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Fermentation of a pollen substitute diet with beebread microorganisms increases diet consumption and hemolymph protein levels of honey bees (Hymenoptera: Apidae)

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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. Newly emerged bees were fed on the diets for seven days. They consumed significantly more fermented than unfermented diet. Hemolymph protein levels 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. Vitellogenin (a key storage protein for honey bees) levels were also increased significantly in bees fed the fermented versus the non-fermented diet. Survival rates were higher for bees fed the fermented versus the non-fermented diet, though the difference was not significant. We conclude that fermentation by beebread-derived microorganisms can improve the acceptance and utility of an artificial protein diet for honey bees.

Introduction

Protein from pollen is needed for brood rearing and for the satisfactory development of adult bees (Crailsheim, 1990; Roulston & Cane, 2000; Hoover et al., 2006). The nutritive value of bee bread for honey bees is higher than that of fresh bee collected, laboratory stored, or frozen pollen, with few exceptions (Hagedorn & Moeller, 1968; Herbert & Shimanuki, 1978; Dietz & Stevenson, 1980; Cremonez et al., 1998; Pernal & Currie, 2002). However, there is recent evidence that processing of pollen by the bees results in pollen preservation and not nutrient conversion (Anderson et al., 2014). Protein levels in the diet affect the resulting protein levels in honey bee hemolymph (Basualdo et al., 2013). Honey bees also ferment pollen to preserve it from harmful microorganisms (Herbert & Shimanuki, 1978; Vasquez & Olofsson, 2009).

During dearth periods, when insufficient pollen is available or when they need to build up colonies for pollination contracts, beekeepers often invest in pollen substitutes to feed their bees (Somerville, 2005). Adequate diet formulation, deterioration during storage, attractiveness to bees and diet costs are major concerns (Herbert et al., 1977). Unfortunately, many nutritional supplements are poorly accepted by bees and have low nutritional value (Schmidt & Hanna, 2006). 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, beebread. Diet efficiency can be measured by various means, including brood and honey production (Herbert et al., 1977; Winston et al., 1983; Paiva et al., 2016) and by measuring the protein levels in bee hemolymph (Cremonez et al., 1998; Cappelari et al., 2009; Basualdo et al., 2013, 2014; Barragan et al., 2016). 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 bee diet should be acceptable to the bees and provide nutrients essential for colony growth and development, bee health and colony production capacity (Herbert & Shimanuki, 1978; Winston et al., 1983; Rousseau & Giovenazzo, 2016). Although various diets have been developed for pollen replacement during dearth periods (Abbas et al., 1995; De Jong et al., 2009; Ellis & Hayes, 2009; Morais et al., 2013a, b), common problems include a lack of attractiveness (Robinson & Nation, 1968; Pernal & Currie, 2002) and inefficient conversion into bee protein compared to beebread (Cremonez et al., 1998). Here, we tested whether fermentation with microorganisms from beebread would improve consumption and utility of a pollen substitute diet, whose components are defined below.

Material and Methods

The feeding trials were conducted in 2012–2013 with unselected Africanized bees in our university apiary in Ribeirão Preto, SP, Brazil (21°10'39'' S, 47°48'24'' W).

Inoculum preparation for fermentation

Inoculum was developed and prepared in our lab. All the glassware and mixing implements were sterilized with 70% ethanol prior to inoculum preparation. Beebread was collected with a metal spatula from 12 brood combs, retrieved from four different colonies. After pooling and mixing, 10 g of freshly collected beebread was added to 300 ml of previously boiled sucrose syrup (50% w/v). This mixture was manually homogenized with a spatula, divided into two equal aliquots in 250 ml amber-colored glass 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.

Diet Preparation

Diets were formulated as follows: 1. Beebread diet -100 g of beebread mixed with 80 ml of distilled water, forming a paste; 2. Sucrose syrup –70% (w/v); 3. Unfermented protein diet - 20 g powdered sugar cane yeast, 16.7 g powdered soy meal., 43.3 g rice meal, 20 g sucrose, and sufficient 50% sucrose syrup to make a paste; 4. Fermented protein diet – same ingredients as diet 3, with 40 ml fermented inoculum added and mixed and then stored in an incubator in a loosely covered plastic food-grade container at 35°C for 28 days. The artificial protein diet contained 25% crude protein in the dry components, before adding the sucrose syrup. Considering the final weight of approximately 180 g (including the sucrose syrup and fermented inoculum, which is about 50% sucrose), the diet paste offered to the bees had approximately 14% protein.

Caged bees and feeding

Combs with emerging worker brood from four Africanized honey bee colonies were placed in an incubator at 34 °C and 80% relative humidity. The workers that emerged within a period of 15–20 hours were collected, mixed as uniformly as possible and groups of 100 of these workers were placed in each plastic confinement cage (8 x 11 x 13 cm) (Morais et al., 2013b).

On days 0, 2, 4 and 6, 4 g of each diet was offered to the bees in plastic feeders (50 ml Falcon centrifuge tubes partially cut lengthwise to fashion troughs) introduced into the cages through a hole drilled in the side. Sucrose syrup (70% w/v) was provided *ad libitum* to all groups. Each diet was tested in eight cages. The bees in the cages were maintained in an incubator in the dark at 34°C and 80% relative humidity.

Quantification of Protein in the Hemolymph

After three and seven days of confinement and feeding, 10 workers were randomly collected from each cage and hemolymph was collected with a pipette from a small incision made with an entomological scissors at the base of the bees' wings. The total protein in the hemolymph was determined by spectrophotometry (Ultrospec 2100 pro - Pharmacia), at 595 nm, using the methodology proposed by Bradford (1976). A standard curve was prepared using bovine serum albumen at 0, 4, 10, 16, 20, and 30 µg protein/µl. The standards and the hemolymph samples were pipetted into 96 well ELISA plates and read in an ELISA microplate reader (Morais et al., 2013b).

Measurements of vitellogenin levels

Soluble hemolymph (proteins were separated by SDS-PAGE, 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 after seven days of confinement and feeding. The hemolymph was collected using the same technique as above, 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 = 121.14) in 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.

Survival rate of caged bees fed natural, fermented and unfermented diets

Twelve confinement cages were prepared, each containing 100 newly emerged bees. Bees in three of these cages were fed with the beebread diet (4 g), three were

fed with sucrose syrup (*ad libitum*), three were fed with the unfermented protein diet (4 g) and three were fed with the fermented protein diet (4 g). The cages were kept in an incubator, under the same conditions of temperature and humidity mentioned above. We counted and removed dead bees from each cage daily. The food was renewed every 48 hours. Survival rates were analyzed by Kaplan-Meier Survival Analysis using SPSS (version 17.0.2).

Determination of the consumption rate and preference for each diet

Ten confinement cages were prepared, each containing 100 newly emerged bees. Each cage was provided with 4 g of unfermented protein diet and 4 g of fermented protein diet, made available at the same time, so that the bees could choose between diets. The cages were kept under the same incubator conditions mentioned above. The experiment lasted eight days and the diet was renewed every two days. Remaining diet was weighed when the protein diet was replaced and on the last day to determine the consumption rate.

Statistical Analysis

Data obtained from the protein quantification in the hemolymph and measurements of vitellogenin levels were compared using ANOVA on Ranks, and pair-wise comparisons were made using the Student-Newman-Keuls test and the t-test. Survival rates were analyzed using Kaplan-Meier Survival Analysis. The mean consumption rates were analyzed using the Wilcoxon Signed Rank Test, in SigmaStat © 3.5.

Results

Total protein levels in the hemolymph of caged workers

The mean levels of protein in the hemolymph of workers in cages fed with protein diets were significantly higher than in those fed sucrose syrup alone. Additionally, bees fed with fermented protein diet had significantly higher levels of protein in their hemolymph than those fed with unfermented protein diet during seven days of confinement (Table 1).

After three days of feeding, the levels of protein in the hemolymph of bees fed unfermented and fermented protein diet were not significantly different from those of beebread fed bees ($p = 0.809$ and $p = 0.437$, respectively). All the groups fed protein diets had higher levels of protein in the hemolymph when compared to bees fed sucrose syrup ($p < 0.01$). After seven days of feeding, both the fermented and unfermented protein diet groups had significantly lower levels of protein in the hemolymph, when compared to workers fed beebread ($p \leq 0.001$ and $p = 0.007$, respectively).

Vitellogenin levels

The density of vitellogenin bands of hemolymph from bees fed the fermented diet was significantly greater (optical density of vitellogenin bands 165.85 ± 6.79 arbitrary optical density units (D.U.) compared to that from bees fed on unfermented diet (123.17 ± 7.71 D.U.; $p = 0.026$, Student-Neuman-Keuls test). These levels were similar to and not significantly different when compared to beebread-fed bees (138.22 ± 5.60 D.U.).

Table 1. Mean and standard deviation of the concentration of total protein (µg/µl) in the hemolymph of honey bees confined in plastic cages at emergence, after feeding on beebread diet, sucrose syrup, unfermented protein diet or fermented protein diet for 3 - 7 days. Pools of hemolymph from 10 bees were analyzed from each of eight cages for each diet. Identical letters in the same row indicate the absence of significant statistical differences. Different letters in the same row indicate significant differences (ANOVA on Ranks and pair-wise comparisons were made using the Student-Newman-Keuls test and the t-test, $p < 0.05$).

Age (Days)	Diets (Concentration of total protein µg/µl)			
	Sucrose Syrup	Beebread Diet	Unfermented Protein Diet	Fermented Protein Diet
3	$14.38^{a} \pm 5.40$	$23.00^{b} \pm 4.35$	$23.65^{b} \pm 6.25$	$26.15^{b} \pm 5.97$
7	$12.14^{A} \pm 3.45$	$54.29^{D} \pm 7.13$	$32.24^{B} \pm 2.40$	$39.46^{C} \pm 6.64$

Survival rates of adult workers confined to cages

Bees fed with the protein diets beebread, unfermented and fermented protein diet survived longer than those fed only with sucrose syrup ($p \leq 0.001$, Kaplan-Meier Survival Analysis, Fig 1). When compared with beebread, fermented and unfermented diet showed no significant difference ($p = 0.188$ and $p = 0.05$), indicating that feeding the artificial diet did not negatively affect adult survival rates. The comparison of the survival rate between bees fed fermented and unfermented diet also showed no significant difference ($p = 0.178$).

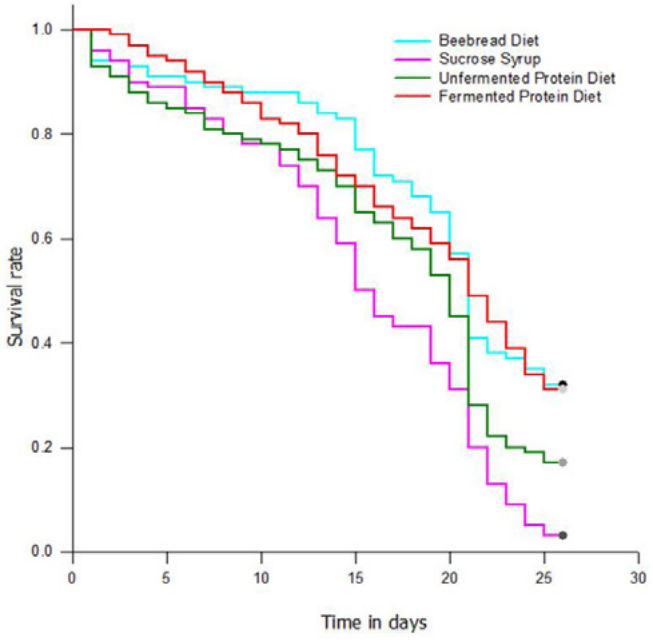

Fig 1. Survival of caged newly emerged bees fed different diets in confinement cages. Diets were beebread, fermented protein diet, unfermented protein diet and sucrose syrup.

Consumption rates and preferences

The mean consumption rates of unfermented and fermented protein diets were 3.5 ± 0.93 mg and 6.1 ± 1.25 mg per bee during eight days, respectively. Fermentation of the diet significantly increased consumption ($p = 0.002$, Wilcoxon Signed Rank Test). In the preference test, when the two protein diets were offered together in the same cage, the bees preferred the fermented over the unfermented diet (2.63 ± 0.75 mg versus 1.09 ± 0.59 mg per bee, respectively; $p < 0.001$, Wilcoxon Signed Rank Test).

Discussion

Fermentation significantly increased diet consumption and the protein levels of bees fed on the pollen substitute diet (Table 1). The highest protein levels were found in bees that consumed beebread, as also found by Cremonez et al. (1998), van der Steen (2007) and Basualdo et al. (2014). A sucrose syrup diet (control) resulted in lower protein levels in the hemolymph, as also found by De Jong et al. (2009) and Morais et al. (2013b).

In *Apis mellifera* Linnaeus the accumulation of storage proteins (vitellogenin) in the hemolymph of adult workers is significantly influenced by nutrition. Consequently, a diet that is able to maintain proteins in the hemolymph both qualitatively and quantitatively helps guarantee the health of the bees (Bitondi & Simões, 1996). We found that workers fed with the fermented protein diet produced vitellogenin at similar levels when compared to beebread fed bees, but significantly higher than in bees fed unfermented diet.

Schmidt et al. (1987) and Manning et al. (2007) observed that differences in the protein levels of pollen affect bee longevity. Herbert and Shimanuki (1978) found that pollen substitutes can efficiently substitute pollen; however, inducing the bees to consume artificial diets can be a major difficulty. They also stated that diets need to be both nutritious and palatable in order to be useful, as also concluded by Mattila and Otis (2006) and Standifer et al. (1973). Ellis and Hayes (2009) found that bees consume more of a fermented diet than an unfermented diet, though they used a probiotic yogurt inoculum instead of beebread microorganisms.

The main microorganisms present in beebread are: bacteria, including the genera *Lactobacillus, Bacillus* and *Agrobacterium*, and fungi of the genera *Penicillium* and *Aspergillus*, as well as yeasts (Gilliam, 1997). These are responsible for the fermentation process. Alterations made by the microorganisms in beebread help preserve, increase palatability and facilitate consumption of bee-collected pollen (Loper et al., 1980; Gilliam et al., 1989; Vasquez & Olofsson, 2009). We used the same microflora to ferment a pollen substitute diet to determine if it would make the diet more attractive and useful for the bees. The bees preferred and consumed more of the fermented diet. On the other hand, Carroll et al. (2017) found that bees preferentially consumed freshly stored pollen (one day old) over pollen that had been stored (and fermented) for a longer period, when choosing what was available in the hive. Beebread from Africanized hives could have a different microflora than that from European bees, as it has been found to be preferred by both types of bees (DeGrandi-Hoffman et al., 2013). Fermenting the diet with bee-derived microorganisms could help correct diet-related gut dysbiosis and protect the bees against pathogens (Maes et al., 2016).

In conclusion, fermenting a pollen substitute diet for bees can make it more useful as a substitute for natural pollen sources, resulting in greater protein levels in the hemolymph and consequently greater brood production in honey bee colonies. This fermentation process requires no specialized equipment and the inoculum can be made from beebread collected from local beehives.

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Authors' Contribution

Study conception and design: JMAD; MMM; DDJ. Acquisition of data: JMAD; APT; RAP. Analysis and interpretation of data: JMAD; TMF; MMM. Drafting of manuscript: JMAD; TMF; MMM; DDJ. Critical revision: JMAD; TMF; MMM; DDJ**.**

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