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Identification of Gut Bacterial Community and Their Effect on the Fecundity of Pumpkin Fly, *Bactrocera tau* (Walker)

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ABSTRACT

The bacteria in fruit flies alimentary tract have been known in advantages in their biology of the insect hosts. Different biochemical, gram reaction and motility tests was performed to identify the mid-gut bacterial community of laboratory host reared pumpkin fly, *Bactrocera tau* (Walker) (Diptera:Tephritidae). Colony characterization of the isolated bacteria was conducted on nutrient agar and MacConey agar plates. Isolated gut-bacterial species viz., *Proteus rettgeri* and *Klebsiella oxytoca* was examined by incorporated with protein (casein:yeast extract:sugar, 1:1:2) and sugar diet to study the effect of bacteria supplemented diets on the ovariole number and egg production of *B. tau*. Eight genera and nine bacterial species were identified under the family Enterobacteriaceae. The identified bacterial genera were *Proteus*, *Klebsiella*, *Streptobacillus*, *Alcaligenes*, *Haemophilus*, *Erwinia*, *Chromobacterium* and *Flavobacterium*. The mean ovariole number per ovary was recorded as 20.66±2.51, 20.56±3.53 and 22.41±3.75 for *B. tau* fed on *P. rettgeri*, *K. oxytoca* incorporated protein diets and only protein diet, respectively. Experimental result revealed no significant influence of gut bacteria added adult diets on egg/female/day of *B. tau* fed on above mentioned diet treatments.

Key words: *Bactrocera tau*, gut bacteria, probiotic, ovariole number, fecundity

INTRODUCTION

Insects form an extremely large group of animals and bear a consequently large variety of associated microbes (Jurkevitch, 2011). Many insects species harvour microbial communities in their digestive system (Dillon and Dillon, 2004). Insect symbionts have been broadly categorized into two main types: primary and secondary symbionts. In many cases, the relationship is mutualistic and the bacteria contribute to the fitness of their insect host (Baumann, 2005; Bourtzis and Miller, 2003; Dillon and Dillon, 2004). Bacterial contribution to fitness, particularly in insects that rely on inadequate food source, is frequently nutritional. These microorganisms may provide certain amino acids (Nogge, 1981), essential vitamins (Douglas, 1998), nitrogen and carbon compounds (Benemann, 1973; Dillon and Dillon, 2004). A significant proportion of this bacterial community was found to actively fix nitrogen within the gut of live adults (Behar *et al*., 2005) and these could be supplementary their host diet with available nitrogen. De Vries *et al*. (2004) reported that the gut microbiota may have alternated between mutualism/commensalism and parasitism in response to changes in their host's diet. Removal of bacteria affects measurable physiological and

behavioural parameters related to fly fitness (Ben-Yosef *et al*., 2008) and conversely, inculcation of original bacteria in the diet contributes to a longer life span (Behar *et al*., 2008b) and enhanced mating competitiveness (Ben Ami *et al*., 2010). Some bacteria also provide resistance against natural enemies and parasites (Oliver *et al*., 2003), promote host immunity (Muniz *et al*., 2006) and enhance social interactions (Dillon *et al*., 2002).

Tephritidae is a large family that includes many fruit pests and these are usually adopted for housing large quantities of bacteria in their digestive tract. The Petri (1909, 1910) described one of the first bacterial symbiotic associations in an insect species, the olive fly, *Bactrocera* (*Dacus*) *oleae* (Rossi). In the genus *Bactrocera* adult fruit flies use a combination of their fluid-centered mode of feeding and their labellar filtering mechanism to feed on fruit juices, leachates and bacteria (Enterobacteriaceae) which constitute their primary source of food in nature (Vijaysegaran *et al*., 1997). The Queensland fruit fly, *Bactrocera* (*Dacus*) *tryoni* (Froggatt) and *Dacus cacuminatus* (Hering), regurgitated and reingested alimentary canal contents which included bacteria (Drew and Lloyd, 1987). Explorations on different fruit fly's associated bacterial community revealed that most of fly's gut microbiota is dominated largely by free-living bacteria of the Enterobacteriaceae, notably by species of *Enterobacter*, *Klebsiella* and *Pectobacterium* (Behar *et al*., 2008b; Daser and Brandl, 1992; Drew and Lloyd, 1987). These components of the bacterial community remain stable throughout the fly's life cycle and between geographical regions and some are inherited vertically (Aharon *et al*., 2013; Behar *et al*., 2008a; Sood and Nath, 2002).

Knowing the intestinal bacteria is important in the context of developing our understanding of symbiotic relationships, multitrophic interactions between insects and plant or animal host and in the developing new strategies for controlling insect pests (Dillon and Dillon, 2004). Behar *et al*. (2008b) reported that the Enterobacteriaceae community within the med fly's (*Ceratitis capitata,* Wiedemann) gut have an indirect contribution to host fitness by preventing the establishment or proliferation of pathogenic bacteria. Hamden *et al*. (2013) proved that the addition of beneficial bacteria (*Klebsiella pneumonia, Enterobacter* spp. and *Citobacter freundii*) to the larvae's diet of *C. capitata* lead to a significant increase in the number of Enterobacteriacae communities inhabiting the sterile male's gut and a subsequent significant increase in the size of males and other morphometric traits and enhanced sexual performance of males at emergence. Community and functional analyses showed that the microbiota of med fly and olive fruit flies contribute to their diet and affect host fitness parameters. The analysis of the microbiota's community structure of mass-reared, sterilized medfly males used in the sterile insect technique revealed a strong reduction in *Klebsiella* spp. compared with non-sterile and wild flies (Yuval *et al*., 2013). Inoculation of sterile males with the gut population affected female mating behviour as they preferentially mated with inoculated versus non-inoculated males. The studies suggested that control can be significantly improved by manipulating symbionts in pest animals.

The Pumpkin fly, *Bactrocera tau* (Walker) (Diptera:Tephritidae) is a polyphagous insect that seriously damages fruit production worldwide including Bangladesh. Females oviposit in unripe fruits, within which the larvae develop, leading to fruit degradation and crop losses. Infestation can lead to total crop failure and to quarantine restrictions. The control strategies remain almost exclusively based on insecticides, despite the awareness of a need for the use of more environment friendly control methods. Although, much research has already been done on the biology (Singh *et al*., 2010), host susceptibility and behaviour (Khan *et al*., 2011) and genetics/molecular phylogeny (Jamnongluk *et al*., 2003) of *B. tau,* yet relatively little is known of its microbial community (Prabhakar *et al*., 2009, 2013; Sood and Nath, 2002, 2005; Sood and Prabhakar, 2009) and their possible impact on fly fitness parameters.

The present study was therefore undertaken to identify the gut bacterial community of laboratory host reared female *B. tau* using different morphological, gram reaction and biochemical tests. The effect of gut bacteria incorporated adult diets (protein and sugar) on the ovariole number and fecundity of *B. tau* was also examined under controlled laboratory condition.

MATERIALS AND METHODS

Insect rearing: Adult *B. tau* culture originated in 2010 from infested sponge gourd, *Luffa cylindsica* (L.) collected from Atomic Energy Research Establishment (AERE) campus. Rearing of *B. tau* was maintained in the Laboratory of Insect Biotechnology Division, Institute of Food and Radiation Biology (IFRB), AERE, Savar, Dhaka, Bangladesh for more than 40 generations using natural hosts. About 3,000 adult flies were maintained in steel framed cages (76.2×66×76.2 cm) covered with wired net. The flies were supplied with protein based diets both in the liquid and dry form viz., (1) Baking yeast: sugar: water at 1:3:4 ratio and (2) Casein: yeast extract: sugar at 1:1:2 ratio. Water was supplied in a conical flask socked with cotton ball. The temperature and the relative humidity of the rearing room maintained at 27±1°C and 75±5% and 14:10 h dark and light cycle.

Isolation and identification of gut bacteria: The gut bacteria of *B. tau* were isolated and identified according to standard method (Cowan, 1981; Holt *et al*., 1994). Ten days old female *B. tau* was collected from laboratory host (sweet gourd) reared population. Flies were dipped in 75% ethanol for disinfection and washed in sterile saline buffer (PBS) prior to dissection. The flies were then cold anaesthetized at 4°C in a refrigerator for 10 min prior to dissection. Gut of the flies was dissected aseptically under laminar flow hood and mid-gut were collected in sterile 4 mL vial containing 2 mL of normal physiological saline. The collected guts were opened with forceps and homogenized with vortex. Homogenized suspensions were serially diluted up to 10^{-2} CFU mL^{-1}. Approximately 100 µL of diluted suspensions were inoculated onto nutrient agar (Oxoid, UK) and MacConey agar by spread plate technique and incubated at 37°C for 24-48 h. On the basis of different colony characteristics about 20 representative colonies were selected for identification. Selected colonies were purified as pure culture through repeated subculturing onto nutrient agar plate and stored at 4-8°C. Gram staining and microscopy were performed to study the morphology of the collected isolates. Isolates were then tested for different biochemical characteristics e.g., sugar fermentation, catalase, oxidate, methyl red, voges proskauer, indol production, citrate utilization etc. Based on overall cultural, morphological and biochemical characteristics, isolated bacteria were identified as per Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Among identified gut bacterial species, *Proteus rettgeri* and *Klebsiella oxytoca* were randomly selected to observe their impact on the ovariole number and fecundity i.e., egg/female/day of *B. tau* fed on bacteria incorporated adult diets.

Preparation of bacteria incorporated of adult diets: Two different gut bacteria viz., *P. rettgeri* and *K. oxytoca* were sub cultured on nutrient agar from maintenance media and then inoculated in a 250 mL conical flask containing 100 mL nutrient broth and inocubated at 37°C for overnight. Cell suspension was centrifuged at 6000 rpm for 10 min and washed twice with 0.01 M sodium phosphate buffer. Finally the cell pellets were suspended with 0.8% NaCl solution and aseptically transferred in test tubes. Cell suspensions were then mixed with protein diet for feeding newly emerged *B. tau*. In the present study newly emerged *B. tau* were given access to six diet treatments viz., (i) Only protein diet (yeast extract:casin:sugar, 1:1:2), (ii) Isolates of *K. oxytoca* and protein diet

(3.8×10^{-6} CFU g^{-1}), (iii) Isolates of *P. rettgeri* and protein diet (3.8×10^{-6} CFU g^{-1}), (iv) Only sugar diet (20% sugar solution), (v) Isolates of *K. oxytoca* and sugar diet (3.8×10^{-6} CFU mL^{-1}) and (vi) Isolates of *P. rettgeri* and sugar diet (3.8×10^{-6} CFU mL^{-1}).

Determination of ovariole number of *B. tau* fed on bacteria added protein and sugar diets: Newly emerged 30 male and 30 female adult *B. tau* were housed in small rearing cages (8×6×12 cm) and supplied with diet treatments mentioned above. Three replicates were maintained for each diet treatment. On day 14 of adult emergence females were collected in small vials (5 mL) and kept in refrigerator for 10 min. Both the ovary of *B. tau* was dissected under stereo microscope and total number of eggs per ovary were counted and recorded. The ovariole development of only sugar fed and bacteria added sugar fed 14 days old adult flies was also determined. Usually, in poly-phagous tephritid fruit flies the paired ovaries consist of about 30-40 polytrophic ovarioles (Fitt, 1990). In mature flies, there is normally only one mature oocyte (egg with shell) per ovariole that is ready for laying, although there may be oocytes in various earlier stag.

Determination of egg per female per day: Three sets of 50 pupae of *B. tau* were collected from stock culture and placed in 55 mm plastic Petri dishes. Each Petri dish was then placed individually inside three small rearing cages and provided with three different types of adult diets viz., only protein, *P. rettgeri*+protein and *K. oxytoca*+protein diet. A plastic container of water with cotton wick was provided inside each cage. Fourteen days after adult emergence, ten male and female *B. tau* from each rearing cage was again placed into small rearing cages (6×6×10 cm). The flies were provided with three types of diets as mentioned above and water was supplied via a cotton wick inserted into a plastic vial (5 mL). Plastic egging receptacles smeared with sweet gourd paste were placed inside the cage for egg collection for 24 h. Eggs were collected for 5 days. Fecundity was determined by counting total number of eggs produced by ten pairs of *B. tau* and divided by 5 days to determine mean eggs per female per day. The experiment was repeated for three times.

Statistical analysis: Data for the ovariole number and fecundity of *B. tau* fed on different bacteria treated diets were analyzed using Analysis of Variance (ANOVA) and Tukey's family error rate was performed using Statistical Software-Minitab USA (version-15). Graphs were created in Microsoft Excel 2007.

RESULTS

Gut bacterial community isolated and identified from ten days old adult female *B. tau* shown in Table 1. Bacterial species identified were *Proteus rettgeri*, *Proteus vulgaris*, *Klebsiella oxytoca*, *Streptobacillus moniliformis*, *Alcaligenes faecalis*, *Haemophilus ducreyi*, *Erwinia herbiloca*, *Chromobacterium lividum* and *Flavobacterium picketti*. All bacterial species belongs to the family *Enterobacteriaceae*. Total viable counts in the gut of *B. tau* were 10^{-5} CFU mL^{-1}.

The influence of bacteria supplemented adult diets on the ovariole number of *B. tau* presented in Fig. 1. No significant differences ($p>0.05$) were recorded among ovariole numbers of *B. tau* fed on *P. rettgeri* and *K. oxytoca* added protein diets and only protein diet. Mean ovariole number were 20.66±2.51, 20.56±3.53 and 22.41±3.75 per ovary of *B. tau* fed on *P. rettgeri*, *K. oxytoca* incorporated protein diets and only protein diet, respectively. Individually ovariole number ranged from 7-34 per ovary depending on diet treatments of adult *B. tau*. No ovariole development was recorded for only sugar and bacteria added sugar fed 14 days old *B. tau* in the present study.

Table 1: Gut bacterial community of adult female *B. tau*

Fruit Fly	Bacterial species
B. tau	*Alcaligenes faecalis*
	Chromobacterium lividum
	Flavobacterium picketti
	Erwinia herbiloca
	Klebsiella oxytoca
	Haemophilus ducreyi
	Proteus rettgeri
	Proteus vulgaris
	Streptobacillus moniliformis

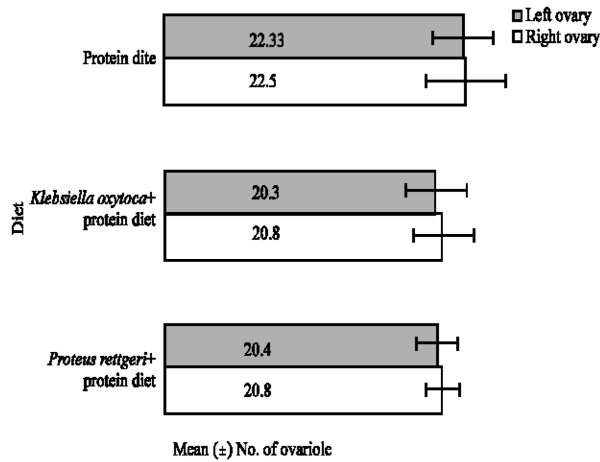

Fig. 1: Mean (±) No. of mature ovariloes of 14 days old *B. tau* fed on *P. rettgeri, K. oxytoca* added protein diets and only protein diets, respectively

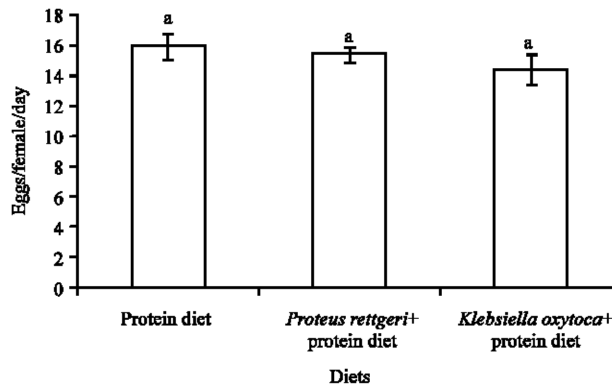

Fig. 2: Egg/female/day of *B. tau* fed on *P. rettgeri, K. oxytoca* added protein diets and only protein diet, respectively. Bars with same letter did not differ significantly ($p>0.05$)

The effect of bacteria added adult diets on the fecundity of *B. tau* shown in Fig. 2. Total egg production per female *B. tau* per day was 16±0.9, 15.5±0.5 and 14.5±1 in number for those fed on only protein, *P. rettgeri*+protein and *K. oxytoca*+protein diets, respectively and did not differ significantly ($p>0.05$).

DISCUSSION

The bacteria associated with different species of Tephritidae have been studied by several authors (Behar *et al.*, 2008a, b; Prabhakar *et al.*, 2013; Sacchetti *et al.*, 2008; Sood and Nath, 2002; Wang *et al.*, 2011). Several bacterial genera viz., *Proteous*, *Klebsiella* identified from gut of *B. tau* in the present study (Table 1) were quiet common for other Tephtriitd fruit flies like, *Bactrocera dorsalis* (Hendel), *B. tryoni*, *C. capiata* (Behar *et al.*, 2008a, b; Thaochan *et al.*, 2009, 2010; Wang *et al.*, 2011). Lauzon (2003) stated that the gut microbiota of these flies is relatively conserved in terms of species composition and comprised mainly various Enterobacteriaceae. Daser and Brandl (1992) also isolated and characterized the gut bacteria from adults of five fruit fly species and noted most of the bacterial strains were Enterobacteria. The uniformity across genera in morphology and in the composition of the microbiota suggested that bacteria are intimately associated with the life cycle of these flies (Ben-Yosef *et al.*, 2008). Using molecular, biochemical and 16S rDNA (rrs gene) analysis (Prabhakar *et al.*, 2009) characterize three bacterial symbionts of *B. tau* viz., *K. oxytoca*, *Pantoea agglomerans* and *Staphylococcus* sp. to determine their taxonomic position. Sood and Nath (2005) reported that antibiotic resistant strains of *Pseudomonas putida* (fruit fly symbiont), *Bacillus subtilis* (fruit fly pathogen) and *Escheichia coli* (non associated strain) could colonize in the gut of *B. tau*. Prabhakar *et al.* (2013) characterized five bacterial spesies of *B. tau* as *Delftia acidovorans*, *Pseudomonas putida*, *Flavobacterium* sp., *Defluvibacter* sp. and *Ochrobactrum* sp. of which four bacterial isolates viz., *Delftia acidovorans*, *Flavobacterium sp.*, *Defluvibacter* sp. and *Ochrobactrum* sp. are reported new records from guts of *B. tau*. Out of eleven types of bacteria isolated from *B. tau* and *B. cucurbitae* infesting cucurbits five were noted common to both the species, viz., *Pseudomonas putida*, *Erwinia herbicola* (*Pantoea agglomerans*), *Cedacea davisae* (*Cedecea davisae*), *Arthrobacter* sp. and *Xanthomonas maltophilia* (*Stenotrophomonas maltophilia*) (Sood and Nath, 2002).

In the present study gut bacteria of natural host (sweet gourd) reared female *B. tau* was identified by conventional morphological and biochemical tests. We revealed the presence of eight bacterial genera among which four viz., *Streptobacillus*, *Alcaligenes*, *Haemophilus* and *Chromobacterium* were different from those reported by earlier mentioned authours worked on gut bacteria of *B. tau*. It appears that bacterial assemblages in the gut of adult tephritids can be varied and inconsistence (Murphy *et al.*, 1994). For instance *K. oxytoca* and *K. pneumonae* have been found in both wild and laboratory *B. tryoni* but not but (so far) together (Drew and Lloyd, 1987; Fitt and O'Brien, 1985). Different environmental conditions and food supply could influence the diversity of the harboured bacterial communities and increase community variations of most of the bacteria species (Behar *et al.*, 2008c; Sood and Nath, 2002; Wang *et al.*, 2011).

In many Tephritid species proteinaceous component is required in the diet for sexual maturation and oogenesis of adult female fly (Carey *et al.*, 2000; Drew and Lloyd, 1987; Meats and Leighton, 2004). Drew *et al.* (1983) established that bacteria of the gram negative family Enterobacteriaceae could serve as an attractant and proteinaceous food for adult *B. tyoni* from a long term laboratory culture. Diets of bacteria, sugar and water gave equal longevity and increased fecundity in *B. tryoni* compared with the conventional diet of autolyzed brewer's yeast, sugar and water. In the present study *B. tau* fed on *P. rettgeri* and *K. oxytoca* added protein diet and only protein diet did not show significant influence of bacteria on mean ovariole number (Fig. 1). The present findings is partially in agreement with the findings of Meats *et al.* (2009) who noted that *B. tryoni* could not produce eggs or mature oocytes on a bacterial diet above the level attained with access to culture medium without bacteria. The present findings also in agreement with the

findings of Halder *et al.* (2013) who reported that bacteria added protein diet had no effect on ovariole number of *B. tau*. However the authors used exogenous bacteria species e.g., *E. coli* and *Lactobacillus lactis* while we used original gut bacteria of *B. tau*, *K. oxytoca* and *P. rettgeri* as probiotics. Bacteria had no effect on the ovarian development and egg production also revealed by several observations. Howard and Bush (1989) noted that the absence of the bacterium did not positively or negatively affect most components of fitness of *Rhagoletis pomonella* (Walsh) and *R. suavis* (Loew) and suggested that larvae of *Rhagoletis* do not depend on gut microflora to provide essential nutrients to detoxify plant secondary components. The bacterial community in the sand fly (*Lutzomiya longipalpis*) larval habitat affects oviposition and larval development, although bacteria are not essential for successful development of *L. longipalpis* (Peterkova-Koci *et al.*, 2012). Ben-Yosef *et al.* (2010) evaluate the presence of bacteria in female olive flies and monitored fecundity-an indirect measure of fitness. The authors reported that bacteria did not affect fecundity when females were fed a nutritionally poor diet of sucrose, or a protein-rich, nutritionally complete diet. However, when females were fed a diet containing non-essential amino acids as the sole source of amino nitrogen, egg production was significantly enhanced in the presence of bacteria. Niyazi *et al.* (2004) reported a significant benefit of probiotic postteneral diets on aspects of behavioural ecology in sterile male *C. capitata*. The inconsistency among the results of different investigations by different authours may be due to differences of the effects of different bacterial species or different strains of the same species on different insect hosts.

The present experimental result on egg/female/day of *B. tau* fed on different bacteria added adult diets and only protein diet partially in agreement with the findings of Singh *et al.* (2010) who reported *B. tau* lay an average 16 eggs day^{-1}. Addition of bacteria in protein diet did not show any effect on the fecundity of *B. tau* in the present trail (Fig. 2). Hendrichs *et al.* (2010) reported that egg development was not sustained by host foliage leachate, Bird droppings, ahid honeydew and to a lesser extend hawthorn fruit leachate, contributed to moderate fly fecundity, whereas preparation of leaf surface bacteria, pollen, insect frass and uric acid did not support significant egg development of *R. pomonella*. Fecundity was greatest where flies were exposed to enzymatic yeast hydrolysate. In our study we also recorded higher number of egg/female/day of fly fed on protein diet but almost similar from those fed on bacteria added protein diet. Bacteria added sugar diet fed fly produce no egg indicate that bacteria had no significant effect on the egg production *of B. tau* and in agreement with the observation of Ben-Yosef *et al.* (2008) who noted that female *C. capitata* feeding on full diet produce significantly more eggs than females on the sugar diet, but the presence of bacteria does not affect numbers of egg produced.

In conclusion, in the present study, a bacterial community composed of *P. rettgeri*, *P. vulgaris*, *K. oxytoca*, *S. moniliformis*, *A. faecalis*, *H. ducreyi*, *E. herbiloca*, *C. lividum* and *F. picketti* was revealed in the female gut of *B. tau*. Enterobacteriaceae constituted the dominant population. Use of selected gut bacteria, *P. rettgeri* and *K. oxytoca* in protein diet did not exert significant influence on the ovariole number and egg/female/day of *B. tau*. Further investigation on the mating competitiveness, longevity and attractancy of *B. tau* to different bacteria supplemented adult diets can be evaluated in both laboratory and semi field-cage trials and may lead to new target for management of this pestiferous fruit fly. Moreover, studies related to approaches as comparative genomics and real-time PCR can be performed to know the molecular mechanisms that underpin symbiont-host interactions.

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