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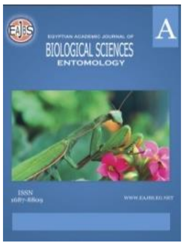

A fermented Pollen Substitute Diet Affects Wax Construction by Honey Bee Workers, (*Apies mellifera* L.)

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ABSTRACT

Pollen substitute diets have become increasingly important for maintaining strong and healthy honey bee colonies. Palatability and nutritional value are key attributes of a good diet. Since beebread, which is pollen fermented by the bees, is the main food of the worker-nurse bees that feed and care for the bee larvae, pollen substitutes should have similar attributes.

In an attempt to simulate this natural food source, an inoculum prepared from beebread was used to ferment a pollen substitute diet.
In this study, it was determined the differences in the wax building by caged workers fed with different diets (sucrose syrup only, bee bread, unfermented pollen substitute diet and fermented pollen substitute diet).
Comb building in honeybees was analyzed with respect to the type of food. The effects on wax combs building were assessed of the presence of beeswax foundation, and of feeding honey bees in cages. Workers fed on unfermented diets produced significantly less wax than did those fed on the fermented diets; although variation in wax production among the treatments was not significant.

We conclude that fermentation by beebread-derived microorganisms could improve the palatability, consumption and nutritional value of the dies and the behavioral activities such as wax building by honey bee workers.

INTRODUCTION

In order to develop artificial protein diets that are nutritious and attractive to bees, it would make sense to make them as similar as possible to their natural proteinaceous food in the hive, bee bread. Honey bees also ferment pollen to preserve it from harmful microorganisms (Vasquez & Olofsson, 2009; Almeida-Dias *et al*., 2018).

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 various means, including behavioral activities as wax building rates.

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 (Winston *et al*., 1983; Rousseau & Giovenazzo, 2016).

The wax of the honey bee (*Apis mellifera* L.) is a complex lipid-based organic compound (natural wax) produced by worker bees using four pairs of specialized wax glands located on the inner side of the 4th to 7th abdominal sternites. Beeswax is secreted in the form of wax scales which honey bees transfer with the forelegs to their mandibles, where wax scales are chewed (salivary secretions added), and then added to the comb being constructed. The main effect of this mandibulation process is a transformation of the texturally anisotropic scale wax into isotropic comb wax (Hepburn *et al*.,2014).

Chemically, beeswax represents a complex organic mixture of more than 300 compounds, of which the fatty acid esters (∼67%), hydrocarbons (∼14%), and free fatty acids (∼13%) predominate (Tulloch, 1980). There are no significant differences in the basic chemical composition of wax originating from different *A. mellifera* subspecies, only small variations related to the proportion of the above mentioned predominant compounds (Fröhlich*et al*., 2000).

This characteristic architectural structure reflects a complex behavior of the honey bees in terms of their self-organization of the hexagonal cell structure, as well as chemical and mechanical modifications of the wax during the wax scale-to-comb transformation pathway (Pirk*et a*l., 2004). Honey bees use beeswax as a construction material for building the comb (also known as honeycomb or comb wax) that serves as food (honey, pollen) storage and as a brood-rearing compartment (provides an infrastructure for rearing brood).

Beeswax is also important for the chemical communication within a honey bee colony; its characteristic chemical composition plays an important role as a source of nestmate recognition cues (Fröhlich *et al*., 2000 ; D’ettorre *et al*., 2006).

Secretion of wax scales is an age-related physiological process that may occur in adult honey bees at any time from day 3 to day 21 post-emergence (Hepburn *et al*., 1991).The wax production phase primarily occurs from day 9 to 21 post-emergence, peaking at day 12 (Hepburn *et al*., 2014). In addition to age, the onset and duration of this phase are influenced by multiple biological and ecological factors including season, comb/storage space needs, honey and pollen availability, and brood-care needs (Hepburn & Magnuson, 1988; Muller & Hepburn, 1992).

Under natural conditions, comb construction depends strongly on the time of the year and on the availability of nectar, with wax production reaching its climax when vegetation blooms and nectar becomes superabundant (Bogdanov, 2016a, 2016b). Many other factors may affect or stimulate wax production, such as outside temperature, the presence/absence of an egg-laying queen, pollen availability, ratio of brood combs, ratio of unoccupied combs, the population of workers or drones, as well as available space in the nest cavity and the bee health (Hepburn *et al.*, 2014). These factors are often intermingled and correlated (Pratt, 2004).

Here, we tested whether using a pollen substitute diet that fermented with beebread microorganisms would improve wax comb construction by the workers as a behavioral effect.

We outline mechanisms that may allow to influence worker's behavior and improving wax building rates.

In this article, we provide a description of the types of wax comb constructions and their growth pattern, also cell properties that are affected by the fermented substitute diet that we prepared.

Comb construction measured by counting the number of built cells of each type (worker, drones & queens); also, by determining the thickness of the constructed hexagonal cells (slightly, intermediate & completely).

MATERIALS AND METHODS

The experiments were carried out in Assiut, Insect Research Laboratory, 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 the samples collection, all the available types of bee bread from hives that placed in different regions in Assiut Governorate at the successive seasons.

Inoculum Preparation for Fermentation:

The inoculum was developed and prepared in our lab 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*). All the glassware and mixing implements were sterilized with 70% ethanol prior to inoculum preparation. After pooling and mixing, 10 g of the mixture of the collected bee bread was added to 300 ml of previously boiled sucrose syrup (50% w/v). This mixture was manually homogenized, was put in 250 ml-colored bottles, and placed in an incubator at 35 °C and controlled relative humidity (70%) for 25 days.

In order to release the CO_2 produced during fermentation, the bottles were briefly opened every 48 h. After the end of this fermentation period, the bottles were sealed and stored at 6–8 °C for up to 20 days. A new inoculum was prepared from freshly collected beebread every 20 days to help reduce contamination with opportunistic fungi and other microorganisms, (Almeida-Dias, *et al.*, 2018).

Diets Preparation:

Firstly, The Samples Were Prepared the Unfermented Diet:

A 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; (Mostafa, 2000**),** and linen oil as an attractive smell.

Then, the fermented diets were prepared as the following;

The same ingredients as the unfermented diet, with 40 ml fermented inoculum for each kilogram of the paste added and mixed well. Then, we stored them in an incubator in a loosely covered plastic food-grade container at 35°C, for 28-days.

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 a piece of the wax foundation were attached to the cage side. We prepared four groups of cages. Newly emerged workers aged 0 - 12 hours were confined in the cages; each cage containing 100 newly emerged bees; five replicates were used for each group. The cages were held also in a dark incubator at 32°C ±1 and 70% RH. (Fig-1).

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).

Fig. 1: wax construction in experimental wooden cages.

Determination Of Wax Building Rate By The Honey Bee Workers:

Wax foundation (wax sheet embossed with the shape of cell bases) that attached to the cage side with dimensions 9 x 5 Cm (area = 45 Cm²), containing 160 bases of worker cells (Fig-2). Given the complex and important role of beeswax in the honey bee colony, it is of crucial importance that the comb foundation sheet on which the workers are building their comb is genuine and uncontaminated and purchased from a trusted source.

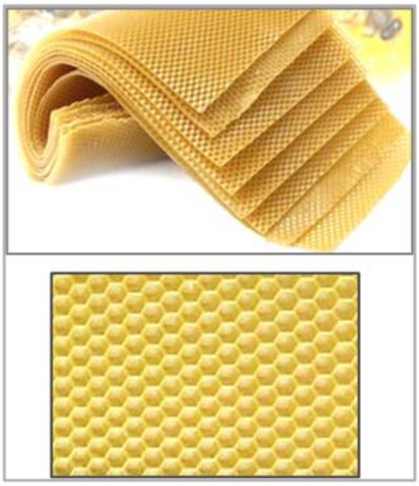

Fig. 2: Wax foundation attached to the cage side with dimensions 9 x 5 Cm (area = 45 Cm²), containing 160 bases of worker cells

After 18 days old of attachment and workers feeding on the different diets, the area of drawn or comb construction (as number of cells), including both the worker-sized and drone-sized cells, were measured on one side of the wax foundation. The worker-sized cells were classified into three types; (slightly, intermediate & completely) constructed cells.

Statistical Analysis:

Data obtained were compared and determined by a computerized analysis EXEL program and using ANOVA on Ranks and Duncan's multiple range test. The mean consumption rates were analyzed using the SPSS Test (version 17.0.2).

RESULTS AND DISCUSSION

Honey bee health is of grave concern for apiculture. The quality of diet influences colony health and strength, improving the performance of different behavioral activities. So that we prepared artificial diets (pollen substitute diets) for providing honey bee colonies with food characterized by appropriate cost, nutritional rich value, palatability and positive impacts on various biological activities to evaluate the effect on the wax construction behavior.

Fermentation significantly increased diet consumption and the health of the bees. The secretion of wax by worker bees was analyzed with respect to the type of food source. The relationship between wax secretion and food diet was determined simply by counting the wax cell construction. Comparison of workers that fed on different diets showed that the workers that feed on the fermented diet (Mixture 28 – days) differed from that which fed on the unfermented diet in total wax cell construction. The workers that fed on the bee bread diet still at the top although the differences were not significant, but the workers that fed on sucrose solution constructed the fewer combs. (Table 1 & Figs.3, 4, 5& 6).

Fig, 3: wax construction in the cages after 18 days of attachment and workers' feeding on the different diets.

The present results showed that the means of total wax cells that were constructed by feeding bees with (sugar syrup and water) stimulates wax production about (75.80) cell was constructed. This strength of sugar water is most similar to plant nectar.

Feeding bees with the fermented diet constructed about (105.80) cell; there is no significant difference comparing to bees fed with bee bread diet that showed extra wax building rate (109.40) cell. Where, the means of total wax cells for the workers fed on unfermented diet, (95.00) cell.

Table-1: Determination of wax cell construction

Wax cell construction 10*16	Non built	Total built	Worker cells: Slightly	Worker cells: Intermediate	Worker cells: Completely	Worker cells: Total worker cells	Drone cells	Queen cell
Control bee bread clover	42	118	39	32	41	112	6	0
	54	106	34	29	38	101	5	0
	57	103	19	28	52	99	4	0
	46	114	26	32	52	110	3	1
	54	106	28	32	43	103	3	0
	50.60 ±6.309A	**109.40 ±6.309A**	**29.20 ±7.662C**	**30.60 ±1.949A**	**45.20 ±6.458A**	**105.00 ±5.350A**	**4.20 ±1.304A**	**0.20 ±0.447B**
Fermented mixture 28-days	51	109	28	32	45	105	2	2
	57	103	25	26	49	100	3	0
	56	104	22	26	54	102	2	0
	54	106	23	31	50	104	2	0
	53	107	23	29	52	104	3	0
	54.20 ±2.3881A	**105.80 ±2.388A**	**24.20 ±2.387C**	**28.80 ±2.775A**	**50.00 ±3.391A**	**103.00 ±2.851A**	**2.40 ±0.548B**	**0.40 ±0.894A**
Unfermented	65	95	30	40	24	94	0	0
	59	101	53	31	17	101	0	0
	69	91	39	34	18	91	0	0
	62	98	45	32	20	97	0	0
	70	90	32	37	21	90	0	0
	65.00 ±5.515B	**95.00 ±7.515AB**	**39.80 ±9.471B**	**34.80 ±3.701A**	**20.80 ±3.271C**	**95.00 ±5.481AB**	**0.00 ±0.000C**	**0.00 ±0.000C**
Sucrose syrup	81	79	53	26	0	79	0	0
	69	91	78	13	0	91	0	0
	90	70	66	4	0	70	0	0
	99	61	50	11	0	61	0	0
	82	78	78	0	0	78	0	0
	84.20 ±11.167C	**75.80 ±11.167C**	**65.00 ±13.304A**	**10.80 ±9.985B**	**0.00 ±0.000D**	**75.80 ±11.644C**	**0.00 ±0.000C**	**0.00 ±0.000C**

The general mean numbers of wax worker cells that were slightly constructed are (29.20 & 24.20), for the workers fed on bee bread diet&Fermented diet, respectively; and (39.80) for the workers fed on Unfermented diet; its top number appeared in the workers fed on only Sucrose syrup (65.00). That is a good indicator for the importance of the pollen substitute diet as an alternative proteinaceous diet for workers to do their best behavioral activities; while the highest number of the constructed wax that was incompletely built (slightly) appeared in the cages where workers fed on sucrose syrup only and low numbers in the diets that contain proteins; also fermentation improve the wax construction behavior, while the lowest number appeared in the cages where workers fed on fermented diets, it’s a mimic to the bee bread diet in number where the difference wasn't significant.

Most of the intermediated constructed wax worker's cells recorded in the (Bee bread diet; Fermented diet & Unfermented diet) (30.60; 28.80 & 34.80), respectively. While the least recorded in the workers fed sucrose syrup only (10.80) cell.

The mean number of wax workers' cells that were completely constructed appeared in the (Bee bread diet & Fermented diet), where (45.20 & 50.00) cells were significantly greater than the other status (Unfermented diet) where (20.80) cells were constructed completely.

That is evidence of the importance of proteins and the fermentation process in improving the wax construction behavior as we mentioned above. Where the worker's wax construction behavior was optimum in the case of the bee bread diet and the fermented diet, compared to the unfermented diet. No completely wax workers cells appeared in the case of sucrose syrup only.

Capped Drone Cells: Larger in diameter and are domed much higher than worker cells. The majority numbers of the drone-sized wax cells that were constructed are (4.80) cells for the workers fed on (Bee bread diet) and (2.40) for the workers fed on the (Fermented diet).

The presence of drone cells in the comb stimulates their drone rearing. It has been suggested that the presence of drones in a colony promotes queen rearing (Ribbands,1953).

The beginnings of constructed queen cells appeared in the workers fed on (Bee bread diet) (0.20) and (0.04) for the workers fed on the (Fermented diet). The process of making a queen cell starts with what is called a "queen cup".

No drone cells or queen cells appeared in the case of workers fed on unfermented diets or sucrose syrup only. The impact of feeding type on drone cells and queen cells construction is dramatic.

Honey bees consume about 3.8 kg of honey to secrete 454 g of wax (Graham- Joe, 1992). So that making sheets of comb foundation with the hexagonal pattern is essential. Such foundation sheets allow the bees to build the comb with less effort, and the hexagonal pattern of worker-sized cell bases makes the process faster.

How can you get the workers to build more wax combs?

In order to build a comb, your bees must secrete wax and to secrete wax, the bees need a source of nectar (or light syrup) to stimulate their wax glands (Hepburn, 1986). The temperature in the hive should be warm enough for the bees to work, the interior colony temperature around the comb-building area must be closer to the natural conditions, somewhere between 30-37°C for the bees to be able to secrete wax and manipulate it to build comb (Hepburn *et al*. 2014); whereas reduced wax production at lower temperatures (e.g., 25.0°C) because at cooler temperatures, the wax becomes less pliable (Williams *et al*., 2013).

Wax construction is also affected by a good source of protein substitute diet for workers with good health, where bees enjoy better health give a better behavioral performance. Building comb takes time and energy; it requires the effort of many bees and good health.

Having fresh comb in the hive promotes healthier bees. For this reason, we want our bees to make some new frames of comb occasionally to hold bee brood, pollen and honey. Another reason for bees to make comb is for the honey harvest. People enjoy eating honeycomb too. Producing fresh honeycomb can be a viable part of honey production.

While we all understand that the bee bread (protein source) and the plant nectar (honey) (carbohydrate source) are very important food for bees, most beekeepers will feed bees at some time. Supplemental feeding can improve the wax building rate. Extra protein feeding can make the workers do their best. Producing beeswax is a vital part of the honey bee life experience, as important – the colony needs to have a good population of healthy young bees for building combs because producing wax requires good bees.

Bees must consume large quantities of honey in order to stimulate the wax glands. Honey is metabolized in fat cells to produce wax. The worker bee has also consumed pollen as a source of proteins in the first few days of her life to promoting fat cell development. Feeding your bees will encourage them to draw out comb.

We investigated how feed type affects the wax comb construction and the differences that arise from feeding on (unfermented diet) and on (fermented diet with bee bread microorganisms), we concluded that fermentation improves wax production rate in honey bees. Since that the prepared fermented diet as similar as possible to the natural proteinaceous food in the hive, (bee bread diet). The wax building rate increased when the workers fed the fermented diet with the sucrose syrup comparing to the sucrose syrup only in a significant change.

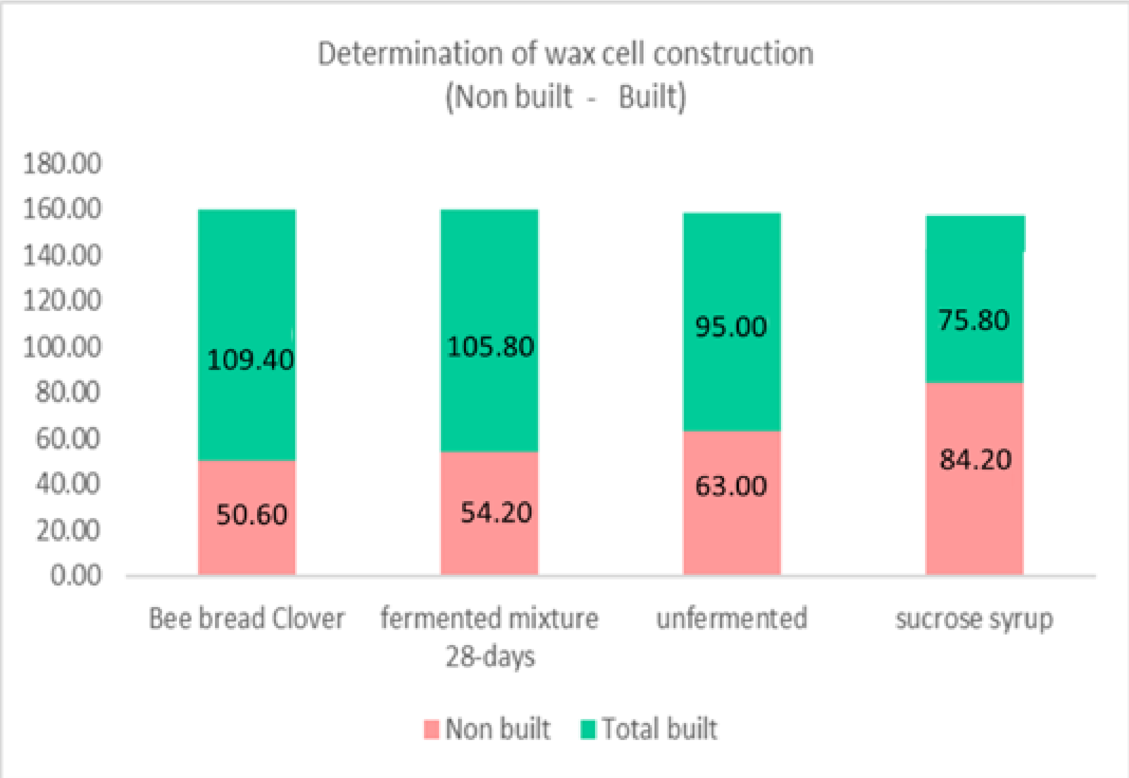

Fig.4: Determination of the mean number of wax cell construction.

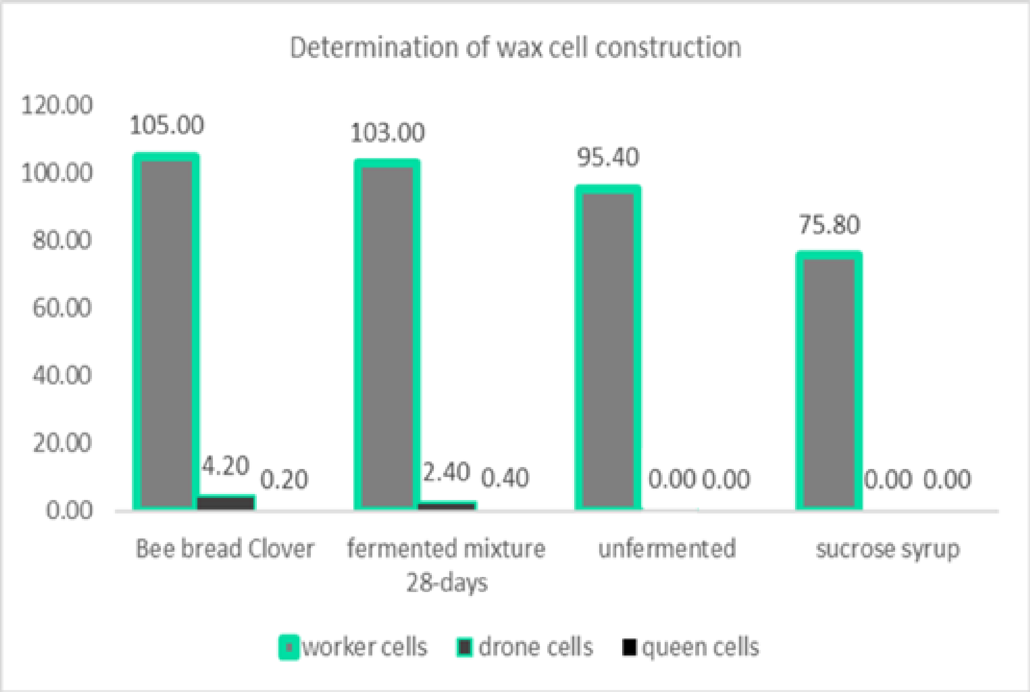

Fig.-5: Determination of the mean number of different types of wax cells

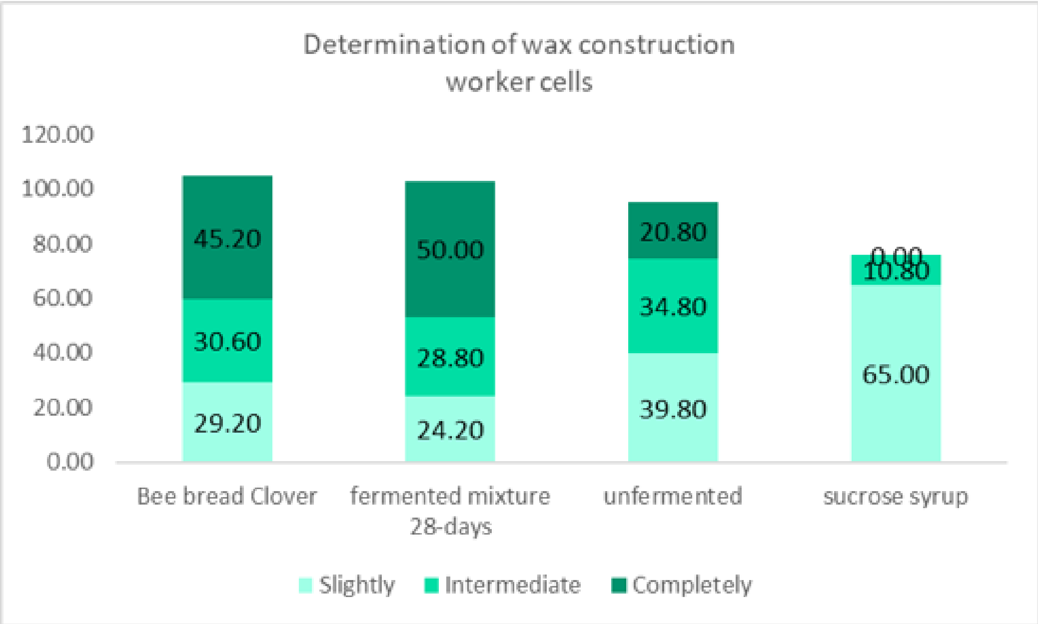

Fig.6: Determination of the mean number of workers wax cell.

Conclusions

It was concluded that the fermentation by beebread-derived microorganisms could improve the palatability, consumption and nutritional value of the dies and the behavioral activities such as wax building by honey bee workers.

Further studies may be by measuring the comb area and weighing comb produced at the end of the experiment. Timing can be investigated experimentally manipulating external factors such as food consumption under laboratory conditions or replicating the experiments at various times of the year, or at different apiaries at different locations.

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