Concentrations and Diversity of Bacteria in the Caribbean Fruit Fly *Anastrepha suspensa*

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Abstract

The Caribbean Fruit Fly (*Anastrepha suspensa*) is an important economic pest of citrus in Florida. Interactions between *A. suspensa* and its microbiota may allow for novel control strategies, but little is known about the concentrations or types of resident bacteria in *A. suspensa*. This is the first study to enumerate and identify bacterial species present in *A. suspensa*. Pupae and adult female (mated) and male flies were surfaced sterilized in 70% ethanol, rinsed twice in sterile H20, and crushed. Fly homogenates were serially diluted in sterile saline, plated onto Brain Heart Infusion Agar with 0.5% yeast extract (BHIA-YE), and incubated for 72 h at 37□C. Based on counts of colony-forming units (CFU) obtained with BHIA-YE, bacterial concentrations in pupae, female flies and male flies averaged 7.8 x 10^3 CFU/mg (n = 13), 6.4 x 10^5 CFU/mg (n = 5), and 2.15 x 10^6 CFU/mg (n = 4) respectively. Five pupal isolates consisted of 4 Gram (-) bacilli and 1 Gram (+) coccus. Twenty adult female fly isolates included: 7 Gram (-) bacilli; 4 Gram (-) cocci; 3 Gram (+) bacilli; and 6 Gram (+) cocci. Most isolates were catalase (+) and oxidase (-). These results indicate adult *A. suspensa* flies harbor higher concentrations of bacteria than do pupae. Identification of isolates, using API 20E strips, found a common bacterial type, *Providencia sp.*, that resided in all female fly subjects.

Introduction

There are many different genre of flies that are now on record. There are 185 species alone that have been discovered in the genus *Anastrepha* (Martinez and Hernandez-Ortiz, 1997). Most of the studies done on *Anastrepha* have covered their anatomy and reproductive system of both males and females (Martinez and Hernandez-Ortiz, 1997). The association of bacteria with fruit flies has been known for nearly a century, but remains little understood to this day.

Communities of microorganisms found in insects are largely diverse, and many insect species have a large enough microbial load to far outnumber their own cells (Dillon and Dillon, 2004). The fitness and longevity of a host can be affected by bacteria inside the host (Brummel et al., 2004). In laboratory-reared insects some of the beneficial relationships between the bacteria and the host have been known to be intermittent or completely absent (Dillon and Dillon, 2004). The bacterial population in any animal can be altered drastically by their environment and diet (Brummel et al., 2004).

The Caribbean fruit fly, *Anastrepha suspensa*, belongs to the tephritid fruit fly family, which are important insects in agriculture because of the destruction of crops. There have not been many studies on the Caribbean fruit fly, which was introduced into Florida in 1965 and infested over 80 kinds of sub-tropical fruits such as cherries and citrus (Dodson, 1982).

There have been few studies done on the bacteria found inside of A. *suspensa* or any other species of *Anastrepha*. In a close relative, *Anastrepha luden (*Mexican Fruit Fly*)*, researchers identified and isolated 18 different bacterial species from the gut (Kuzina et. al., 2000). The majority of the investigations on the role of associated bacteria in fruit flies include studies on obligate symbiotic relationships in larval growth and development, larvae nutrition, and bacteria as an adult food source (Kuzina et. al., 2000). Determining the bacteria present within the fruit fly would be economically beneficial because this could lead to possible population control methods. Strategies for control may be obtained by studying the relationship between the fruit fly and its resident bacteria.

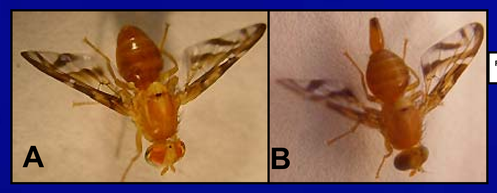

Fig.1. *A. suspensa*: male (**A**) and female (**B**)

Objectives

The objectives of the present study were to:

- optimize the culture medium for determining concentrations of viable, culturable microorganisms present in *A. suspensa;*
- determine and compare microbial concentrations present in pupae and non-virgin male and female adult flies *A. suspensa*; and
- identify resident microorganisms present in *A. suspensa*

Methods

Medium Optimization. To maximize colony counts, three different enumeration media (e.g., Brain Heart Infusion (BHI) Agar, Blood Agar, and Plate Count Agar) were tested.

Bacterial Enumeration. *A. suspensa* pupae were obtained from mass-rearing facility in Florida. 24 hours after emergence, flies were separated by sex. Upon reaching sexual maturity males and females were allowed to copulate for 10 min; following copulation, flies were held in a -70ºC freezer until processed.

Each fly was transferred aseptically to a sterile tea strainer using sterile forceps. Flies in the tea strainer were processed as follows: 1) immersion and agitation in 70% ethanol for 2 min; 2) a 30-sec wash (immersion and agitation) in sterile DRO water; and 3) a second 30-sec wash in sterilized DRO water. The surface sterilized fly was then transferred to a sterile 1.5 mL tube, and 1.0 mL of 0.9% NaCl (saline) was added. The fly was then macerated for 5 min using a sterile pestle. Another 0.5 mL of saline was added, and the contents vortexed for 20 sec to ensure a homogenous fly puree.

Dilutions were performed by aseptically transferring 1.0 mL of the fly puree into a 9 mL of sterile saline. After vortexing for 20 sec, serial 1:10 dilutions were performed using 9-mL saline dilution blanks. Following serial dilutions, 0.1 mL from a dilution was aseptically transferred to a enumeration medium and spread evenly over the entire agar surface using a sterile glass hockey stick. For each dilution plated, triplicate plates were inoculated. Following inoculation, plates were incubated at 37ºC for 72 hours and the number of colonies counted.

Identification of Isolates. Preliminary identification was to initiated by choosing colonies that differed morphologically and isolating the organisms. 20 isolates were vobtained from the non-virgin female flies. For all 20 isolates the following tests were performed: 1) determination of Gram reaction via Gram staining, 2) catalase test to detect presence of catalase enzyme , 3) oxidase test to detect presence of cytochrome oxidase enzyme.

The identification process was continued by streaking isolates onto BHI plates and incubating these plates for 24 hrs at 37ºC. One colony from each plate was transferred to 9.0 mL of sterile saline and mixed thoroughly until the solution was slightly cloudy. Using a sterile pipette, the solution was transferred into each tubule of an API 20E identification strip; strips were incubated at 37ºC for 24 hrs. After incubation, each tubule was assayed according to standard APIC protocols (Biomérieux, France) and the isolates identified.

Results

- In initial studies to determine which enumeration medium produced the greatest colony counts, it was found that Brain Heart Infusion (BHI) media produced the highest viable counts (**Fig. 2**).
- Enumerations were used for the comparison of the microbial loads in the male flies, female flies and pupae. It was found that male flies had the largest microbial load, and the pupae had the smallest microbial load (**Fig. 3**). The average weights of the male flies, female flies, and pupae were 9.0, 12,, and 9.0 mg, respectively.

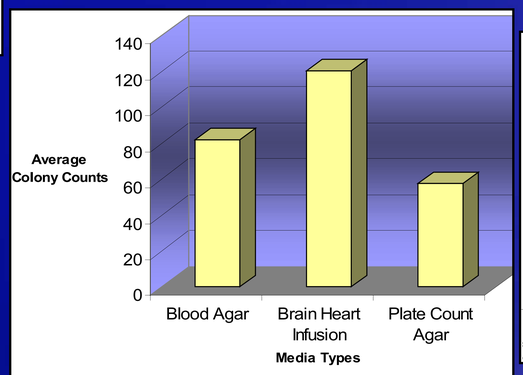

Figure 2: The average colony counts are shown for the three types of media that were tested. These results were obtained using female flies 4 and 5.

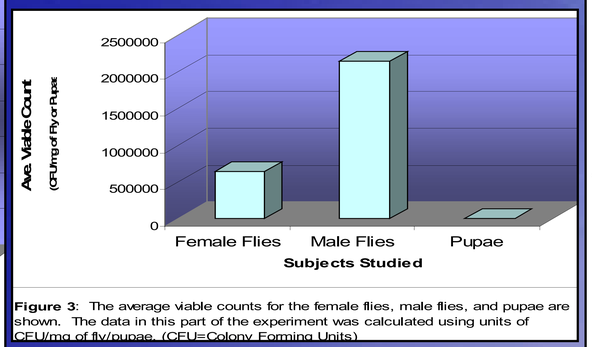

Figure 3: The average viable counts for the female flies, male flies, and pupae are shown. The data in this part of the experiment was calculated using units of CFU/mg of fly/pupae. (CFU=Colony Forming Units)

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- Preliminary identification performed on isolates that were obtained from the enumeration plates were gram stains, catalase and oxidase tests.
  - Gram stains showed five pupal isolates consisted of 4 Gram (-) bacilli and 1 Gram (+) coccus, and twenty adult female fly isolates included: 7 Gram (-) bacilli; 4 Gram (-), cocci; 3 Gram(+) bacilli; and 6 Gram (+) cocci (**Fig 4**).
  - Catalase test results showed 18 catalase (+) and 1 catalase (-) from female fly isolates, and 5 catalase (+) pupae isolates.
  - Oxidase test results showed 18 oxidase (-) and 1 oxidase (+) from the female fly isolates, and 4 oxidase (-) and 1 oxidase (+) pupae isolates.
- Using API strips (**Fig. 5**), identifications were obtained for 11 of the 20 isolates collected. The results obtained can be found in **Table 1.**

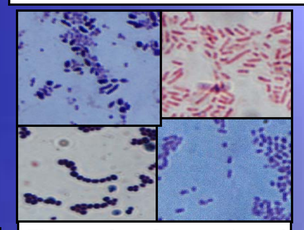

Figure 4: Gram stains of bacterial isolates from flies.

Table 1: Identification results from API 20 E

The Fly # is read (media type/sex of fly and fly the plate) number/the plate it came from/colony number from

Fly #	Identification
BH-Female(1)-P3-C1	*Providencia alcalifaciens Providencia stuartii*
BH-Female(2)-P3-C2	*Providencia alcalifaciens Providencia stuartii*
BH-Female(3)-P3-C1	*Providencia alcalifaciens Providencia stuartii*
BH-Female(3)-P3-C2	*Providencia alcalifaciens Providencia stuartii*
BH-Female(4)-P1-C1	*Providencia alcalifaciens Providencia stuartii*
BH-Female(5)-P3-C1	*Providencia alcalifaciens Providencia stuartii*
BH-Female(5)-P3-C2	*Klebsiella oxytoca Enter. agglom. IND+*
PCA-Female(5)-P2-C1	*Klebsiella oxytoca Enter. agglom. IND+*
PCA-Female(4)-P1-C2	*Enter. Aerogenes Serratia liquefaciens*
BH-Female(4)-P2-C1	*Enter. aerogenes Serratia liquefaciens*
BH-Female(3)-P3-C2	*Ent. agglum. IND(-) Ent. amnigenus 1 Ent. intermedium*

Figure 5: API-based identification of bacterial isolates from flies. Image **A** shows an unused API strip, **B** is a used API strip from the most common bacterial type found, and **C** shows the colony morphology, on a streak plate.

Summary

- Enumeration studies showed that male flies contained the highest concentrations of viable, culturable microbes. Pupae compared to the male and female flies had significantly fewer bacteria.
- Of the 15 isolates, only 11 could be identified using the API 20E system. The most common bacteria isolated were *Providencia alcalifaciens/Providencia stuartii*, which was found in all female flies processed. In order to distinguish between the species of *Providencia*, more tests are needed. In a study on the Mexican Fruit Fly, *Anastrepha luden*, 18 different bacterial species were identified: *Enterobacter*, *Providencia*, *Serratia*, and *Staphylococcus* were the most common genera (Kuzina et. al., 2000).
- Currently, the Florida Department of Agriculture and Consumer Services, has been using many strategies to manage and control the Caribbean fruit fly (Weems Jr. H.V. and Heppner J.B., 2001). One control method used has been the spraying of groves and adjacent areas with pesticide bait. The studies that once emphasized the insect-bacteria symbiosis are now geared towards the insect-microbial pathogen relationship, to assist in production of microbial insecticides (Dillon R.J. and Dillon V.M., 2004).
- The identification of the bacteria that reside in the Caribbean fruit fly provides insight into the significance of microorganisms in the life of the fruit fly. This knowledge may prove important to creating a biological control method of eradication of fruit fly pests.