 0.05$ (one-way ANOVA, LSD test). N= 5,30 bees for each cohort.

Studies found that colonies provided with vitamin C experienced a decrease in average bee losses during the winter by approximately 33%. [11] . A study conducted by [12]showed that supplementing a high dose (2 g / kg) of vitamin C to an artificial diet improved the rearing of honeybee brood. The researchers concluded that this increase in brood production can be attributed to the antioxidative properties of vitamin C. showed significant differences in the mean body weight, total protein and carbohydrate content of newly emerged drones, and the sperm count of mature drones due to adding vitamin C to bee nutrition [13]. they have concluded that adding vitamin C to spring sugar syrup feeding to bee colonies can increase brood area, colony population, worker body weight, and protein content. The optimal level for adding vitamin C appears to be around 2000-6000 mg/L of sugar syrup[14] .

Immunity experiments

From the graph below (Figure 6), we can see that generally implants from bee workers consumed different diets of vitamin C had significant effect ($F_{5,44} = 7.86$, p= 0.000) where the encapsulation index was 64.54 and 63.63 % in P: F and 8mg of vitamin C respectively while the rate in concentration 16,2,4 mg of vitamin C reached 54.07, 57.15 and 60.40 respectively while it was27.46 in paste sugar

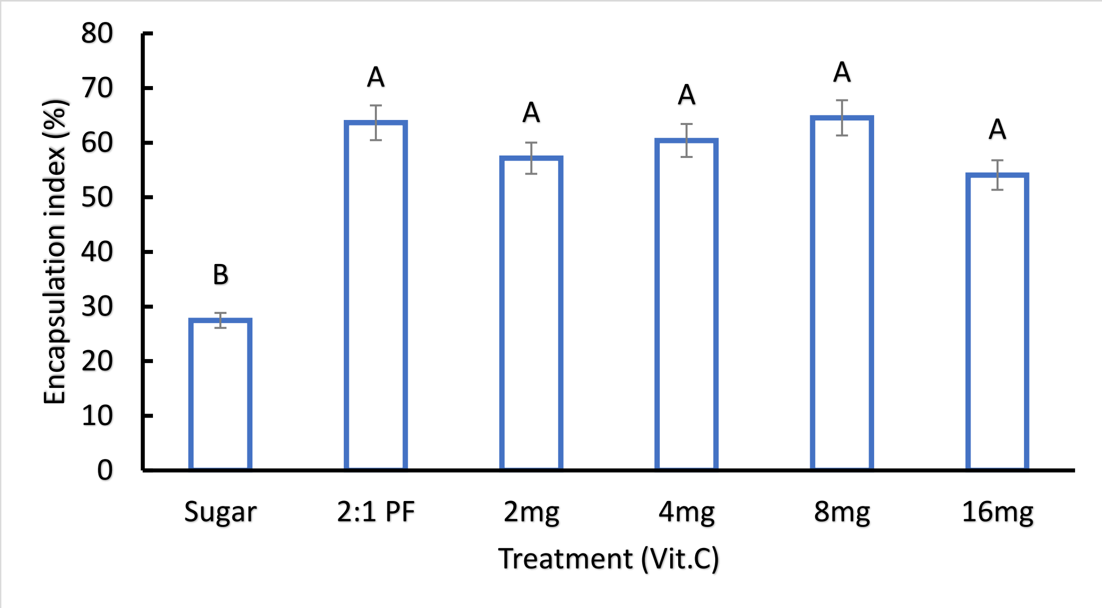


Figure (6): Immunocompetence of 14-day-old honeybee workers as affected by feeding on different pastes of vitamin C diets. The level of encapsulation of a nylon thread implant in honeybees was measured as a percentage of hemocytes/melanin area on the filament. Means labeled with the same letter do not differ significantly according to the LSD procedure.

Regarding the melanization (Figure 7), we can see that generally, implants from bee workers who consumed different diets of vitamin C had a significantly ($F_{5,44}$ = 1.51, p= 0.000) greater degree of melanization than those fed on vitamin C. The maximum percentage of melanization index was 70.39 and 68.33 % in 8 and 2 mg respectively. In comparison, the rate in the concentration of 4 mg of vitamin C reached 61.88 %, where the minimum percentage was 49.80 % at 16mg of vitamin C. In contrast, the percentage in control 2:1 PF and sugar was 64.79 and 34.62 %, respectively.

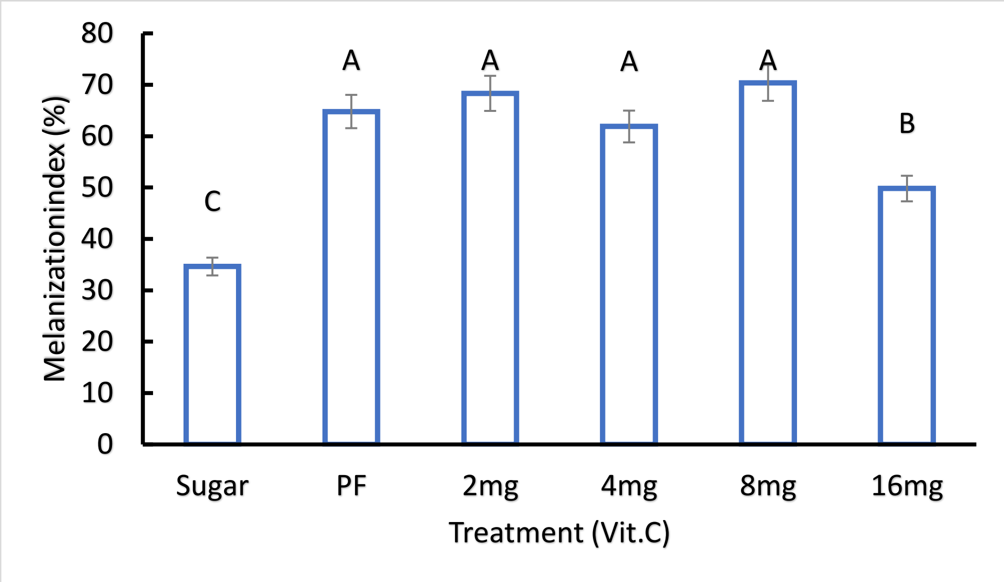


Figure (7): Immunocompetence of 14-day-old honeybee workers as affected by feeding on different pastes of vitamin C diets. The level of melanization of a nylon thread implant in honeybees was measured as a percentage of hemocytes/melanin area on the filament. Means labeled with the same letter do not differ significantly according to the LSD procedure.

We tested the relationship between the encapsulation index of 14-day-old bees and the melanization index. The results showed a significant positive correlation between encapsulation and melanization in bees feeding different vitamin C diets (Pearson correlation, r = 0.565, p = 0.000) (Figure 8).

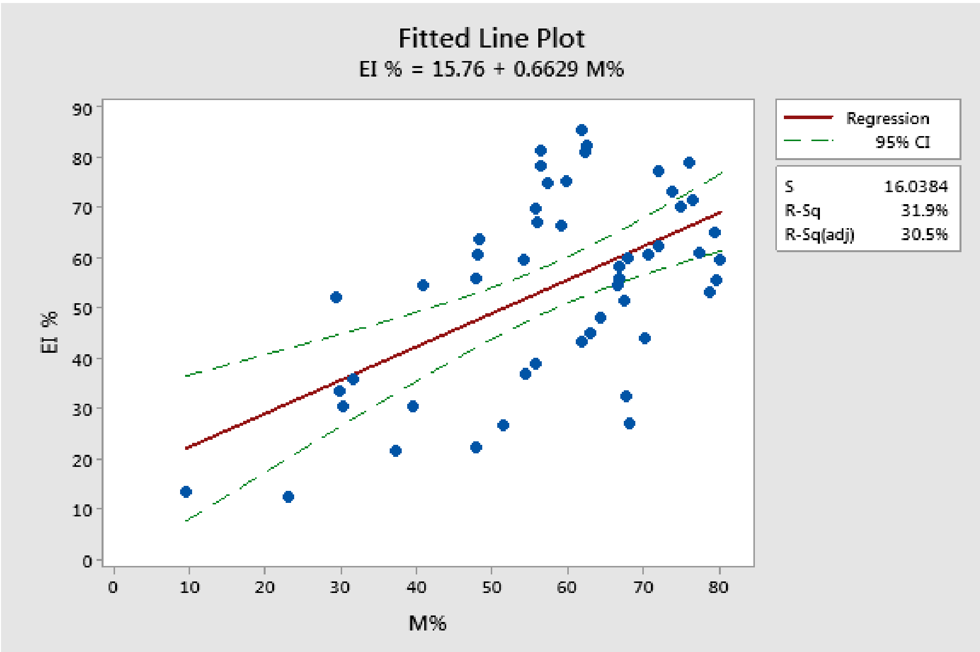


Figure (8): Pearson correlation between encapsulation and melanization of nurse bees (14-day-old) feeding different concentrations of vitamin C diet in the laboratory.

Study found that supplementing colonies with vitamin C improved their antioxidative defenses and reduced the negative impacts of *Varroa* infestation on the bees [15]. Vitamin C in the honeybee diet significantly reduces infestation levels and potentially enhances certain aspects of carbohydrate metabolism in infested worker bees. Consequently, this dietary addition could improve apiary conditions [16]. The research demonstrates the beneficial effects of a vitamin-enriched diet on improving honeybee colony health and reducing the impacts of Varroa mites and disease during the critical overwintering period [17] .

Found that supplementing the honey bee diet with vitamin C had a beneficial effect on the antioxidative system of the developing brood, leading to healthier emerging adult workers and reduced winter colony losses [18]. Supplementation of vitamin C can potentially maintain the redox status and offer protective effects against the peroxidative damage induced by sub-lethal concentrations of the neonicotinoid insecticide imidacloprid in *Apis cerana* indica worker bees[11] .

Studied by [19]changes in vitellogenin and stress protein levels in honey bees under different diets. This study provides valuable insights into the effect of nutritional changes on stress and overwintering ability in honeybees, suggesting that changing nutrient levels can affect bee immunity. The protein content in the diet of adult worker honey bees can trigger metabolic responses, influencing the production and expression of antimicrobial peptides. This demonstrates the significance of pollen nutrition for honey bee immunity [20]. using vitamin C or Echinacea purpurea extract as safe additives can boost the survival of bee colonies during the winter period,The most promising results in this study regarding recovery time after exposure to low temperatures, survival rates after narcosis, and hemocyte counts were observed with these two materials. After winter, the strength of bee colonies fed with sugar mixed with vitamin C and Echinacea purpurea extract was approximately equal to their strength before winter [21] .

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