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Full Length Research Paper

Distribution and characterization of microbial communities in *Chrysoperla zastrowi sillemi*, an important predator of sap sucking insect pests

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Common green lacewing, *Chrysoperla zastrowi sillemi* is one of the important biological control agents and is used effectively to manage various insect pests. Chrysopid predators are found to harbor many endosymbiotic yeasts and bacteria. Keeping this in view, a study on the distribution of yeast and bacteria in the adult diverticulum and larval gut was conducted using transmission electron microscopy (TEM) and molecular techniques. TEM showed the presence of a load of bacterial cells towards the periphery of inner side of the epithelial lining and the dividing bacterial cells in the larval gut. Numerous oval and kidney shaped yeast fauna were found to be distributed within the lumen and diverticular folds of the diverticulum of adult. Our study reveals the presence and distribution of yeast and bacterial cells from the adult diverticulum and gut of larva. Microbial isolates were identified by sequencing 16S rRNA gene for bacteria and ITS region of yeast including partial rRNA genes ITS-1 and partial 5.8S rRNA gene for yeast, respectively which revealed the presence of yeast isolates namely *Kodamaea ohmeri*, *Torulaspora delbrueckii, Wickerhamomyces anomalus* and bacterial isolates namely: *Enterobacter hormaechei*, *E. cloacae* and *Enterobacter* sp. as most common from adult and larvae, respectively.

Key words: Diverticulum, diverticular folds, epithelial lining, lumen, yeast, bacteria.

INTRODUCTION

Lacewings are one of the important biological control agents that are used effectively to manage various insect pests especially sucking pests in different agro-ecosystems (Cannard et al., 1984; Carvalho et al., 2002; Symondron et al., 2002; Venkatesan et al., 2009; Henry et al., 2010). It has long been considered as a promising candidate for pest management worldwide due to its wide prey range and geographical distribution, resistant to insecticides, voracious larval feeding capability as well as ensured commercial availability (New 1975; Tauber et al., 2000; Mc Ewen et al., 2001; Medina et al., 2003; Pathan et al., 2008; Sayyed et al., 2010). Adult lacewings feed solely on nectar, pollen and plant secretions containing sugar (Hagen, 1950; Hassan, 1974; Freistas, 2002) although a few are predatory (Coppel and Mertins, 1977). There is growing interest to understand microbial diversity like symbiotic yeast and bacteria because yeasts are known to provide amino acids, vitamins, degradation of xenobiotic compounds, play a role in host finding and fermentation of food (Dowd, 1989, 1991; Vega and Dowd, 2005; Peter et al., 2012). Symbiotic bacteria are known to fix the atmospheric nitrogen as well

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synthesize other essential nutrients (Dillon and Dillon, 2004; Nardi et al., 2002; Lilburn et al., 2001; Breznak, 2000) and enhancing the internal defence mechanism against toxic compounds. In this regard the chrysopid predators are also found to harbor many endosymbiotic yeasts like symbionts in adults which may provide essential amino acids that are normally absent in their diet (Hagen and Tassan, 1966, 1972). Hagen et al. (1970) identified the yeasts *Torulopsis* sp. in *Chrysopa carnea*, Nguyen et al. (2007) isolated five novel *Candida* sp. from Neuropteran, some of the yeasts like *Metschnikowia noctiluminum, Candida picachoensis* and *C. pimensis* were obtained from gut of adult lacewings (Woolfolk et al., 2003; Suh et al., 2004; Nguyen et al., 2006). Chrysopid predators harbor bacteria in their mid gut region which degrades the digestive residues (Mc-Dunnough, 1909; Spiegler, 1962; Jepp, 1984). Therefore in the present study we described the internal anatomy of the diverticulum of adult as well as midgut of both larvae and adult using TEM and also characterized the yeast and bacterial microflora in *C. z. sillemi* through molecular studies.

MATERIALS AND METHODS

Collection of Chrysopid predator

Adults of lacewings collected from various cotton fields of states (district) namely; Tamil Nadu (Coimbatore), Darward (Karnataka), AndhraPradesh (Guntur), Bangalore (Karnataka), Delhi, Haryana (Sirsa), Punjab (Ludhiana), Rajasthan (Sriganganagar & Udaipur), lab population, Gujarat (Anand), Orissa (Bhuvaneshwar) and Maharashtra (Nagpur). The collections were made in the early morning hours (7-9 am).

Rearing of Chrysopid predator

The adults were transferred in to the oviposition chambers (14 × 9 cm) covered with muslin cloth. Cotton swabs dipped in water and the other with 50% honey, protein mixture [Protein X® (PFIZER Ltd, Mumbai, India): commercial yeast (Gloripan, DEV, INC, China): honey: sucrose in the ratio of 1:1:1:1] and castor pollen grains was provided for adult and provided with perforated brown paper for facilitating egg laying. Eggs were collected every two days interval and kept for hatching with UV treated (to prevent them from hatching) *Corcyra cephalonica* (Stainton) eggs and the containers (14 × 9 cm) were covered with perforated brown paper. Freshly emerged larvae were secured individually in glass vials (4 × 2.5 cm) plugged with cotton. *Corcyra* eggs were provided in the vials for larvae alternate days. Entire rearing was carried out at 26±1°C, 65±5% RH, photoperiod of 14L: 10D in a plant growth chamber.

Transmission electron microscopy (TEM)

Single population collected from Coimbatore (Tamil Nadu) named as CZS-1 was taken for TEM analysis. Larvae (n= 15) and adults (n= 18) of *C. z. sillemi* were processed according to standard protocols (Woolfolk et al., 2004). The third instar larvae were dissected to obtain the intact midgut after removing the fat bodies.
Similarly the adults were dissected to obtain diverticulum. The image of such dissected samples was digitized using Auto Montage software (Leica Stereozome microscope M205A) (Figure 1A and 1B). The gut tissues were fixed in 3% glutaraldehyde and subjected to TEM studies. Briefly, tissues fixed in 3% glutaraldehyde for 24 h were washed in 0.1M phosphate buffer (pH=7.2-7.4), post fixed in 1% osmium tetroxide, washed and dehydrated 1 h each with 70, 80, 90 and 100 % ethanol. After dehydration, the tissues were cleared in propylene oxide, followed by infiltration over night in the mixture of propylene oxide and epoxy resin (resin mixture contains Araldite CY212 resin, DDSA, Dibutyl Phthalate and DMP30), subsequently transferred to pure araldite and finally embedded using flat embedding moulds and kept at 60°C for 48 h for polymerization. Plastic blocks containing the specimen were cut under Leica EMUC6 Ultra microtome using glass knives. Initially 1 µm thick sections were cut and stained with 1% Toluidine blue for light microscopic examination (Carl Zeiss, Primostar), later 70 nm thick ultrathin sections collected on copper grids were stained using Uranyl acetate and lead citrate. The stained grid containing specimens were scanned under Tecnai G^2 Spirit Bio-twin TEM at 80 KVA and representative areas were captured using *Mega View-III* digital CCD camera.

Isolation of microflora associated with Chrysopid predator

Thirteen field-collected populations of *C. z. sillemi* adults and larvae were separated individually into sterilized glass tube (4.5 × 2.5 cm) and kept in 95% ethanol for 2-3 min to disinfect the surface; followed by a wash with saline solution (0.5%). Further, the adults were dissected in a sterile condition and obtained intact tissues of larval gut and adult diverticulum which were used for isolation of microorganism. The tissues were placed in a micro centrifuge tube containing 100 µl of sterile saline solution (0.9% NaCl) and were then mashed. The suspension thus obtained was spread on to YPDA (Yeast Peptone Dextrose Agar) media (consisting of 10 g of yeast extract, 20 g of peptone, and 20 g dextrose and 15 g of agar in 1 L of distilled water) and was incubated for 48 h at 25°C. The colonies obtained were streaked in to media plates to obtain pure culture. Single yeast and bacterial colonies were purified at least twice. Pure culture were then inoculated into YPDA broth and incubated to obtain reaches optimum growth.

Molecular characterization of resident microflora of Chrysopid predator

Yeast and bacterial genomic DNA were isolated from the pure culture by slightly modified method (Kim et al., 1997). The extracted DNA was used as template for amplification of ITS & 16S r RNA.

Characterization of yeast isolates

For yeast isolates, the primers ITS-1F (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS-1R (5’-GCTGCGTTCTTCATCGATGC-3’) (White et al., 1990) were subsequently used for the amplification of partial rRNA genes and ITS-1 and partial 5.8S rRNA gene. The conditions for amplification were initial denaturation at 94°C for 3 min followed by 30 cycles of 1min at 94°C, 1 min at 60°C and ending with an extension 1 min at 72°C (C1000™ Thermal cycler). The mixtures consisted of total volume of 50 µl containing 10x-reaction buffer, 10 mM dNTP, and 2 µl of each primer and 1U *Taq* polymerase. Each PCR was performed with a total of 10 µl of genomic DNA.

Characterization of bacterial isolates

For bacteria isolates the primers 16S-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-R (5'-

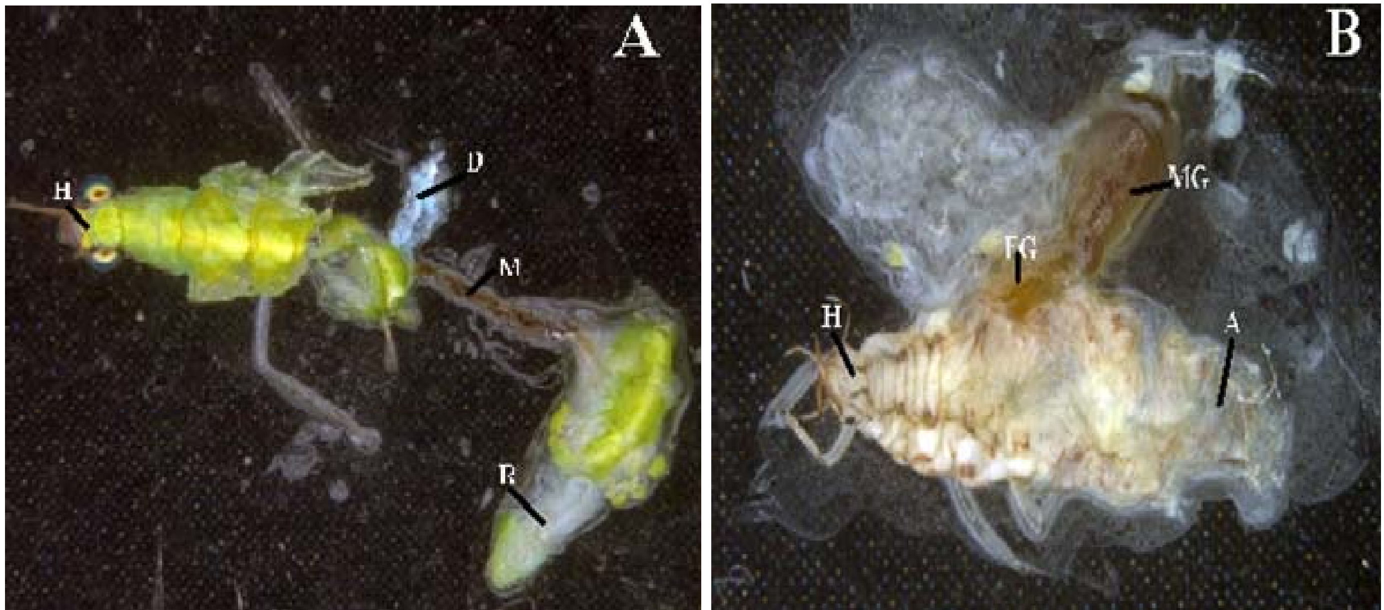

Figure 1. (A) Gut of *C. z. sillemi* adult. H, Head; D, diverticulum; MG, midgut; R, rectum. (B) Gut of *C. z. sillemi* larva. H, Head; FG, foregut; MG, midgut; H, head; A, abdomen.

CGGTGTGTACAAGACCC-3') (Universal primers) were subsequently used for the amplification of 16S r RNA gene. Standard PCR conditions were followed for amplification (O Neill et al., 1991).

The resulting PCR product was electrophoresed in a 1.5% TBE - agarose gel, and a 100 bp ladder was used to size products. The resulting PCR product was purified by PCR purification kit (Min elute spin column PCR purification kit) and were sequenced by Sanger's dideoxynucleotide sequencing. All sequence data were analyzed using basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov). The partial rRNA gene sequence including sequences for the 18S rRNA, ITS 1 and 5.8S rRNA genes and 16S rRNA gene were deposited in GenBank.

RESULTS

Collection of chrysopid predator

Field collected chrysopid predators were identified as *Chrysoperla zastrowi sillemi* based on morphology, behavior and acoustic analysis (Henry et al., 2010) and were named as CZS-1, CZS-2, CZS-3, CZS-4, CZS-5, CZS-6, CZS-7, CZS-8, CZS-9, CZS-15, CZS-19 and CZS-20. Lab population was named as CZS-10.

Rearing of chrysopid predators

Different stages like larvae, pupae and adults were collected from field to maintain the uniform cultures and to obtain all stages for the studies *C. z. sillemi* was successfully maintained under laboratory conditions and diet.

Internal anatomy of larval gut and adult gut and diverticulum

TEM showed a thick epithelial lining surrounding the lumen and the lumen harbor load of bacterial cells towards the periphery of inner side of the epithelial lining and the dividing bacterial cells (Figure 2; 1-4), confirming that bacterial cells are widely distributed within the lumen of the gut of larvae.

The adult diverticulum was folded internally and numerous kidney, oval and other shapes of yeast fauna were found distributed within the lumen of the diverticulum and between the diverticular folds of the diverticulum of adult (Figure 3: 1-6). The numerous shape of yeast is due to the ascospores production, different species of yeast produce different types of ascospores revealing that the diverticulum harbors different species of yeast.

Non-dividing bacterial fauna were observed in the lumen of adult gut lined by epithelia (Figure 4: 1-4). TEM analysis of adult gut showed the distribution of bacterial cells but not the yeast cells.

Molecular characterization of yeast and bacteria

Characterization of yeast

The amplified product checked against the 100 bp ladder on 1.5% agarose gel stained with ethidium bromide (1%) showed an amplification length of ≈175 bp for yeast isolates isolated from diverticulum of adults from strains

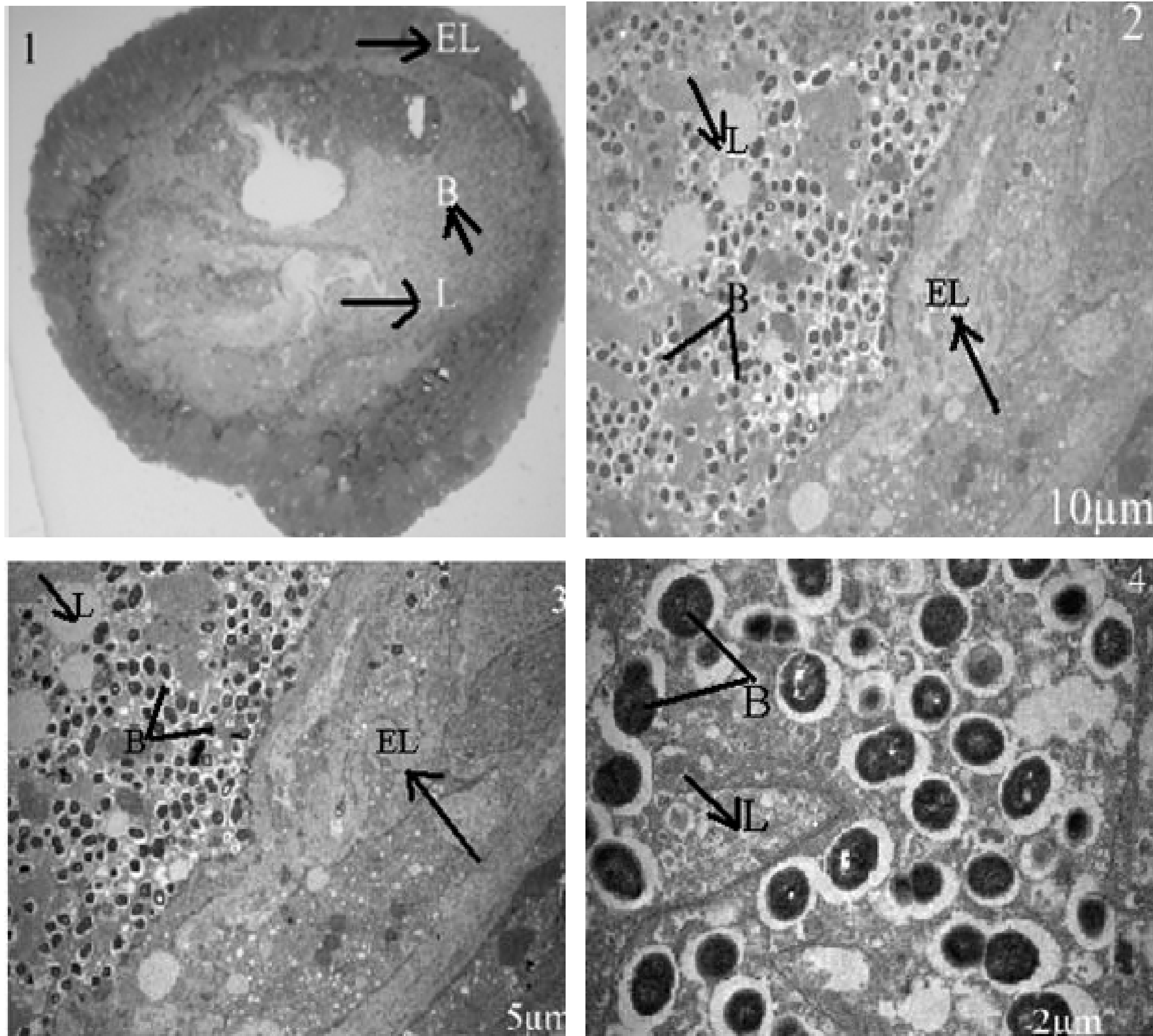

Figure 2. (1) Light microscopic view of semi ultra thin section of larval midgut stained with toluidine blue showing L-lumen, EL-Epithelial lining; B-Bacterial cells; (2-4) Transmission electron micrographs of larval gut of *C. z. sillemi*; Dividing bacterial (B) oval cells observed in the lumen (L) lined by epithelial lining (EL). Scale bar.10, 5 and 2 µm.

CZS-1, CZS-5, CZS-9, CZS-15 and CZS-16. Similarly ≈ 300 bp for stains CZS-4, CZS-7 and CZS-10.

Eight yeast isolates were identified from adult diverticulum, the sequence when analysed by BLAST revealed the yeast genera as well as species and the percentage matching was found to be 100, 99, 100, 97, 99, 99, 97 and 97%, respectively. The yeast isolates were identified as *Wickerhamomyces anomalus* (strain CZS-1 & 5), *Pichia anomala* (CZS-2, 8 &15), *Candida blankii* (CZS-3), *Can. apicola* (CZS-2), *Torulaspora delbrueckii* (CZS-4), *Zygosaccharomyces rouxii* (CZS-7), *Kodamea ohmeri* (CZS-9 &16), *Can. pimensis* (CZS-10). *W. anomalus* was most commonly found yeast isolate in populations (CZS-1, CZS-2, CZS-5 and CZS-8). The characterization of yeast confirms that the diverticulum of adults harbour various species of yeast.

Characterization of bacteria

Bacterial isolates showed amplification length of ≈1200bp when checked on the 1.5% agarose gel stained by ethidium bromide (1%). Seventeen bacterial isolates were isolated from larval gut of strains CZS-1, CZS-2, CZS-3, CZS-6, CZS-8, CZS-9, CZS-10, CZS-15, CZS-19 and CZS-20.

The bacterial isolates were found to be *Enterobacter cloacae* (CZS-1 & 8), *Enterobacter* sp. (CZS-1,2,3 & 8), *Pantoea dispersa* (CZS-2), *Bacillus* sp.(CZS-3 & 9), *Agrobacterium tumefaciens* (CZS-6), *Enterobacter hormaechei* (CZS-8, 9 & 10), *Enterobacter asburiae* (CZS-9), *Bacillus cereus* (CZS-9), *Enterococcus faecium* (CZS-10), *Empedobacter* sp., (CZS-10), *Lactococcus garvieae* (CZS-15), *Enterococcus gallinarum* (CZS-19), *B. subtilis*

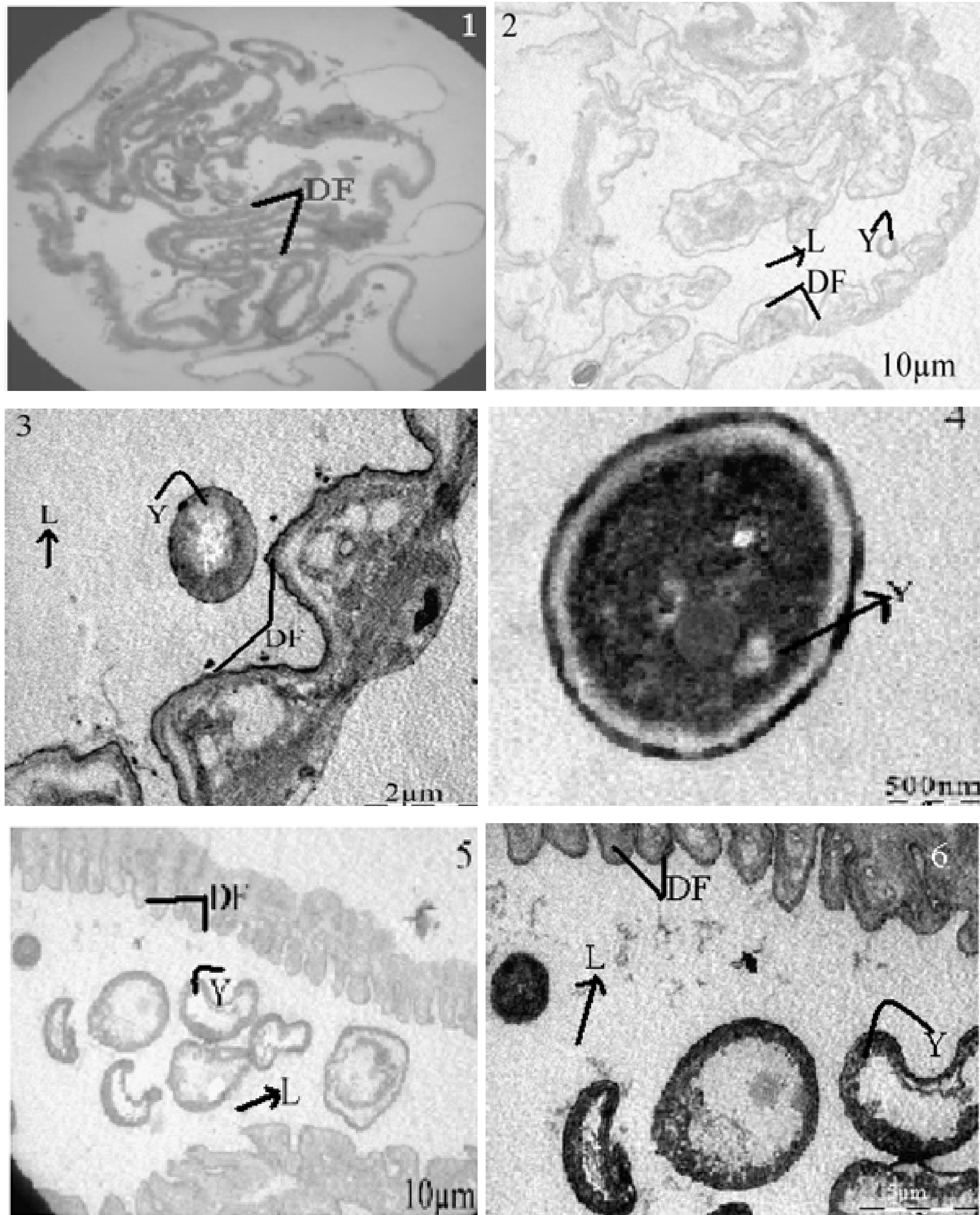

Figure 3. (1) 1 µm thick semi thin section showing diverticulum. (2-6) Transmission Electron micrographs of adult diverticulum of *C. z. sillemi* shows oval and kidney shape yeast (Y) cells observed in the lumen (L) lined by diverticular folds (DF). Scale Bar.10 µm, 5 µm, 2 µm and 500 nm.

(CZS-20), *Enterococcus faecalis* (CZS-20), *B. pumilus* (CZS-20), *Enterococcus* (CZS-10), *Leclercia adecarbxylata* (CZS-15) and their percentage matching were 90-100%. *Enterobacter* sp. was most commonly found bacterial isolates in populations (CZS-1, CZS -2, CZS -3 & CZS- 8). The sequences of these bacteria and yeast isolates were submitted to Gen Bank and accession numbers were obtained (Table 1).

DISCUSSION

TEM studies showed the presence of yeast and bacteria in larval gut and adult diverticulum and similar observations were made by (Chen et al., 2006; Woolfolk et al., 2003). We have observed yeast cells of different shapes like spherical, kidney and irregular; Woolfolk et al. (2003) however, observed only spherical shape.

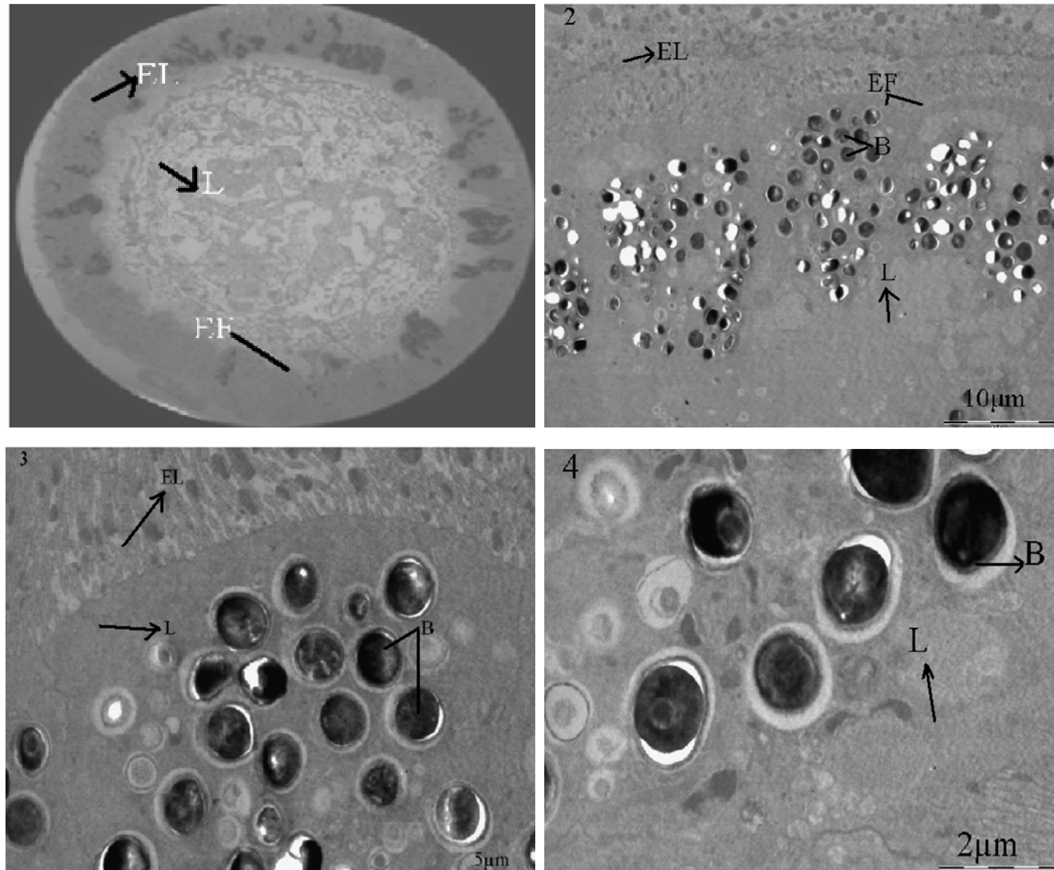

Figure 4. (1) Light microscopic view of 1µm thick semi thin section stained with toluidine blue showing adult gut having epithelial line (EL), epithelial folds (EF) and lumen (L). (2-4) Transmission Electron micrographs of adult gut of *C. z. sillemi* shows oval shaped bacteria (B) present in the lumen (L) lined by epithelial lining (EL) and Epithelial folds (EF). Scale Bar.10 µm, 5 µm, 2 µm and 500 nm.

Bacterial symbionts are present intracellular in bacteriocytes which are often present in certain organs like caeca of the digestive tract, malphigian tubules, appendages of the reproductive tract (Buchner, 1953; Peterson et al., 1994; Kikuchi et al., 2009) in some insects where as in *Chrysoperla carnae* the bacteria were observed free in the lumen (Chapman, 1985). Our study reveals that *C. z. sillemi* harbored bacterial cells in the lumen but not in specialized structures like bacteriocytes.

There was no instance of yeasts in the eggs and larvae. For further confirmation whether adult's posses yeasts in their gut upon eclosion from pupae no instances of yeast were found. Only the field collected adult chrysopids showed the presence of yeast isolates. This indicates that the yeast are transients and have been acquired through the diet and can be indicated as facultative endosymbionts and transmission through is not obligatory. This is in accordance with (Hagen et al., 1970) who suggested that *C. carnea* obtain yeasts from environment and they did not observe any yeasts from larvae. However, this result was contradicted by (Woolfolk et al., 2004) who isolated yeasts from the field collected larvae of *C. rufilabris* and could not isolate from the newly eclosed adults.

Yeast symbionts in the diverticulum provide the amino acid, valine to adult females which may increase the fecundity of the same (Hagen et al., 1970). Further, Woolfolk et al. (2004) observed that the diverticulum was associated with large tracheal trunk which could involve gas exchange in order to support the activity of yeast and other obligate anaerobes or facultative anaerobes (Barnett et al., 1990; Canard et al., 1990; Woolfolk et al., 2004). Similarly we had isolated most of the yeasts from the diverticulum and not from gut.

Several yeasts were isolated from the adult diverticulum, however, *Kodamaea ohmeri*, *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* were found to be prevalent in many populations. Chrysopids are found to have association with variety of micro flora namely *Torulopsis* sp. (Hagen et al., 1970, 1972); *Candida multigemmis* (Buhagiar) Meyer and Yarrow; *T. multigemmis* (Johnson, 1982); *Metschnikowia chrysoperla*, *C. picachoensis* and *C. pimensis* (Suh, 2004; Gibson et al., 2005); *M. pulcherrima* (Woolfolk et

Table 1. Diversity of yeast and bacterial isolates from *C. z. sillemi.*

Population	Yeast isolate from adult diverticulum and accession number	Bacterial isolate from larval gut and accession number
CZS-1 (Coimbatore)	*Wickerhamomyces anomalus* (JQ061141)	*Enterobacter cloacae* (KC 333898), *Enterobacter* sp., (KC 333890), *Enterobacter* sp., (KC 333891)
CZS-2 (Dharwad)	Not observed	*Pantoea dispersa* (JX873957), *Enterobacter* sp., (JX873958)
CZS-3 (Guntur)	*Candida blankii* (JQ340778)	*Enterobacter* sp., (JX 873959), *Bacillus* sp., (JX 873960)
CZS-4 (Bangalore)	*Torulaspora delbrueckii* (KC507190)	Not observed
CZS-5 (Delhi)	*Wickerhamomyces anomalus* (JQ340781)	Not observed
CZS-6 (Sirsa)	Not observed	*Agrobacterium tumefaciens* (KC 333915)
CZS-7 (Ludhiana)	*Zygosaccharomyces rouxii* (JQ410172)	Not observed
CZS-8 (Sriganganagar)	Not observed	*Enterobacter* sp., (KC407909), *E. hormaechei* (KC 333906), *E. cloacae* (KC 333907), *Enterobacter* sp., (KC 333908)
CZS-9 (Udaipur)	*Kodamaea ohmeri* (KC473466)	*Bacillus* sp., (KC 333897), *E. asburiae* (KC 333899), *E. hormaechei* (KC 333901), *B. cereus* (KC 333903)
CZS-10 (Lab population)	*Candida pimensis* (KC473468)	*E. faecium* (KC 333895), *E. hormaechei* (KC 333894), *Empedobacter* sp., (KC 333902)
CZS-15 (Anand)	*Pichia anomala* (KC473464)	*Lactococcus garvieae* (KC 333889)
CZS-19 (Bhuvaneshwar)	Not observed	*Enterococcus gallinarum* (KC 333905)
CZS-20 (Nagpur)	Not observed	*B. subtilis* (KC 333910), *Enterococcus faecalis* (KC 333911), *B. pumilus* (KC 333997)

al., 2004). *T. delbrukii* was isolated from the gut of female *Chauliodes rastricornis* (Neuroptera) in LA, USA. Yeast isolates belonging to the *Metschnikowia* were isolated from the digestive tracts of lacewings (Nguyen et al., 2006). *Kodamaea ohmere* was isolated from gut of female *Corydalus cornutus* (Neuroptera) (Nguyen et al., 2007) found *M. pulcherrima* was predominant yeast found in the alimentary canal of *Chr. rufilabris* which are acquired from the environment. However, we did not observe *M. pulcherrima* in Indian populations of *C. z. sillemi* may be due to the difference in cropping pattern and diversity grown in different countries.

Our study reveals that bacterial isolates *viz.*, *Enterobacter hormaechei*, *E. cloacae* and *Enterobacter* sp. were observed as most common from larvae which are culturable. Some of the facultative symbionts like Enterobacteriaceae are found in aphids, whiteflies, tsetse flies and mealy bugs (Baumann, 2005; Chiel et al., 2007;

Darby et al., 2001; Moran et al., 2005; Novakov, 2007; Russell et al., 2003; Sauer et al., 2000; Weiss et al., 2006). The facultative mutualists play a role in insect host like enhancing the host reproduction, providing protection against natural enemies of host (Oliver et al., 2008; Piel, 2002; Scarborough et al., 2005; Holt et al., 1994). Though TEM analysis revealed the presence of bacteria in adult gut which were unculturable on the media indicates that both the larvae and adult of *C. z. sillemi* harbor different types of bacterial fauna showing that the bacteria are facultative.

However, Woolfolk et al. (2004) isolated *E. areogenes* and *E. cloacae* from the adult diverticulum. Chen et al. (2006) observed the midgut structure and contents of *Chrysoperla carnea* larvae and is hypothesized that the several species of *Chrysoperla* including *Chr. carnea* (Stephens), *Chrysopa oculata* Say, *Ceratochrysa cubana* (Hagen), and the alimentary canal is closed between the midgut and the hindgut. Hence, microflora present in the larval gut may decompose the digestive residues in the midgut.

Conclusion

The study reveals the distribution of microbial diversity of *C. z. sillemi*, as the larvae showed the presence of bacteria and the adults showed the presence of yeast. The route of transmission may be horizontal. This is the first study established the presence of yeast and bacterial cells in adult diverticulum and larval midgut of *C. z. sillemi* through TEM and molecular studies. Further, it is suggested to study on the role of yeast on the fitness attributes of the adult predator especially on reproducing capacity and bacteria on insecticide degradation in larvae as the larvae of the predator was found to have high resistance to various groups of insecticides.

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