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Investigation on endosymbionts of Mediterranean flour moth gut and studying their role in physiology and biology

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ABSTRACT

The bacterial flora of flour moth and their role in the physiology of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) were investigated. The gut flora of *E. kuehniella* was isolated on Luria Bertani Agar (LBA), followed by incubation at 28 °C respectively for 48–72 h. The isolates were identified using 16S rRNA sequencing. Altogether five bacterial species of different genera were identified as *Bacillus infantis*, *Streptomyces cacaoi*, *Janibacter* sp., *Stenotrophomonas* sp. and *Acinetobacter* sp. The fourth instar larvae reared on artificial diet without any antibiotic showed higher efficiency of conversion of digested food (ECD) and efficiency of conversion of ingested food (ECI) (16.068 ± 0.568% and 14.455 ± 0.448%), respectively. Approximate digestibility (AD) and Consumption index (CI) of the fourth instars larvae were higher on artificial diets containing tetracycline. The higher activity of alpha-amylase and general proteases were observed in the midgut of larvae reared on artificial diet containing antibiotic chloramphenicol. Total protein and lipid value were higher respectively in larvae that were reared on artificial diet containing streptomycin sulfate and artificial diet containing tetracycline. It seems that antibiotics had no significant effect on enzymatic activities of *E. kuehniella*. The larvae performed well in tetracycline treatment as per larval developmental duration compared with other treatments. Therefore, the gut bacteria do not seem to play a crucial role in the survival and development of the *E. kuehniella*. However, the question of adaptation of this pest species to hosts rich in protease inhibitors, such as flour has to be examined and ascertained.

1. Introduction

The Mediterranean flour moth, *Ephestia kuehniella*, is a serious cosmopolitan pest of stored products, especially flour (Brindley, 1930). The larvae cause serious losses both to the quantity and quality of stored products through feeding, webbing, and fecal matter (Hansen and Moran, 2011). The use of synthetic fumigants (e.g., phosphine and methyl bromide) has been the prevailing control strategy against pest infestation in stored products. However, insect resistance to phosphine is a serious problem, and in some countries control failures have been reported in field situations (Collins et al., 2002). Methyl bromide has been classified as an ozone depleter and therefore is being phased out (Rajendran and Sriranjini, 2008). Owing to unfavorable effects of these conventional fumigants, alternative pest control tactics are being developed (Ayvaz and Karaborklu, 2008).

Microbial diversity in the digestive system of caterpillars (Lepidoptera) has been a recent focus of attention using both traditional culturing and culture-independent techniques (Shannon et al., 2001; Sittenfeld et al., 2002; Broderick et al., 2004; Erturk and Demirbag, 2006). Insects gut contains bacteria that can produce some digestive enzymes and detoxifying enzymes (Genta et al., 2006), contribute to host-detoxification (Dillon and Dillon, 2004) and attribute resistance to pathogen invasion (Dillon and Charnley, 1991, 1995, 1996). There are reports on the presence of *Acinetobactor*, *Arthrobactor*, *Stenotrophomonas*, *Bacillus cereus*, *Planococous* and Bacillaceae bacteriums in the digestive tract of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Saraf et al., 2015).

The survey of literature showed that except a study on the effect of Wolbachia on reproductive and life-history traits in *E. kuehniella* (Sumida et al., 2017), there has been no study so far on gut symbionts and their probable role in *Ephestia kuehniella* development and physiology.

Many entomologists have thoroughly studied the development

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of new strategies based on the interaction of bacteria and insects (Indiragandhi et al., 2008a, 2008b). The nutritional contributions of the gut microbiota may increase host insect survival under sub-optimal dietary conditions. They may improve digestion efficiency and provide digestive enzymes or vitamins (Dillon and Dillon, 2004). Growth, development and reproduction of insects strongly depend on the quality and quantity of food eaten by insects (Scriber and Slansky, 1981). According to Bernays and Chapman (1994), chemical compounds found in food, affect survival, growth and reproduction of the insects. Protease inhibitors in plants are a part of proteins in plants that are considered as a defense mechanism against insects (Jouanin et al., 1998). Possible role of protease inhibitors in plant protection was investigated for the first time long ago (Mickel and Standish, 1947). These researchers observed that the larvae of some insects can grow on soybeans. Later it became clear that the presence of trypsin inhibitor in soybean, prevents *Tribolium castaneum* (H.) (Coleoptera: Tenebrionidae) from normal development on this plant (Lipke et al., 1954). Therefore, the insects that can still live and grow on the plants with these inhibitors have some mechanisms to overcome them (Chapman, 2013).

There is still inadequate information on the microorganisms that are associated with Lepidoptera (Indiragandhi et al., 2008a, 2008b; Thakur et al., 2016). Regarding the impact of antibiotics on nutritional and physiological aspects of the *E. kuehniella* as yet no information is available. Therefore, this study is a first report on bacterial flora in *E. kuehniella* and then we try to evaluate the effect of various antibiotics on physiological performance in this moth and pave the way for further investigation in manipulating this pest through digestion.

2. Materials and methods

2.1. Insect rearing

Ephestia kuehniella eggs were procured from Plant Pests and Disease Research Institute of Iran. They were transferred to a growth chamber set at 25 ± 1° C, 70 ± 5% relative humidity and 16 to 8 h L/D. After hatching from the egg, larvae were provided artificial diets (wheat flour 43 gr, yeast 6 gr, glycerine 20 ml).

2.2. Isolation of gut flora of Ephestia kuehniella (cultured bacterial)

The larvae were selected from the rearing jars. They were starved for 6 h, then sterilized by dipping into 75% ethanol for 2 min, and followed by rinsing twice with sterilized water on a clean bench. The whole gut of each larva was dissected and put into a sterile centrifuge tube (1.5 mL), and then 200 μL sterile phosphate-buffered saline (PBS) solution (pH 7.4) was added. Both the samples were streaked on media Luria Bertani Agar (Tryptone Bacteriological 10 gr, Yeast Extract 5 gr, NaCl 10 gr and Agar 15 gr) incubated at 28 °C for 48–72 h. Initial identification of strains was based on color, shape, gram-positive or negative, and biochemical tests. Isolates obtained on Luria Bertani Agar were purified by repeated sub culturing on fresh media (Visotto et al., 2009). Twenty five pure cultures were selected for preliminary tests.

2.3. DNA extraction and PCR amplification

In order to make the bacterial suspension, 25 μL Distilled water was poured into tube (200 μL capacity) and bacteria were transferred to it. The samples were subjected to boiling at 105 °C for 10 min, cooled on ice, and centrifuged at 10,000 × g for 10 s and then stored at −20 °C. Following centrifugation, the upper phase containing DNA about 3–5 μl. Aliquots of 2 μl of template DNA were used for PCR. Bacterial 16S rRNA universal primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT- 3′) were used to perform polymerase chain reaction (PCR) amplification (conditions: 93 °C for 5 min; 93 °C for 45 s, 55 °C for 45 s, 72 °C for 1min and 30 s, 34 cycles; and 72 °C for 7 min). The bacterial removal efficacy was detected by 1.5% agarose gel electrophoresis. To get an accurate result, PCR amplification and electrophoresis were conducted 5 times, although no variations detected.

2.4. Nutritional indices

Nutritional indices were determined using fourth instar larvae that was reared for one generation (from first instar to the end of third instar larva). Experiments were carried out for 3 days. A gravimetric technique was used to determine weight gain, food consumption, and the amount of feces produced. The fourth instar larvae were starved 6 h prior to the start of experiments to exude gut contents. Nutritional indices were measured on the dry weight basis. After measuring the weight of the larvae, they were introduced to artificial food, and the weights of the larvae were recorded before and after feeding until they stopped feeding. Food utilization rates were then calculated based on the following formulas of Waldbauer (1968):

Approximate digestibility (AD) = (E − F)/E × 100 (%)
Efficiency of conversion of ingested food (ECI) = P/E × 100 (%)
Efficiency of conversion of digested food (ECD) = P/(E − F) × 100 (%)
Relative growth rate (RGR) = P/TA (mg/mg. day)
Consumption index (CI) = E/A.

where: A = dry weight of the insect over unit time (mg),E = dry weight of food consumed (mg), F = dry weight offeces produced (mg), P = dry weight gain of insect (mg), T = the duration of the experimental period (day).

2.5. Biochemical assessments

2.5.1. Sample preparation

The fourth instar larvae were selected from those reared on diet containing antibiotics from first instar to the end of third instar larva and their guts were removed by dissection under a stereomicroscope in ice cold buffer (Saline buffer). Five larval guts were placed in 2 ml of distilled water or buffer related to each test and then samples were homogenized. The homogenates were centrifuged at 4 °C for 10 min. The resulting supernatants were transferred into new micro tubes and frozen at-20 °C until further use.

2.5.2. Assay of α-amylase activity

The α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955). Soluble starch (1%) (Merk, Darmstadt, Germany) was used as substrate. A 30 μL of the enzyme were incubated for 30 min at 35 °C with 80 μl universal buffer (glycine, mes (2-[morpholino] ethansulphonic acid), succinate, NaOH, double distilled water) and 50 μl soluble starch. In order to stop the reaction, 100 μl DNS was added and the mixture was heated in boiling water for 10 min. DNS is a color reagent and the reducing groups released from starch by α-amylase action were measured by the reduction of 3,5-dinitrosalicylic acid. All assays were performed in four replicates. Absorbance was measured at 545 nm after cooling in ice for 5 min.

2.5.3. Assay of α- and β-glucosidase avtivity

α- and β-glucosidase activities were assayed according to the method of Silva and Terra (1995) with slight modification. Assays