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Evaluating the effectiveness of incorporating natural nutritional supplements into candy paste to improve hygienic behavior in honey bee colonies (*Apis mellifera* L.)

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Abstract
Hygienic behavior (HB) in honey bees (*Apis mellifera* L.) is a sophisticated social immunity mechanism and a primary natural defense against various brood pathogens and parasites, including American Foulbrood (AFB) and the *Varroa destructor* mite. This behavior involves the detection, uncapping, and removal of diseased or dead larvae by worker bees before the infection spreads to healthy brood. The researchers conducted their experiment during the Spring season of 2025 at the research apiary located in the College of Agriculture, Kirkuk University. The researchers studied how natural additives, including gelatinous Aloe Vera powder, peanut butter, and Salep, affected the basic candy paste to elicit vital bee behavior. The study tested three natural additives: Aloe Vera powder, peanut butter, and Salep applied to basic candy paste, to determine their effects on bee hygienic behavior. The study employed the Pin-prick Assay to evaluate the cleaning efficiency of worker bees. The results showed that the treatments produced statistically significant differences at P values below 0.05. The treatment that used Aloe Vera as a supplement achieved the best cleaning performance by reaching a cumulative cleaning rate of 98% with an error margin of 1.2% by the third day after treatment. The peanut butter treatment achieved 94.33% cleaning efficiency with a 2.1% error margin, while Salep achieved 87% cleaning efficiency with a 1.8% error margin. The control group reached its minimal cleaning effectiveness by achieving 80% cleaning efficiency, which included a 3.5% error margin. The research evidence shows that natural supplements containing bioactive enzymes, amino acids, and antioxidants will improve workers' neuro-sensory responses, which will result in better health and behavioral immunity for the entire colony.

Keywords: Hygienic behavior, honey bee, Aloe Vera, pin-prick assay

Introduction
Honey bees (*Apis mellifera L.*) are indispensable components of global biodiversity and agricultural productivity. They provide essential pollination services for approximately one-third of the human diet, contributing billions of dollars to the worldwide economy via growing yields of crop production [9]. Worldwide honey bee populations face their most severe threats today because these threats have become classified as "Colony Collapse Disorder" (CCD). The existing threats to bees include habitat destruction, pesticide risks, the spread of *Paenibacillus larvae*, and the *Varroa destructor* parasitic mite. Hygienic behavior is a genetically and physiologically regulated trait that allows worker bees to identify and remove infested or dead brood before the infectious stage is reached [15]. Social immunity in colonies depends on two factors, which are their current nutritional state and their existing physical strength. The hypopharyngeal and mandibular glands, which produce pheromones and hive-duty enzymes, require proper protein and lipid intake for their development [4, 12]. Bees experience nutritional difficulties during times when flowers stop producing nectar and pollen and when their surrounding conditions undergo severe changes. Beekeepers use supplementary feeding methods, which include candy paste that contains sugar and water or honey as their primary feeding method. The standard candy recipes do not provide the essential micronutrients, enzymes, and antioxidants that people require for their complete well-being. Furthermore, various innovative approaches have been explored in regional contexts to enhance the biological, physiological, and behavioral performance of honey bee colonies, including the application of magnetized water [8] and the evaluation of insect growth regulators on physiological metrics [10].

The study investigates the "Booster Effect," which occurs when natural additives are introduced to products. Scientists selected *Aloe* Vera because it contains acemannan and multiple bioactive enzymes that have the ability to strengthen immune systems and fight bacteria. Peanut butter served as the primary ingredient because it delivers essential plant-based lipids and proteins that create "fat bodies" (vitellogenin reserves) in bees. Researchers included Salep from Orchis species because its special polysaccharide content improves feed taste and keeps its original structure. The research develops a chemical-free environmental solution that enhances behavioral immunity in honey bee colonies through specific nutritional methods.

Related Work

The hygiene behavior (HB) has been known to be among the most important forms of social immunity in honey bee colonies (*Apis mellifera L.*). HB includes the detection, uncapping and removal of infected, parasitized or dead brood by worker bees, which minimizes the dissemination of pathogens and parasites within the colony. A number of studies have found that honey bee colonies with high amounts of hygienic behavior are more resistant to brood diseases and parasite attacks due to *Varroa destructor* mite. Hygienic behavior is stated to be a hereditary trait and an effective factor which positively affects colony health, productivity and longevity as stated in [15]. The connection between hygienic behavior and resistance to disease was studied as well. It was shown by [17] that hygienic colonies have better defense reactions against brood diseases and parasitic mites and hence better colony performance. In a similar way, it was demonstrated by [7] that the colonies with high hygienic behavior levels had greater abilities to minimize *Varroa* mite population via brood removal. Thus, the development of hygienic behavior as a sustainable replacement for chemical control of honey bee diseases is essential. Nutrition is another crucial aspect of honey bee physiology and behavior. [3] concluded that adequate nutrition is critical for ensuring immune competence, glandular development, brood production, and the longevity of workers. Malnutrition can result in poor colony health and reduced efficiency of social immune functions. In addition, [4] found that colonies fed nutritionally rich diets showed lower pathogen levels and better physiological state than those with restricted diets. Nowadays, much attention is paid to using natural supplements for improvement of colony health and productivity [12]. Highlighted the significance of pollen diversity and bioactive components in bee nutrition and increased colony resilience. Environmental-friendly natural products with antioxidants, polysaccharides, vitamins, and amino acids have been gaining popularity recently as alternative to synthetic stimuli and antibiotics. These components can be helpful in improving digestion, boosting immunity, and behavioral response in worker bees. Among natural supplements, *Aloe vera* has become one of the most popular ones because of its high content of polysaccharides, enzymes, vitamins, and antioxidant components [11]. pointed out that improved nutrition can play an important role in enhancing hygienic behavior and resistance to diseases in bee colonies. The components of *Aloe vera* have been shown to contribute to improvement of immune response and physiological activity in biological systems. However, information about the effect on hygienic behavior of honey bees is scant. Protein and fat-based nutritional supplements have been used for investigation as possible enhancers for colony development [2]. showed that supplement diets rich in proteins and other essential nutrients increased brood rearing and colony development. Peanut butter can be considered as an easily obtainable source of plant proteins, fatty acids, and energy compounds that may promote the development of glands of nurse bees and brood production. But there is a lack of information about the influence of peanut butter on hygienic behavior. Although a lot of papers have been published concerning honey bee nutrition and hygienic behavior, there is no much information about the effect of *Aloe vera*, peanut butter, and Salep when they are included in the formulation of candy paste. The majority of previous researches were devoted only to disease resistance or nutritional effect separately, and only some studies investigated both behavioral immunity and brood development under field conditions. The aim of the current research was to investigate the effectiveness of these natural nutritional supplements on hygienic behavior and colony development in honey bees in Iraq.

Materials and Methods

Study Site and Environmental Conditions

The experiment was carried out in the beekeeping experimental station at the College of Agriculture, Kirkuk University, Salah al-Din Governorate, Iraq. The experiment was carried out during spring season (March–May 2025), where the temperature was from 22 to 28 °C and relative humidity was 45–60%.

Colony Standardization and Preparation

Twenty-five healthy colonies of local hybrid species (Carniolans; *Apis mellifera* carnica), with young, mated sister queens in each one, were chosen for this experiment. In order to maintain consistency among the colonies, each colony was made uniform in terms of its population size, *i.e.*, five frames of bees, with at least three frames with brood and two frames of food (pollen and honey). The colonies were divided into five treatments, each group containing five colonies.

Experimental Diets and Preparation

The control candy paste was prepared using a mixture of base candy paste consisting of 500g of powdered sugar, 100g of crushed pollen, 50g of cinnamon powder, and 50ml of rose water that was mixed with 100ml of water. The experiments were prepared as stated below:

The colonies were randomly assigned to five treatment groups as follows

- **T_1:** Negative Control (Foraging only, without supplementary feeding).
- **T_2:** Basic Candy Paste (500 g powdered sugar + 100 g crushed pollen + 50 g cinnamon powder + 50 mL rose water).
- **T_3:** Candy + Salep (Basic candy paste supplemented with 50 g natural Salep powder).
- **T_4:** Candy + Peanut Butter (Basic candy paste supplemented with 50 g pure unsalted peanut butter).
- **T_5:** Candy + *Aloe vera* (Basic candy paste supplemented with 50 g laboratory-prepared *Aloe vera* gel powder).

Hygienic behavior was measured by the "Pin-prick" method [16]. In each colony, a square area of a brood comb with 100 sealed cells (with pupae) was chosen. Each cell was pierced with a sterile needle to kill the pupa without causing excess leakage of body fluids. The comb was put back to its respective hive. The

number of cells where wax capping was removed and the dead pupa had been cleaned was determined after 24, 48, and 72 hours.

5. Assessment of Sealed Brood Area: In determining the secondary biological effects of the specialized nutrition pastes on colony productivity, the sealed brood area was determined per experimental colony. Two measurements were done; first before the beginning of feeding (day 0) and secondly after 21 days of the treatment, representing the complete development period of the worker bees. The measurement of the sealed brood area was done using the standard wire grid system with a cm2 grid system. The grid was then placed over the brood frames, and the number of squares containing the sealed brood counted. The difference between the sealed brood area at day 21 and the sealed brood area at day 0 was determined for each treatment block.

Statistical Analysis

Analysis of the data involved the use of Randomized Complete Block Design (RCBD). The analysis of variance (ANOVA) test was carried out to check the significance of treatments. Duncan's Multiple Range Test (DMRT) (Pprec0.05) was conducted to compare means of the different supplements. All calculations were performed using the SPSS statistical software package. The overall experimental design and evaluation procedures are illustrated in Figure 1.

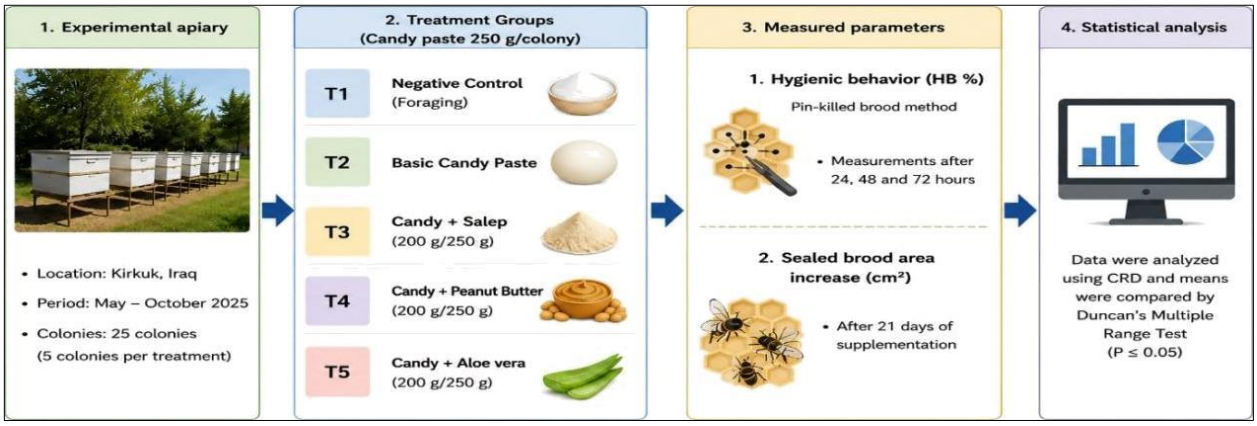

Fig 1: Experimental design of the study Figure 1. Experimental design of the study showing colony allocation, nutritional treatments, measured parameters, and statistical analysis procedures.

Results and Discussion

Quantitative Analysis of Cleaning Efficiency

The experimental data revealed that the addition of natural supplements significantly accelerated the expression of hygienic behavior. While all groups showed an upward trend in cleaning over the 72 hours, the rate of removal was significantly higher in the supplemented groups than in the control group.

Table 1: Effect of Nutritional Supplements on Hygienic Behavior Percentage (%) of Honey Bee Colonies (Mean±SE)

Treatment Group	24 h (Day 1)	48 h (Day 2)	72 h (Day 3)
Negative Control (Foraging)	12.50±2.10^{d}	42.00±3.40^{e}	80.00±3.50^{c}
Basic Candy Paste	18.00±1.50^{cd}	55.00±2.80^{d}	86.00±2.40^{bc}
Candy + Salep	22.00±1.80^{c}	62.50±2.20^{c}	87.00±1.80^{b}
Candy + Peanut Butter	28.50±2.00^{b}	72.00±1.90^{b}	94.33±2.10^{ab}
Candy + Aloe vera	35.00±1.40^{a}	78.50±1.20^{a}	98.00±1.20^{a}

Values are presented as mean±standard error (SE). Means within the same column followed by different superscript letters differ significantly according to Duncan's Multiple Range Test at $P \leq 0.05$.

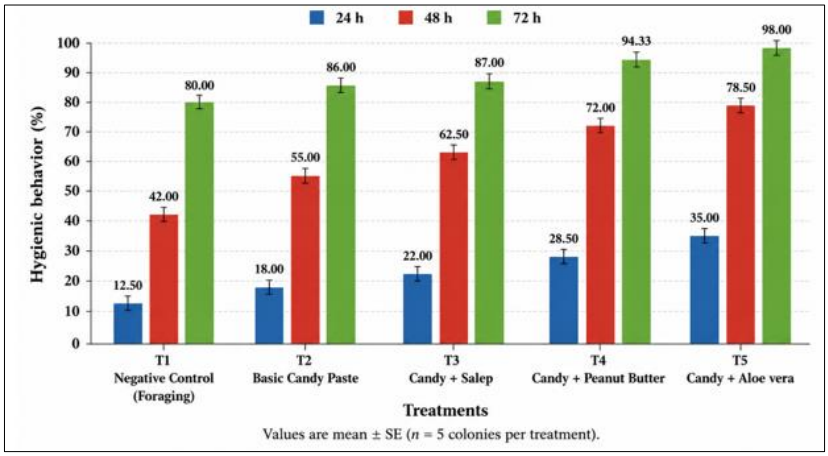

Fig 2: Effect of nutritional supplements on hygienic behavior (%) of honey bee colonies after 24, 48 and 72h of treatment.

Effect of Nutritional Supplements on Brood Rearing Activity

The experimental data revealed that targeted nutritional intervention not only accelerates behavioral immunity but also exerts a highly positive influence on the reproductive and colonybuilding dynamics of honey bee colonies. As shown in Figure 2, colonies supplemented with *Aloe vera* and peanut butter exhibited higher hygienic behavior percentages than the control group throughout the observation period. All treatment groups exhibited varying degrees of positive progress in brood development over the 21-day observation period.

Table 2: Effect of Different Nutritional Supplements on the Sealed Brood Area (cm²) of Honey Bee Colonies (Mean±Standard Error)

Treatment Group	Initial Brood Area (cm2)	Brood Area After 21 Days (cm2)	Net Increase (cm2)
Negative Control (Foraging)	2510.00±115.0	2750.00±130.5 d	240.00±25.1 d
Basic Candy Paste	2530.00±98.5	3210.00±145.2 c	680.00±48.3 c
Candy + Salep	2505.00±105.0	3390.00±112.8 c	885.00±35.6 c
Candy + Peanut Butter	2520.00±110.0	4250.00±165.4 a	1730.00±75.2 a
Candy + Aloe vera	2515.00±88.0	4080.00±122.1 b	1565.00±52.4 b

Note: Means within the same column followed by different lowercase letters are significantly different ($P<0.05$) according to Duncan's Multiple Range Test.

The analytical results compiled in Table (2) indicate a statistically significant expansion ($P<0.05$) in the sealed brood area across the nutritionally supplemented groups compared to both the basic candy paste and the negative foraging control. It should be emphasized that the treatment T_2 (Candy + Peanut Butter) produced the best performance, obtaining the largest increase in sealed brood area (1730.00±75.2: cm²), and next came T_1 (Candy + *Aloe* Vera) with 1565.00 \pm 52.4 cm². The outstanding effectivity of peanut butter as a supplement can be explained by the biochemical properties of this product. As is known, peanut butter contains an especially large amount of plant lipids and essential amino acids. Macro- and micronutrients are the basic components for the formation of the hypopharyngeal and mandibular glands of the nurse bees. In turn, the developed glands have the increased capacity to produce royal jelly that stimulates the queen's egg-laying speed. It means that this biological process contributes greatly to the broodrearing productivity. The above conclusion is in agreement with [2], who showed that the diets rich in lipids and proteins were able to effectively enhance the vitality and brood-rearing capability of honey bee colonies during crucial seasons. At the same time, the high speed of the brood development in the group fed with *Aloe* Vera can be attributed to the specific 'Booster Effect' due to acemannan polysaccharides and the corresponding bioactive enzymes. As is evident from Table 1, while *Aloe vera* is known to function as a neuro-stimulant that strengthens olfactory sensory organs so as to boost hygienic behavior, it also aids in maintaining gut integrity, maintaining microbial equilibrium and improving nutrient absorption in worker bees. This helps in channeling more energy and physiology to raising broods and growing their numbers. Such a dual approach further strengthens the fundamental principles proposed by [3] regarding the indissoluble link between the nutritional state of honey bees, behavioral immunity and survival.

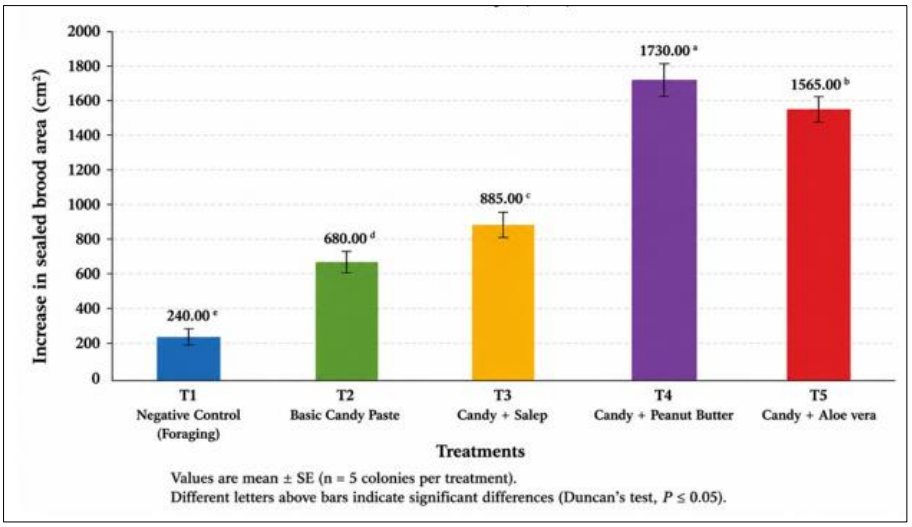

Fig 3: Effect of nutritional supplements on sealed brood area increase after 21 days (cm²)

As is clear from Figure 3, nutrition supplements had a significant impact on the development of the sealed brood area of honey bees. The highest increase in the sealed brood area was observed for the colonies which were fed on peanut butter, then those on *Aloe* Vera and Salep. On the contrary, the negative control showed the lowest brood development.

4.2 Discussion

The *Aloe* Vera treatment (T_1) provided the fastest and most efficient outcome as 98% of the total removal was observed on the third day [11]. Such high efficiency could be explained by the fact that *Aloe vera* contains special polysaccharides (acemannan) and antioxidants that might work as neurostimulators thus increasing olfactory sensitivity of the worker bees. Studies conducted by [1] show that there is a direct correlation between nutrition and antenna sensitivity to VOCs produced by decaying brood [17]. Peanut Butter treatment (T_2) provided 94.33% success and became the second best treatment option. It confirms the importance of lipids as the crucial elements for physiological functioning of honey bees. High

content of essential fatty acids and proteins in peanut butter probably helped house bees maintain their vitality and perform such activities as uncapping and cleaning for prolonged periods of time. Stable immune system functioning of *Apis mellifera* needs significant lipid reserves [2]. The effects observed in Salep and Basic Candy were moderately positive compared to the negative control treatment. However, the basic mixture was more beneficial than foraging from natural sources due to the presence of pollen and cinnamon, which ensured an adequate "cushion" of nutrients necessary for foraging. The lack of bioactive elements in these two groups contributed to slow reactions in comparison with *Aloe vera* and peanut butter groups. It can be concluded that the timing of the hygiene response is as important as the percentage itself. The supplementary groups managed to exhibit high rates of cleaning within 48-72 hours, thus increasing their chances of interrupting the development of the American Foulbrood infection cycle [5].

Conclusions and Recommendations

Conclusions

- Specific nutritional supplements are an extremely efficient way to improve behavioral immunity (hygienic behavior) in honeybee hives.
- The strongest of these nutritional supplements is the *Aloe vera* gel powder, which improves the rate of the hygienic activity significantly.
- Using natural additives, which are also inexpensive, such as *Aloe vera* and peanut butter, represents a possible substitute for using chemicals and antibiotics.

Recommendations

- **Beekeeping Practices:** It is necessary for beekeepers to incorporate *Aloe vera* (concentration of 10%) in their feeding regimen in the autumn/winter seasons to guarantee the optimal functioning of hygienic behavior in the coming spring.
- **Further Research:** Future research works need to be conducted on the histology of the hypopharyngeal glands along with the estimation of hemolymph vitellogenin level to formulate a physiological perspective of the supplements' working.
- ***Varroa* Management:** A Follow up study should be performed in order to confirm the decline in the *Varroa* mite infestation rates due to the supplements.

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Author Contributions

- **Omar Ali Daham:** Conceptualization, methodology, investigation, and data collection.
- **Samara Alzuhairi:** Data analysis, manuscript writing, reviewing, and editing.
- **Omar Ismaeel:** Experimentation assistance, field supervision.
- **Ahmed Alsabte:** Statistics analysis, supervision, and manuscript reviewing.

Conflict of Interest

The authors declare no conflict of interest.

Informed Consent

Not applicable.

Ethical Approval

This study did not involve human participants or vertebrate animals requiring ethical approval.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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