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Optimisation of a banker box system to rear and release the parasitoid *Habrobracon hebetor* (Hymenoptera: Braconidae) for the control of stored product moths

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Abstract

Pyralid moths, such as *Ephestia kuehniella* (Zeller) or *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), are among the pests of most concern in mills and food industries worldwide. One option for their control, which presents an alternative to the application of insecticides, is the release of natural enemies. *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) is a larval parasitoid of pyralid moths that is commercially available for augmentative release in storehouses. They are delivered as adults that limits their performance. To improve their quality when released at the target location, a banker box has been developed consisting of a rearing box that optimises the release of the parasitoid. In the present study, the non-pest larvae *Galleria mellonella* (L.) has been used as a host, substituting for *E. kuehniella* larvae which was used in the previous design. The best results were obtained when a mixture of two larval sizes of the host were offered to the female parasitoid, producing five times more adults than with *E. kuehniella* larvae.

Quality of the released parasitoids was optimal because they were delivered in the pupal stage inside the rearing box and adults began to emerge *in situ*. The banker box released adult parasitoids over a prolonged period of approximately 25 days at the target location. The use of this banker box may significantly help in the biological control of stored product moths.

Key words: *Ephestia kuehniella*, *Galleria mellonella*, biological control, parasitoid, pyralidae.

Resumen

Los pirálidos *Ephestia kuehniella* (Zeller) y *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) se encuentran entre las plagas más preocupantes de los molinos y las industrias alimentarias de todo el mundo. Una alternativa a la aplicación de insecticidas para su control es la liberación de enemigos naturales. *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) es un parasitoide de larvas de pirálidos que está disponible comercialmente para su introducción en almacenes de la industria agroalimentária. Estos parasitoides se envían normalmente como adultos lo que limita su efectividad. Para mejorar su calidad cuando se liberan en el destino, se ha desarrollado una "banker box" consistente en una caja de cría, que permite mejorar la calidad del parasitoide liberado. En este estudio, hemos mejorado esta "banker box" utilizando como huésped la larva *Galleria mellonella* (L.), especie que no es plaga en almacenes, en sustitución de las larvas de *E. kuehniella* que se utilizaron en el diseño anterior. Los mejores resultados se obtuvieron cuando se ofreció a la hembra del parasitoide una mezcla de dos tamaños de larvas del huésped, produciéndose cinco veces más adultos que con las larvas de *E. kuehniella*. La calidad de los parasitoides liberados fue óptima porque se distribuyeron en la fase de pupa, y los adultos comenzaron a emerger ya *in situ*. La "banker box" liberó

parasitoides durante un período prolongado de aproximadamente 25 días. El uso de esta "banker box" puede ayudar a mejorar significativamente el control de las polillas que atacan los productos alimenticios almacenados.

Palabras clave: *Ephestia kuehniella*, *Galleria mellonella*, control biológico, parasitoides, pirálidos

Introduction

Pyralid moths, such as the Mediterranean flour moth *Ephestia kuehniella* (Zeller) and the Indian meal moth *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), are among the most destructive pests in mills and food-processing facilities in Europe (Eliopoulos et al., 2002; Mohandass et al. 2007; Trematerra and Gentile, 2010). Eggs of both species are laid on the flour and grain surface, and larvae are often burrowed within the silk produced. The moths may breed hidden inside the machinery and tubing systems of mills and food-processing facilities, resulting in obstructions due to the accumulation of silk, exuviates, faeces and dust (Belda et al., 2011). Moreover, their metabolic activity increases moisture in and temperature of stored products, providing favourable environmental conditions for mold development, which decreases food quality and may be harmful to human health (Gorham, 1979; Nopsa et al., 2015). To control these and other insect pests, manufacturers and farmers have commonly relied on the use of pesticides. However, due to the hazards associated with their use, introduction of legislative restrictions over the last decade have limited the application of contact insecticides and fumigants in the food industry. In addition, consumers are more conscious of the effects of pesticides on their health and on the environment, and are frequently searching for non-chemical alternatives (Fields and White, 2002; Hagstrum and Subramanyam, 2009; Phillips and Throne, 2010). One option for controlling storage pests is to use biological control through the release of natural enemies (predators, parasitoids or enthomopathogens) (Schöller et al., 1997), a strategy that is well-developed in Integrated Pest Management (IPM) programs against plant pests of greenhouse crops.

Habrobracon hebetor (Say) (Hymenoptera: Braconidae) is a gregarious ectoparasitoid considered to be one of the best potential control agents for Lepidopteran pests in food storage environments. This cosmopolitan species is naturally encountered

in mills and alimentary industries worldwide. It parasitizes species such as *P. interpunctella* or *E. kuehniella*, although it is also known to attack other pyralid species, such as *Galleria mellonella* (L.) (Lepidoptera, Pyralidae), which are not related to the food storage environment (Aamer et al., 2015; Alam et al., 2014; Amir-maafi and Chi, 2006; Belda and Riudavets, 2013; Eliopoulos and Stathas, 2008; Golizadeh et al., 2017; Grieshop et al., 2006; Press et al., 1982; Saadat et al., 2014). Females of *H. hebetor* preferentially attack last instar larvae by paralysing them with venom before laying a variable number of eggs on or near the surface of the immobilised larvae. Paralysed host larvae are then used as a food source for both developing wasps and for adult females (Akinkurolere et al., 2009; Ghimire and Phillips, 2014; Kryukov et al., 2017; Yu et al., 2003).

Habrobracon hebetor is commercially available and is commonly released in the adult form. However, the short life span of adult parasitoids and the possible damage caused during transportation highlights the need to optimise release methodologies (Kehrli et al., 2005). To overcome this problem, Lucas et al. (2015) designed a rearing box that can be delivered to the target premises before the start of adult moulting. Once in place, adults can emerge gradually from the rearing box as they moult from the pupae and disperse at their own pace. With delivery of the rearing box, the risk of damage to the adults caused by transportation is eliminated, since they travel as immature forms that are far less fragile. A rearing and releasing box for control of the granary weevil *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) by the parasitoid *Lariophagus distinguendus* (Forster) (Hymenoptera: Pteromalidae) has also being developed by Niedermayer and Steidle (2013). Lucas et al. (2015) reared *H. hebetor* on *E. kuehniella,* which presents a certain risk of contamination in food processing facilities from the accidental escape of hosts that may occur. The aim of the present study was to optimise the rearing box system

designed by Lucas et al. (2015) by testing *G. mellonella* as an alternative host. This is a specific pest of honeybee colonies, which do not cause problems in food storage facilities. In addition, their larvae are significantly bigger than that of *E. kuehniella*, which could improve parasitoid production. We tested the production of *H. hebetor* when large larvae or a mix of medium-sized and large larvae of *G. mellonella* were offered to the parasitoid females in comparison with offering large larvae of *E. kuehniella*.

Materials and methods

1. Insect colonies

Colonies of *E. kuehniella* and *H. hebetor* were started with individuals from samples collected from stored-product facilities and mills in North-eastern Spain. The colony of *G. mellonella* was started with individuals provided by Dr. F. García del Pino (Universitat Autònoma de Barcelona). *E. kuehniella* was reared in l-L glass jars with 250 g of a mixture of white wheat flour and 7% yeast by weight. *G. mellonella* was reared in l-L glass jars with a diet consisting of 70 g baby cereal, 5 ml vitamin, 30 g sugar, 30 ml glycerine, 35 ml water, 30 g wheat germ and 5g yeast. *Habrobracon hebetor* was reared on third to fourth instar larvae of *P. interpunctella*. To increase egg loads, adult parasitoids were provided with honey impregnated in absorbent paper. All colonies were maintained and experiments performed in controlled conditions at 28 ± 2 ºC, 70 ± 5% Relative Humidity and a photoperiod of 16:8 h of light:dark.

2. Bioassay

The parasitoid was reared in a plastic box (11 cm high × 11 cm diameter) containing 20 lepidopteran larvae (*E. kuehniella* or *G. mellonella*) and their respective diets. This box was covered with a thin mesh for ventilation. Three females and two males of *H. hebetor* (0 to 48-h old) were added and a paper strip moistened with honey solution

was included as their feed. Eleven days after the introduction of parental parasitoids, the new generation of adults started to emerge.

The production of the rearing box was evaluated in a Plexiglas cage (15 cm high × 15.5 cm wide × 22 cm long with a hole in the lid and covered with mesh for ventilation) containing the rearing box and another similar box containing 10 fourth instar larvae of *E. kuehniella*, as described in Lucas et al. (2015). This pest box was included to encourage the emerging parasitoids to leave the rearing box. The pest box was open, to allow parasitoids to enter and find the hosts. A thin layer of tanglefoot was painted on the opening to prevent moth larvae from escaping.

When the new generation of parasitoids was ready to emerge, the mesh covering the rearing box was replaced by a lid containing 50 holes, each with a 1.6-mm diameter, to allow the exit of new adults. On this same date, the pest box was added to the Plexiglas cage. This was denoted as time 0 in the evaluation of the rearing box production. Four days later, the first count was conducted. For that purpose, all parasitoids outside the rearing box were counted, sexed and retired from the system, and the pest box was replaced by a new one. All pest boxes recovered from the Plexiglas cage were covered with a thin mesh and maintained until the emergence of the host or the parasitoid. Four more counts were performed after 7, 14, 21 and 25 days. At the final count, the number of *H. hebetor* adults and the number of lepidopteran adults present inside the rearing box were also counted.

Three different host treatments were considered: fourth instar larvae of *E. kuehniella* (EK), fourth instar larvae of *G. mellonella* (GM) and a mixture of second and fourth instar larvae of *G. mellonella* (GM2). A rearing box with each host treatment, but

without parasitoids, was used as a control treatment. Fifteen replicates were carried out for each parasitoid treatment and five for the controls.

3. Data analysis

The following variables were evaluated: total number of *H. hebetor* adults produced and their sex ratio; percentage of *H. hebetor* adults leaving the rearing box in total on each sampling date and the proportion that was females; percentage of mortality of *E. kuehniella* larvae in the control treatment and in the pest box (mortality in the pest box was corrected by mortality of controls); percentage of 'host profitability' in the rearing box (host-induced mortality by the parasitoid was also corrected by host mortality in the controls).

Data on the total number of emerged parasitoids did not comply with the requirements of parametric tests, and the Kruskal–Wallis analysis of variance, a non-parametric equivalent of analysis of variance (ANOVA), was used to compare the treatments; when significant, this test was followed by a pairwise Mann–Witney *U*-test. The *p*-values were corrected for multiple comparisons using the Bonferroni technique. The proportion of emerged *H. hebetor* females was evaluated using a chi-square test. The percentage data were arcsine transformed and the analysis of variance was used to compare treatments. When significant, means were compared by the Tukey test ($P > 0.05$). Statistical analysis was performed using the statistical package JMP 8.0.1 (JMP 2009).

Results

The designed banker box successfully produced parasitoids with all treatments tested. The production of adults was dependent on the treatment ($\chi^2 = 15.76$; df = 2; $P < 0.001$): significantly more parasitoids were obtained when second and fourth instar larvae

179 of *G. mellonella* were offered than when only fourth instar larvae of *G. mellonella* or
180 fourth instar larvae of *E. kuehniella* were offered (Fig. 1). The percentage of *H. hebetor*
181 adults that left the rearing box also differed between treatments ($F_{2, 42} = 48.44$; $P < 0.001$).
182 At the end of the experiment, more than 98% of parasitoids were found outside the rearing
183 box with treatments GM and GM2, while only 81% of parasitoids left the rearing box
184 with the EK treatment (Fig. 2).

185 The banker box system released adult parasitoids over a period of approximately
186 25 days and this release significantly decreased over time with all treatments ($F_{4, 210}$ =
187 100.73; $P < 0.001$): more than 98% of parasitoids dispersed from the rearing box during
188 the first 14 days (Table 1). Adult parasitoids that left the rearing box produced high host
189 mortality in the pest box. This host mortality in the pest box was correlated with parasitoid
190 production over time ($R = 0.37$; $P < 0.001$): after 4 days of the start of parasitoid
191 emergence, 100% of pest mortality was observed, after one week 98.3 ± 0.7% and after
192 fourteen days 57.8 ± 5%.

193 The sex ratio of the emerged *H. hebetor* adults was female biased in the GM2
194 treatment group (58.5% of females; $t = 2.03$; df = 14; $P = 0.031$), while no differences
195 were observed between sexes with the other two treatments (55.9% and 57.1% of emerged
196 females for EK and GM, respectively; $t = 1.08$; df = 14; $P = 0.150$ for EK and $t = 1.33$;
197 df = 14; $P = 0.102$ for GM). The proportion of females that left the rearing box decreased
198 with successive counts over time ($F_{2, 37} = 6.49$; $P = 0.004$ for EK, $F_{2, 36} = 3.52$; $P = 0.040$
199 for GM and $F_{2, 40} = 8.86$; $P < 0.001$ for GM2). However, the pace of emerging females
200 was different in the two host species. In the EK treatment group, this decrease was rapid
201 after three days. In contrast, with the GM and GM2 treatments the percentage of females
202 produced was similar during the first week, decreasing sharply there after (Fig. 3).

Host survival in the absence of parasitoids varied between treatments. The percentage of hosts that developed to adult stage was 64 ± 1% for EK, 67 ± 1% for GM2 and 97 ± 1% for GM. The percentage of hosts killed in the rearing box (host profitability), corrected by the specific larvae mortality observed in the absence of the parasitoid, differed between treatments ($F_{2, 42} = 15.27$; $P < 0.001$). When *E. kuehniella* larvae were offered as the host, a higher percentage of larvae were killed (85%) than when the host was *G. mellonella* (47% for GM and 62% for GM2) (Fig. 4).

Discussion

In the present study, we successfully optimised the banker box system designed by Lucas et al. (2015) by using *G. mellonella* larvae of mixed ages. We selected *G. mellonella* as the host for two main reasons: (1) this species has large larvae, which have been suggested to be qualitatively superior for parasitoid fitness (Akinkurolere et al., 2009; Ghimire and Phillips, 2010a; Godfray and Shimada, 1999) and (2) this species does not present a risk of contamination in mills and grain industries. Larger larvae could be more suitable than smaller ones for several reasons. In the case of gregarious parasitoids, such as *H. hebetor*, larval competition is common and this should be reduced with larger larvae (Boivin and Martel, 2012; Rasool et al., 2017; Taylor, 1988). Large larvae have less refuge opportunities, being more exposed to attack by parasitoids (Akinkurolere et al., 2009) and expecting a higher oviposition rate. A higher fecundity of *H. hebetor* was observed in *G. mellonella* compared to *E. kuehniella* (78.3 eggs/female and 66.3 eggs/female, respectively) (Amir-maafi and Chi 2006). However, larger larvae could also have drawbacks. When Ghimire and Phillips (2010b) compared the performance of *H. hebetor* on twelve different lepidopteran species (among them were our two host species, *E. kuehniella* and *G. mellonella*), they observed that, despite having greater oviposition response in large host larvae, survival rate was a lower. In our results, a significant

increase in production was observed when larval *G. mellonella* of mixed ages were offered. One explanation is that large larvae, although inducing a higher oviposition rate, exhibit greater defensive behaviour; this presumably increases mortality of the immature parasitoids and lengthens the developmental time (Milonas, 2005). The number of parasitoid offspring was reduced drastically when *H. hebetor* was reared on the larger host *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) in relation to the smaller host *G. mellonella* (Rasool et al., 2017) In fact, in our study, a number of *G. mellonella* larvae were observed with melanisation rings and rapid decomposition. This is a typical consequence of cellular encapsulation, which is an innate defensive immune response in some Lepidoptera species (Kryukova et al., 2011).

When using *G. mellonella* as a host more than 98% of the parasitoids left the rearing box, compared to the 81% observed with *E. kuehniella*. It may be that *G. mellonella* is a non-preferred host, forcing the parasitoid to leave the rearing box as it was attracted to the odors of *E. kuehniella* larvae in the pest box.

Yu et al. (2003) suggested that *H. hebetor* females can vary in clutch size and the resulting progeny sex ratio to optimise the host, so that the overall sex ratio could be stabilised. Like many parasitic Hymenoptera, female *H. hebetor* develop from fertilised eggs and males from unfertilised eggs (Benson, 1973). Some theoretical models proposed that the changes in the progeny sex ratio of gregarious parasitoids that generate a female bias are based on host quality (Charnov et al., 1981; Ghimire and Phillips, 2014). In our study, only the treatment with mixed-age larvae presented a significant female bias, suggesting GM2 to be the most optimal treatment. This same treatment produced significantly more progeny than the other tretaments.

The proportion of female offspring leaving the rearing box from the total number of emerged females in our study decreased with time, which suggests that females are more prone to leave the rearing box than males. Newly emerged females in the rearing box mate as soon as they moult to adult forms with their sibling males and, afterwards, they look for a host where to lay their eggs. They will leave the rearing box if they do not find a suitable host for egg-laying inside. With the EK treatment, few or no suitable host larvae were left by the parental generation of the parasitoid, as is shown by the high percentage of host profitability (Fig. 4). But, with GM and GM2 treatments containing large larvae of *G. mellonella,* host profitability was significantly lower (*H. hebetor* left more than 15% of *G. mellonella* alive in the rearing box) and newly emerged females of the parasitoid still laid some eggs on those hosts. This lower host profitability could be for several reasons, including the presence of refuges offered by the diets (*E. kuehniella* diet offers less refuge opportunities than the diet of *G. mellonella)*, the host size (*H. hebetor* may need less large larvae to oviposit the same amount of eggs compared to smaller larvae) and the host preference of the parasitoid (Ghimire and Phillips, 2010b). Finally, the parasitoid venom may have been depleted more quickly when females attempted to subdue the larger host *G. mellonella*, resulting in less effective attacks, as reported by Ghimire and Phillips (2010a).

In summary, the results obtained in this study indicated that *G. mellonella* could be a suitable host for rearing *H. hebetor* in the conditions of a banker box system. This species, when offered in mixed ages, resulted in a higher parasitoid production that was biased toward females, and a significantly higher number of parasitoids left the banker box compared to *E. kuehniella*. Additionally, by using *G. mellonella* as a host instead of *E. kuehniella,* we eliminated the risk of contamination by accidental escape of host larvae.

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391

392

393 **Table 1.** Mean percentage of released *H. hebetor* adults (± SE) from the rearing box at
394 each sampling date and for each treatment.

	% *H. hebetor* released				
Treatment	**4 days**	**7 days**	**14 days**	**21days**	**25 days**
EK	55 ± 6	24 ± 5	20 ± 6	0.2	0.4
GM	42 ± 6	37 ± 4	19 ± 7	1	1 ± 1
GM2	52 ± 6	34 ± 6	12 ± 3	1 ± 1	0.3
Mean	50 ± 3a	32 ± 3ab	17 ± 3b	1c	1c

395 Mean values followed by the same letter are not significantly different ($P < 0.05$).

396

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399

400 **Figures**

401 **Fig. 1.** Mean number of *Habrobracon hebetor* adults (± SE) emerged from each
402 treatment: *E. kuehniella* fourth instar larvae (EK), *G. mellonella* fourth instar larvae (GM)
403 and *G. mellonella* fourth and second instars (GM2). Mean values followed by different
404 letter denote statistically significant differences ($P > 0.05$).

405

406 **Fig. 2.** Mean percentage of *H. hebetor* adults (± SE) that left the rearing box with each
407 treatment: *E. kuehniella* fourth instar larvae (EK), *G. mellonella* fourth instar larvae (GM)
408 and *G. mellonella* fourth and second instars (GM2). Different letters next to error bars
409 denote statistically significant differences ($P > 0.05$).

410

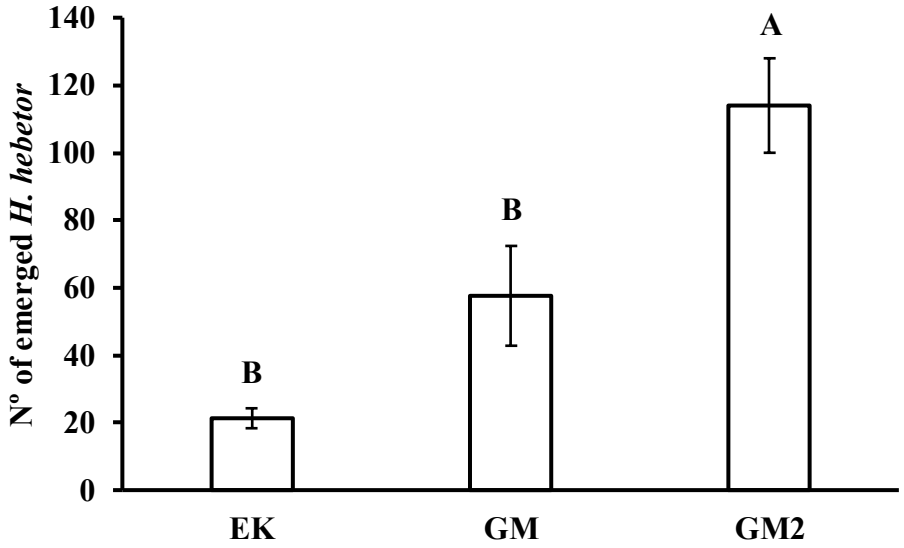
411 **Fig. 3.** Mean percentage of females (of the total emerged females) (± SE) leaving the
412 rearing box at each sampling date (3, 7 and 14 days after the emergence of adults started)
413 per treatment: *E. kuehniella* fourth instar larvae (EK), *G. mellonella* fourth instar larvae
414 (GM) and *G. mellonella* fourth and second instar larvae (GM2). Different letters above
415 error bars denote statistically significant differences between each treatment ($P > 0.05$).

416

417 **Fig. 4.** Mean percentage host mortality (± SE) in the rearing box (host profitability) with
418 each treatment: *E. kuehniella* fourth instar larvae (EK), *G. mellonella* fourth instar larvae
419 (GM) and *G. mellonella* fourth and second instars (GM2). Different letters above error
420 bars denote statistically significant differences ($P > 0.05$).

421

422 Figure 1.



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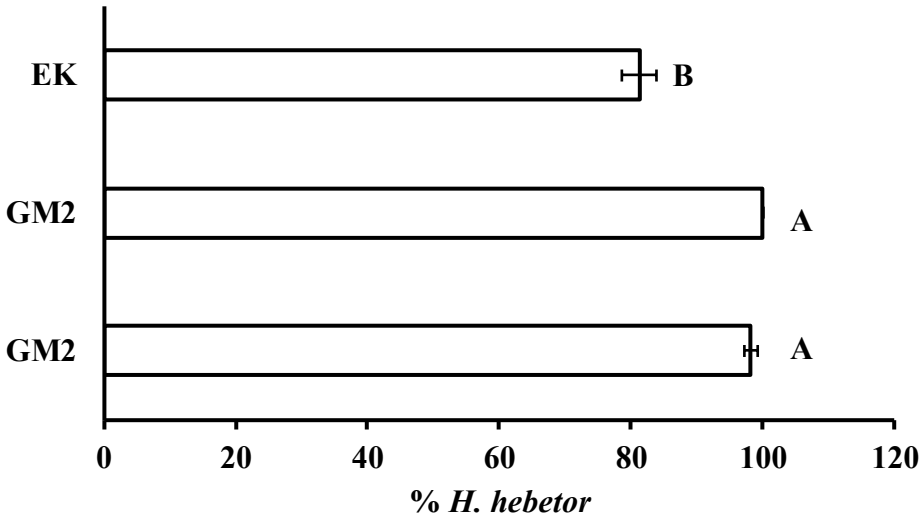
426 Figure 2.



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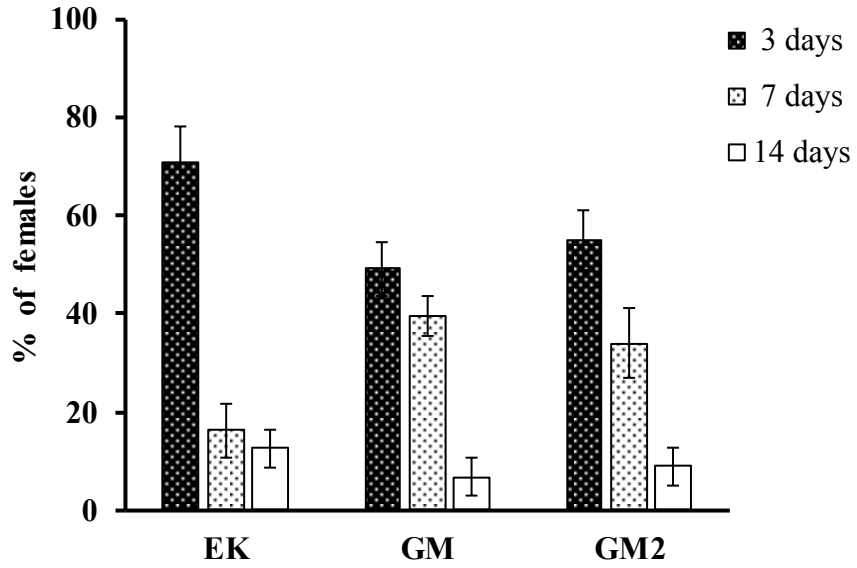
430 Figure 3.



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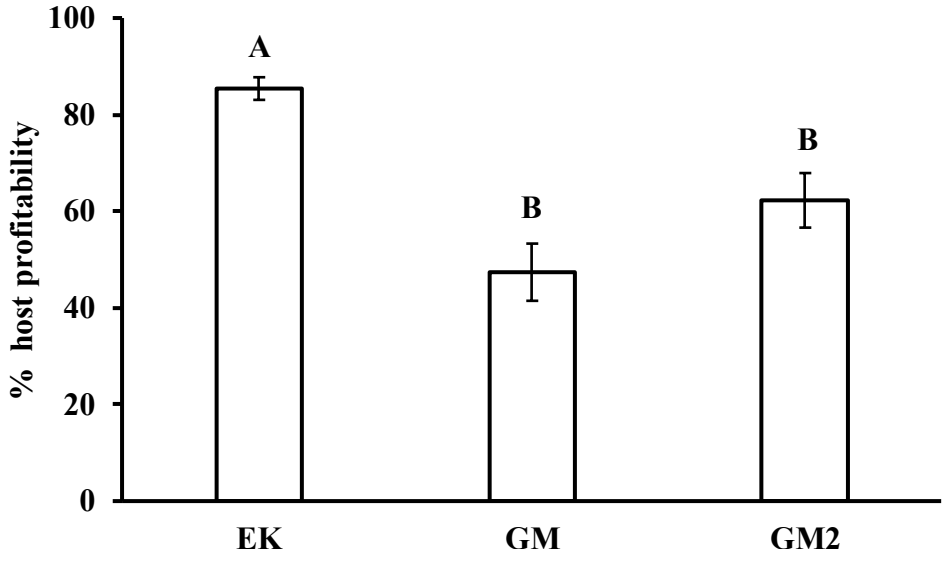
432 Figure 4.



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