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# Fermented pollen substitute diet affects the lifespan of honey bee workers under the effect of food consumption rate and vitellogenin expression

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**Abstract.** This study determined the longevity of caged workers fed with different diets (carbohydrate only, bee bread, unfermented pollen substitute diet, and fermented pollen substitute diet). Survival rates were higher for bees fed the fermented versus the non-fermented diet, though the difference was not significant. The honey bees consumed significantly more fermented than an unfermented diet. Hemolymph proteins were significantly higher in bees that had been fed a fermented versus an unfermented diet. Though still significantly lower than in bees fed on beebread, where vitellogenin (an essential storage protein for honey bees) levels were increased significantly in bees fed the fermented versus the non-fermented diet and were similar to and not significantly different when compared to beebread-fed bees. We conclude that fermented by beebread-derived microorganisms can improve the nutritional value, acceptance, and utility of an artificial protein diet and lifespan of the honey bee's workers.

***Keywords:*** *Pollen substitute diet, bee bread, fermentation, vitellogenin, honey bee lifespan*

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## 1. Introduction

As the most economically important pollinator, Honey bees are indispensable for maintaining global ecological stability and agricultural production. However, the colony losses worldwide in the past decade threaten agricultural production and food supply (Garibaldi et al., 2011a, b). Generally, the availability and quality of food fundamentally determine the distribution and scale of bee populations (Plascencia and Philpott, 2017). Previous reports have demonstrated that climate change and human activity may affect food availability and diversity, partially responsible for honey bee colony health (Donkersley et al., 2014; Ziska et al., 2016). The growth, development, productivity, and health of a honeybee colony are dependent upon fulfilling larvae and adult workers (Brodschneider and Crailsheim, 2010). The survival and quality of larvae and adult workers are of prime importance for the productivity and health of a colony. In general, a honeybee colony requires macronutrients (i.e., proteins, carbohydrates, and fats) and micronutrients (i.e., vitamins and minerals) for the growth and development of healthy larvae and adults (Crailsheim et al., 1992; Brodschneider and Crailsheim, 2010).

Protein levels in honey bee hemolymph affected by protein levels in the diet, as found before (Basualdo et al., 2013). Beekeepers usually used pollen substitute diets to feed the bees (Somerville, 2005). However, many diets are poorly accepted by bees and have low nutritional value; this was proved by (Schmidt and Hanna, 2006). Suitable diet formulation, deterioration during storage, attractiveness to bees, and diet costs are significant concerns (Herbert et al., 1977). The prepared diet should be nutritive and attractive to bees; it should be as similar to the natural proteinaceous food in the hive, beebread; bees ferment the pollens to protect them from harmful microorganisms (Herbert & Shimanuki, 1978).

Nutrition is involved in regulating multiple aspects of honey bee biology such as caste, immunity, lifespan, growth, and behavioral development. Diet efficiency can be measured by measuring the protein levels in bee hemolymph (Barragan et al., 2016; Almeida-Dias et al., 2018). Generally, natural forage is better for bee health and production than artificial diets (DeGrandi-Hoffman et al., 2010, 2016), but it is not always available in sufficient quantity (Somerville, 2005). A good diet should be palatable

and acceptable to the bees and provide nutrients essential for colony growth and development, bee health, and colony production capacity (Rousseau and Giovenazzo, 2016). Although various pollen substitute diets prepared before as indicated by (Ellis and Hayes, 2009; Morais et al., 2013a, b); the same problem was found to include lacking attractiveness (Pernal and Currie, 2002); another problem was found, the bee hemolymph proteins was lower compared to beebread (Cremonez et al., 1998). Here, we tested the diet we prepared by fermentation with microorganisms from beebread that would improve the consumption and survival rates of the workers.

The lifespan of a honey bee depends on the type of bee it is. The life span of worker honey bees ranges from five to seven weeks. The first few weeks of a worker's life are spent working within the hive, while the last weeks are spent foraging for food and gathering pollen or nectar. The life span of the honey bee is mainly determined by pollen consumption and protein abundance, as well as the honey bee's level of activity. Variation in worker behavior and lifespan correlates with vitellogenin (Amdam and Omholt, 2003). In a highly regulated manner, this protein is expressed during development and adult life by both sexes and all behavioral groups of honey bees. The primary site of synthesis is the fat body, which lines the body wall as a single cell layer composed of trophocytes and oenocytes. From the fat body, vitellogenin is secreted into the hemolymph (blood). We summarize what is currently known about honey bee vitellogenin and its effects on worker life histories. We outline mechanisms that may allow vitellogenin to influence worker phenotypic outcomes and discuss how this protein has become a central regulatory element during honey bee social evolution.

## 2. Materials and methods

### 2.1. Experiment conducted area

The experiments were carried out in Assiut, Insect Research Laboratory of Plant Protection Research Institute. The experiment was conducted with the first hybrid of Carniolan honey bee (*Apis mellifera carnica* Pollmann) workers in October 2019, after collecting all the available types of bee bread from hives in different regions in Assiut governorate at the successive seasons.

### 2.2. Inoculum preparation for fermentation

Our lab prepared the inoculum from a mixture of all the available types of bee bread that we collected (Clover…*Trifolium alexandrinum*; Maize … *Zea mays*; Bean …*Vicia faba*; Fennel … *Foeniculum vulgare*; Anise … *Pimpinella Anisum*). We used 70% ethanol for sterilization before inoculum preparation. After pooling and mixing, 10 g of the mixture of the collected bee bread was mixed with 300 ml of sucrose syrup (50% w/v) that was previously boiled and mixed well this mixture manually, then put in 350 ml - colored bottles, and placed in an incubator at 35 °C and relative humidity (70%) for 25 days. CO2 produced from the fermentation process was released by open the bottles every 48 h. The bottles were stored at 6–8 °C in order to be saved for up to 20 days, where anew one was prepared from freshly collected beebread every 20 days to help reduce contamination with other microorganisms (Almeida-Dias et al., 2018).

### 2.3. Diets preparation

We first prepared the unfermented diet with 4.5-part powdered sugar, 3-part powdered soy meal, 1-part powdered yeast, 0.5-part powdered milk, sufficient previously boiled water to make a paste (Moustafa et al., 2000)**,** and linen oil as an attractive smell. Then we prepared the fermented diets by mixing 40 ml fermented inoculum with a kilogram of the unfermented diet we prepared before. Then we stored them in an incubator in loosely covered plastic containers at 35 °C, for 28-days.

### 2.4. Preparation of bee cages and bioassay protocol

Experimental wooden cages were prepared for the experiment, every cage (15 × 15 × 5) Cm dimensions with a glass side and other was covered with black muslin, was provided with a vial of tap water and another vial of sugar solution 1:1 (w: v), food source and pieces of the wax foundation were attached to the cage side. We prepared two sets of groups. Newly emerged workers aged 0 - 12 hours were confined in the cages, each cage containing 100 newly emerged bees; one set for consumption rate and longevity; another for hemolymph collecting and protein analysis. Five replicates were used for each group. The cages were also held in a dark incubator at 32 °C ±1 and 70% RH (Figure-1& 2).

The cages were continuously supplied with water, sucrose solution, and food source; they were divided into four groups depending on the food source they were introduced as follows:

Group ‹ 1›, cages contained bee bread diet as a control: (A)
Group ‹ 2 ›, cages contained fermented diet (mixture 28-days): (B)
Group ‹ 3 ›, cages contained unfermented diet: (C)
Group ‹ 4 ›, cages contained only sucrose solution 1:1 (w: v): (D)

**Figure 1**. Experimental wooden cages.

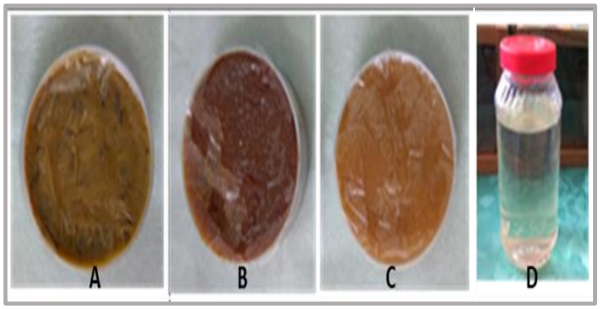


**Figure 2**. The four-food source that was introduced in the cages.

**2.5. Measurements of vitellogenin levels and other types of amino acids soluble in hemolymph**

To study the effect of the different food sources, we measured the vitellogenin level in the workers' hemolymph at different ages; about 5-7 workers were randomly collected from each cage every three days of feeding. This procedure was repeated six times at three-day intervals. Workers' hemolymph was collected with injection in the thorax using syringe 3 cm using EDTA anticoagulant (Ethylene Diamine Tetra Acetic disodium salt). Approximately 2 µL of hemolymph was collected from each individual. Only transparent hemolymph was used in these studies. The hemolymph (2-3 µL/bee) was transferred from the syringe to a 1.5-mL Eppendorf tube, and the volume was adjusted to 60 to 80 µL. The samples were stored on an ice container at –20 °C for further analysis (Figure 3).

**Figure 3**. Workers were removed from each cage every three days of feeding for hemolymph collecting

Proteins were separated by SDS-PAGE (sodium dodecyl sulfate electrophoresis) according to the method of Laemmli (1970) in a 7.5% polyacrylamide gel; 0.5 µl of hemolymph was obtained from a pool of 10 workers from each cage every three days of feeding. This procedure was repeated six times at three-day intervals. The hemolymph was collected, then mixed and centrifuged at 4000 rpm for 4 min at 4 ºC, added to sample buffer, and subjected to a constant current of 15 mA at 7–10 °C. The buffer was made from 3.03 g of Tris PM = 121.14in 50 ml of distilled water; the pH was adjusted to 6.8, and the volume completed to 100 ml with distilled water; 1.25 ml of this solution was added to 0.5 ml 70% (w/w) sucrose, and 3 ml distilled water, 1.2 g bromophenol blue, and 0.25 ml mercaptoethanol. After electrophoresis, the gels were stained with 1% Coomassie Brilliant Blue dissolved in a

solution of glacial acetic acid, ethanol, and distilled water (1:5:5 v/v/v), which was also used for the gel discoloration, then the gel was scanned.

**2.6. Determination of the food consumption rate and preference for each diet**

Each cage was provided with 10 gm of bee bread diet, 10 g of unfermented diet, and 10 g of fermented diet and was offered to the workers in the cages in plastic feeders. Another group of cages was with only sucrose solution—five cages for each group as replicates. The cages were saved in the same incubator conditions as we indicated before. We provided Sucrose syrup (50% w/v) continuously to all groups.

Every three days, the amount of food/cage was compared to the number of live bees existing in each cage during the investigation. Food consumption was calculated and represented as (Mg/bee/3 day). This procedure was repeated every three -days intervals, and the diets were renewed if consumed. To evaluate the dry weight, we used the desiccation by the oven; the decrease in weight of the food was calculated to correct the quantity of food consumption (Figure 4).

**Figure 4**. Food consumption by the honeybee workers.

**2.7. Determination of worker's longevity and survival rate**

In each cage, every three days, dead bees were counted and removed. The LT 50 (The time required to reach 50% mortality) was estimated.

**2.8. Statistical analysis**

Survival rate values in the day(s) of bee workers fed were determined by a computerized analysis EXEL program and analyzed using Survival Analysis. The diet consumption was statistically analyzed by the SPSS Test (version 17.0.2). ANOVA compared the proteins in the hemolymph data and results of vitellogenin levels on Ranks and the t-test.

**3. Results and discussion**

**3.1. Determination of the food consumption rate (gm) and preference for each diet**

The total food consumed by the workers was calculated in the dry weight (mg/bee/3 days) (Table 1 and Figure 5 & 6). The workers consumed significantly more fermented than an unfermented diet. The amount of food consumption by the workers differed significantly over all the compared age periods, and the honeybee workers consumed different amounts of food at the several diets. The workers started to consume a considerable amount at the first period after emergence (1-3 days), then decreased gradually from the (4-6 days) towards the progressing period till the end of the observed periods at (15–18 days) for the most diets. Similar results were also obtained by Jaycox and Parsi (1981), who reported that mass consumption of pollen begins when workers are from 42 – 52 hours old and reaches a maximum around day-live of worker age and then decreased to a low level over time.

**Table 1**. Determination of food consumption rate

| Age | 3-days | 6-days | 9-days | 12-days | 15-days | 18- days | Grand mean | Deviation % |
|---|---|---|---|---|---|---|---|---|
| Control Bee bread Clover | 0.1580 | 0.1250 | 0.0150 | 0.0060 | 0.0130 | 0.0130 | | |
| | 0.2090 | 0.1150 | 0.0130 | 0.0170 | 0.0130 | 0.0290 | | |
| | 0.2420 | 0.0410 | 0.1120 | 0.0110 | 0.0060 | 0.0020 | | |
| | 0.1850 | 0.1010 | 0.0130 | 0.0110 | 0.0120 | 0.0150 | | |
| | 0.2110 | 0.0890 | 0.0110 | 0.0040 | 0.0120 | 0.0130 | | |
| | **0.2010 ±0.0032** | **0.0942 ±0.0033** | **0.0328 ±0.0044** | **0.0098 ±0.0005** | **0.0112 ±0.0003** | **0.0144 ±0.0009** | **0.0606 ±0.0021 A** | **0.00** |
| FermentedMixture 28- days | 0.0780 | 0.1510 | 0.0550 | 0.0580 | 0.0260 | 0.0280 | | |
| | 0.0770 | 0.1100 | 0.0210 | 0.0150 | 0.0090 | 0.0130 | | |
| | 0.0860 | 0.1260 | 0.0450 | 0.0490 | 0.0140 | 0.0080 | | |
| | 0.1020 | 0.0990 | 0.0480 | 0.0500 | 0.0150 | 0.0060 | | |
| | 0.0660 | 0.1200 | 0.0090 | 0.0190 | 0.0220 | 0.0120 | | |
| | **0.0818 ±0.0013** | **0.1212 ±0.0019** | **0.0356 ±0.0019** | **0.0382 ±0.0019** | **0.0172 ±0.0007** | **0.134 ±0.0009** | **0.0512 ±0.0015 AB** | **- 0.94** |
| Unfermented | 0.0530 | 0.0140 | 0.0210 | 0.0040 | 0.0030 | 0.0020 | | |
| | 0.0460 | 0.0120 | 0.0140 | 0.0080 | 0.0020 | 0.0020 | | |
| | 0.0630 | 0.0240 | 0.0230 | 0.0020 | 0.0090 | 0.0040 | | |
| | 0.0670 | 0.0290 | 0.0060 | 0.0030 | 0.0080 | 0.0030 | | |
| | 0.0590 | 0.0200 | 0.0040 | 0.0050 | 0.0390 | 0.0030 | | |
| | **0.0576 ±0.0008** | **0.0198 ±0.0007** | **0.0136 ±0.0009** | **0.0044 ±0.0002** | **0.0122 ±0.0015** | **0.0028 ±0.0000** | **0.0184 ±0.0007 C** | **- 4.22** |

The superiority of food consumption was recorded for the workers fed by the bee bread diet (0.0606 mg/bee/3day), and there were no significant differences between them and the workers fed by the fermented diet (0.0512 mg/bee/3day). At the same time, the lowest food consumption was noticed for the workers fed by the unfermented diet (0.0184 mg/bee/3day). Also, the deviation in the total consumption of each diet from that of the bee bread diet (control) was estimated. The percentages of deviation from the control diet were (-0.94 and -4.22%) in the case of (fermented and unfermented diets), respectively. We concluded that fermentation significantly increased diet consumption. According to Almeida-Dias et al. (2018), fermentation of a pollen substitute diet with beebread microorganisms increases diet consumption and hemolymph protein levels of honey bees.

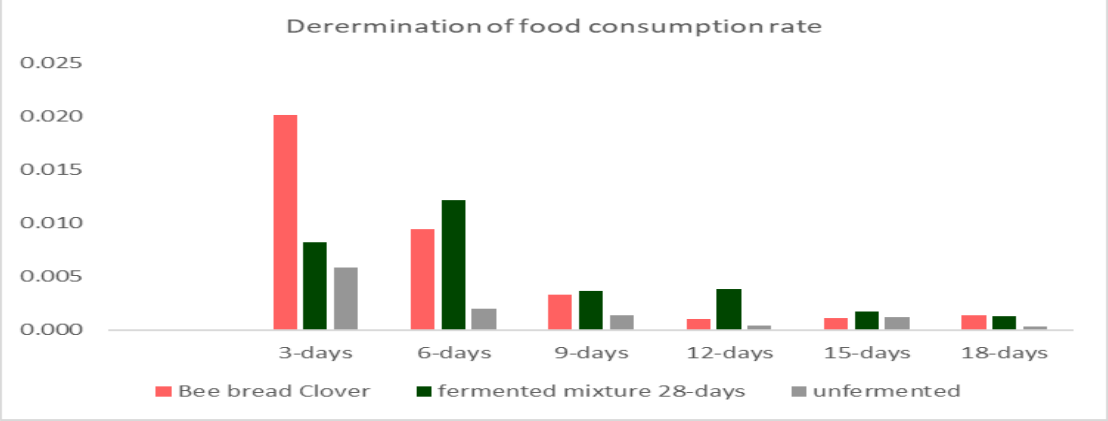


**Figure 5**. Determination of food consumption rate

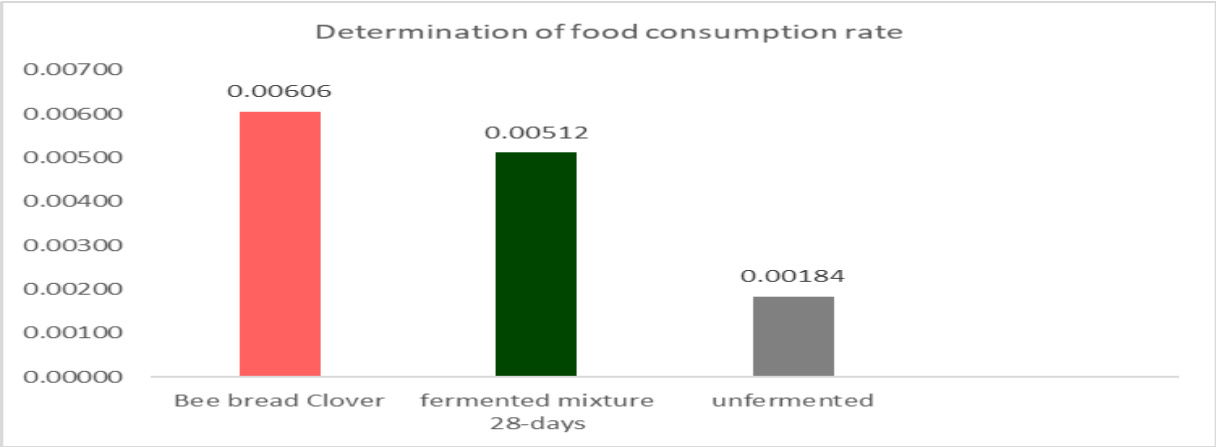


**Figure 6**. Mean of food consumption rate

### 3.2. Measurements of vitellogenin levels and other types of amino acids soluble in hemolymph

The determination of protein concentration in the hemolymph of honey bees is an accurate method to evaluate the efficiency of protein diets. The data show considerable variability in protein content in the hemolymph of bees feed on different even though consumption was similar. Some differences seen in the figure might originate from the average variance in protein staining (Figure 7). Here, we establish and compare the molecular properties of vitellogenin from honeybee hemolymph. Vitellogenin levels were increased in bees fed the fermented than the unfermented diet significantly. The density of vitellogenin bands from bees fed the fermented diet was broader than bees fed on an unfermented diet, significantly by the optical conversation. These levels were similar and not significantly different when compared to bees fed on beebread.

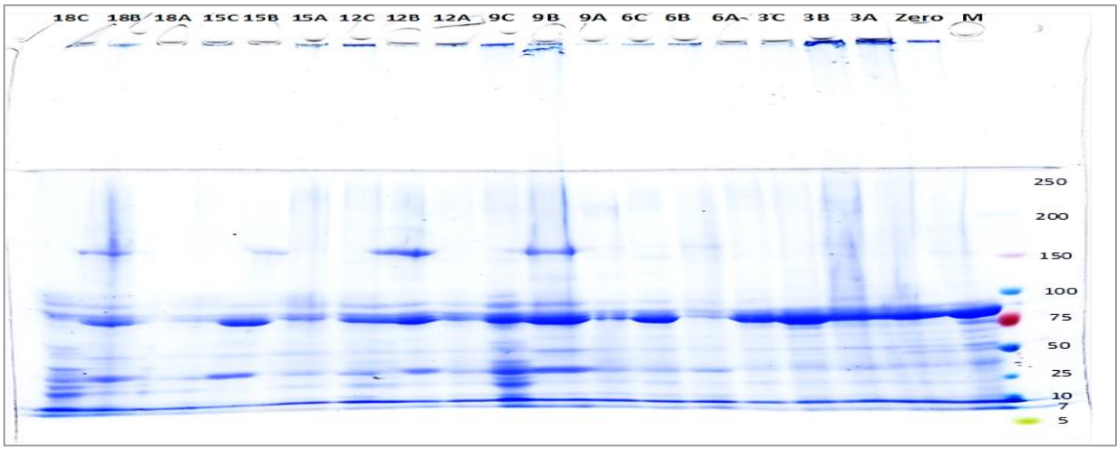


**Figure 7**. Measurements of vitellogenin levels soluble in hemolymph using SDS-gel page (0, 3, 6, 9, 12, 15, 18 days of bee age; Control bee bread, B- Fermented diet, C- Unfermented diet)

Vitellogenin expression was influenced by diet and age. The results indicated that at the end of metamorphosis/time of emergence, the honeybee hemolymph became depleted of proteins, and only a few proteins remained at a higher concentration. According to the marker used, the central band with the highest density is a product at (~ 75 kDa) at zero-day of the bees age, and its density decreased at 3-days of bees fed the bee bread diet, and the fermented diet and the density decreased more at 9, 12, 15-days of the bees fed bee bread, at 6-days of the bees fed the fermented diet and at 15-days of the bees fed the unfermented diet.

Anew bands appear at (~ 150 kDa), it did not appear at zero-day of bee age, it appeared only in the hemolymph of bees fed the fermented diet at 6-days in low-density density increased at 15- and 18-days of bees age. At (~ 80 kDa), it did not appear at zero-day of bee age; it appeared in low densities in most cases and slightly increased at 9-days of the bee age that fed the fermented and unfermented diets. Consequently, As the band (~55 kDa) remained unchanged, and the lower product (~50–55 kDa) slightly increased at 9-days of bees age that fed the fermented diet, a single product (~40 kDa) seems to be unique at 3-days of bee age and 6-days of bee age and slightly increased in the hemolymph of bees fed the fermented diet at 9, 12, 15, 18- days, and only at 9-days in the bees that fed the unfermented diet. Additional protein bands between (~ 5–20 kDa) and around (~ 25 kDa) for all treatments were revealed; they showed no difference between the hemolymph of bees fed the bee bread and the fermented diet.

Comparing Vitellogenin protein band densities for the most common bands demonstrated that the protein concentrations differ significantly from each other according to the different diets and the honey bee age. The vitellogenin is stored in the fat body then secreted into the hemolymph; it is a lipoglycoprotein. Vitellogenin plays great physiological and behavioral activities and longevity in workers (Wheeler and Kawooya, 1990).

The vitellogenin recognized full-length 180-kDa vitellogenin and the lighter fragment of 150 kDa (Seehuus et al., 2007) and a 40-kDa fragment (Havukainen et al., 2011) in honey bees in the fat body, hemolymph, and brain, in hemolymph, a band of approximately 75 kDa was detected. Havukainen et al. (2011) used ion-exchange–purified hemolymph to detect different vitellogenin bands of 40, 150, and 180 kDa. However, in raw hemolymph extract, a band of approximately 70 to 75 kDa was detected. He also found that this protein may represent a degradation product of the full-length or 150-kDa vitellogenin. Both the full-length and the 150-kDa fragments are post-translationally modified by phosphorylation and glycosylation.

Subsequently, vitellogenin might serve as a potential biomarker for neonicotinoid and other pesticide exposure in bees. Juvenile hormones and vitellogenin hormonally regulate the transition of nurse bees to foragers. During the life course of workers, vitellogenin levels in the hemolymph and fat body drop, and these reduced concentrations influence several aspects of the life history of bees (Münch and Amdam, 2010). Levels of vitellogenin in hemolymph and fat bodies are highest in the long-living winter bees and lowest in the short-living foragers (Seehuus et al., 2006). Vitellogenin governs various physiological aspects, including development, behavior, life span, and immunity (Münch et al., 2008; Peso et al., 2016; Salmela et al., 2015) is considered to be a general marker for honey bee health.

Vitellogenin has an independent and positive influence on worker lifespan; this effect may be explained by a nutritive role of the vitellogenin protein (Engels et al., 1990) and its positive influence on oxidative stress resilience cell-based immunity (Seehuus et al., 2006). Thereby, vitellogenin is among the most multifunctional life-history regulators known in honey bees and is likely instrumental for colony social organization. As vitellogenin protein levels decline, titers of the life-shortening juvenile hormone (JH) increase, and workers show immune senescence, susceptibility to oxidative stress, and reduced survival, in addition to a higher probability of abandoning nest-tasks in favor of foraging for nectar (rather than pollen) from flowering plants (Amdam et al., 2004; Guidugli et al., 2005; Seehuus et al., 2006). The specific, pleiotropic effects of vitellogenin on honeybee physiology, longevity, and food-related behavior suggest that this protein can suppress insulin/insulin-like signaling (IIS) in workers so that vitellogenin acts as an antioxidant to promote longevity in bees, this hypothesis explored, as well as related roles of insulin–IGF-1 signaling and juvenile hormone (Hunt et al., 2007).

### 3.3. Determination of worker's longevity and survival rate

The half-life time was estimated as LT 50 (number of days required for 50 % of the bee to die). The mortality percentages and LT 50 were illustrated in Figure 8. Data revealed that caged bees fed by the sucrose syrup only shortened the longevity of honeybee workers (LT 50, 12.90 days) compared with those fed by the bee bread and the fermented and unfermented diets. The results also indicated that the half-life time of the workers fed the bee bread, fermented and unfermented, was similar primarily, recording, LT 50., (24.33, 22.76, and 19.82 days), respectively. However, the survival rate was still higher for the fermented than the unfermented diet, though the difference was not significant.

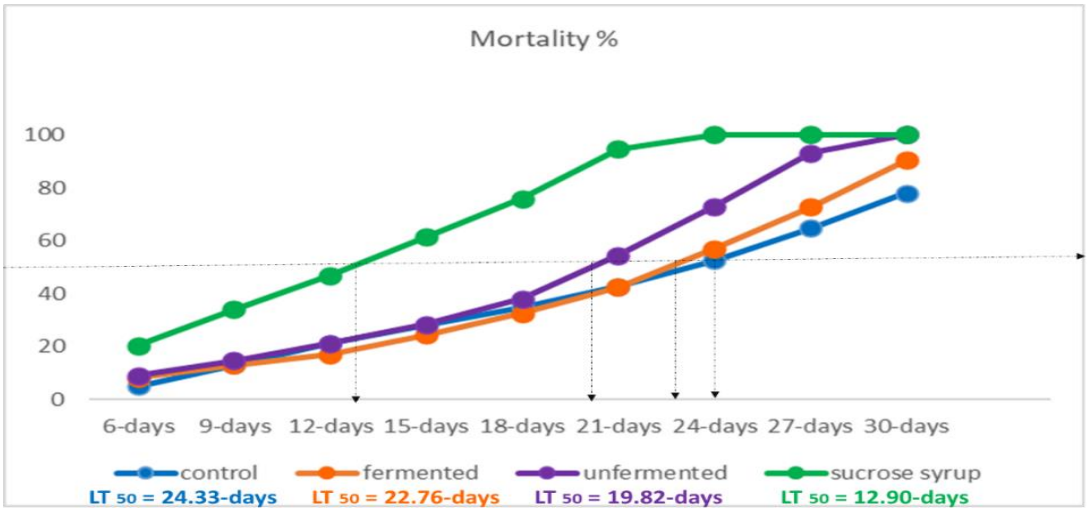


**Figure 8.** Workers longevity (The half-life time LT 50)

Dukas (2008) and Münch et al. (2013) found that physiological specialization enabling bees to perform different tasks, but the 3 major worker types (nurse, forager, and winter bee) differ markedly in life span. Foragers, one type of worker bee, typically die within 2 wk and are the shortest-lived individuals, whereas bees continuing nursing can have life spans longer than 50 d. The longest-lived workers, winter bees, survive from late summer to next spring. Only the queen lives longer, surviving 2 to 3 yr and up to 5 yr.

The highest levels of proteins were found in bees that consumed beebread (Cremonez et al., 1998; van der Steen, 2007). Fermentation increased diet consumption and the protein levels of bees. Vitellogenin in the hemolymph of workers is affected by the diet contains. A pollen substitute diet can increase proteins in the hemolymph, improving the bee immunity and health (Bitondi & Simões, 1996). We found that vitellogenin produced in workers fed the fermented diet was the same as bees fed on bee bread but higher than in bees fed an unfermented diet significantly.

As Manning et al. (2007) found that the differences in the protein levels affect longevity. The previous results by Ellis and Hayes (2009) found that bees consume more fermented than unfermented diet, but they used yogurt inoculum instead of beebread microorganisms.

We used the bee bread microorganisms in a pollen substitute diet fermentation to ensure the diet would be more attractive and healthier for the bees. The main microorganisms present in beebread are responsible for fermentation, increased palatability, and pollen consumption (Gilliam, 1997; Gilliam et al., 1989). *Lactobacillus, Bacillus,* and *Agrobacterium*, and fungi of the genera *Penicillium* and *Aspergillus*, as well as yeasts. Fermenting the diet with bee bread microorganisms could protect the bees against pathogens (Maes et al., 2016).

On the other hand, Carroll et al. (2017) found that bees consumed freshly stored pollen over pollen that had been fermented.

A pollen substitute diet that fermented can improve the diet's efficiency and make it more beneficial for bees where it increases protein levels in the hemolymph and the bee's survival rate become longer. We found that workers fed with control bee bread showed the best survival, followed by workers fed with fermented diet, and workers fed with unfermented diet had the shortest life.

The fermentation of the diet by bee bread microorganisms affected the vitellogenin compared to the unfermented diet. These results proved that fermentation makes the workers live longer. Vitellogenin is also a good indicator that can improve the survival rates of honey bees. Vitellogenin can be an excellent immune improver and a good antioxidant (Seehuus et al., 2006). Paoli et al. (2014) show that young bees require higher protein intake than older adult workers. For honeybees, the main source of carbohydrates is floral nectar, while pollen satisfies the nutritional requirements for protein, lipids, sterols, and micronutrients (Wright et al., 2018).

Future studies could help evaluate how adequate pollen substitute is to the productivity of vitellogenin and understand how vitellogenin can apply to processes of the honey bee brain. Application in the hive to see; if this can increase longevity, pollination, and productivity, without harming the bees or the hive.

## 4. Conclusions

We conclude that; fermented pollen substitute diets are more effective than unfermented. It can improve the palatability and consumption rate of the artificial diets and increase the vitellogenin (The most important protein of workers hemolymph) to make the lifespan of the honey bee workers longer.

**Conflicts of interest.** There are no conflicts of interest.

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