Indian Journal of Environmental Sciences 21(2) 2017, pp. 94- 100 ISSN 0971-8958
Green Earth Foundation

STUDIES ON GUT ASSOCIATED BACTERIA OF *CHRYSOCORIS STOLLI* WOLF

M.N.V. Vijayalakshmi, C. Sakunthala, J. Kavipriya, U. Vijiyalakshmi, and K. Sankaraiyah*
Department of Zoology, Kanchi Mamunivar Centre for Post Graduate Studies, Lawspet, Puducherry - 605008
*Corresponding Author

ABSTRACT

The aim of the present study was to identify and characterize the gut associated bacteria in *Chrysocoris stolli*. The bacterial flora of *C. stolli* was determined by using both culture dependent and culture independent tools. In total, 30 bacterial isolates were characterized based on their morphological, biochemical, physiological, and molecular characteristics. Among 30 isolates, 23 were gram-negative bacteria, 16 catalase producers, 3 lipase producers, 22 amylase producers, 12 cellulase producers and all 30 isolates were ammonia producers. The bacteria isolated from *C. stolli* was identified as *Citrobacter europaeus* (CSI27) by using 16S rRNA sequence.

Key words: *Chrysocoris stolli,* gut associated bacteria, 16S rRNA partial sequence, *Citrobacter europaeus* (CSI27)

INTRODUCTION

Earth is the planet of insects as they are found in almost every corner of the earth. Insects belong to the class insecta are a major group of arthropods consisting of more than 1 million insect species which are far more than any other animal species and are 72.8% of all animals (Dillon and Dillon 2006). One of the major features of insects is their ability to survive in different ecological conditions (Kehinde et al. 2011). Due to their wide spread distribution, insects are inevitably associated with extremely large variety of microscopic life forms, including viruses, bacteria, fungi, protozoa, nematodes and multicellular parasites. Although some of these microorganisms exhibit a rather wide host range, many associations are highly specialized and involve not only certain insect species but also particular life stages of the insect host (Aronson et al. 1986). The success of insects is due to their remarkable adaptability to a vast array of terrestrial habitats, including those that are strongly limited or imbalanced in nutrients and to their ability to face pathogens (such as bacteria). Nevertheless, insect success is also due to the collaboration with bacteria in term of symbiosis since bacteria play crucial roles in the biology and life cycle of most insects' species, affecting nutrition, development, reproduction, immunity, defense against natural enemies and speciation (Moran and Baumann 2000, Moran 2001, Moran 2006). Microorganism is a rich source of new metabolites with a wide variety of biological activities and some of them display significant practical applications (Chellaram and Priya 2012). The gut microbe interactions are diverse and include antagonism, commensalism, and mutualism and range from obligate to facultative (Douglas 2009). In many insect species, gut possess different types of bacteria which are transient and do not remain in the gut during all life stages. However in some cases, a variety of permanent microorganisms are present that supply essential nutrients to their host and some possess obligate microbial endosymbionts that benefit the insects (Bridges 1981). Symbiotic associations between different organisms are of great importance for evolutionary and ecological processes (Smith 1989). Bacteria are particularly valuable symbiotic partners owing to their huge diversity of biochemical pathways that may open entirely new ecological niches for higher organisms (Sapp 1994).

No work has been done to explore the bacterial diversity in the adult *Chrysocoris stolli.* This study is of great significance because multiple varieties of symbiotic bacteria do exist in the gut of the *Chrysocoris stolli* and performing different functions. And also there may be a possibility for the presence of new bacterial strain. Therefore, in this study, an attempt has been made to investigate bacterial flora of gut of the *Chrysocoris stolli*, by using morphological and biochemical tests, and also 16S rRNA gene sequencing.

MATERIALS AND METHODS

Insect Collection and Gut Dissection

The adult insect (*Chrysocoris stolli*) was collected from its host plant (*Croton bonplandianus*) in Lawspet, Puducherry

in the month of March 2017. The insect was surface sterilized in 70% ethanol for 1 minute and rinsed in sterile water before dissection. The insect was dissected inside a sterile laminar flow hood using sterilized dissection scissors, needle and forceps. The head and last abdominal segment of insect were severed, and pressure was applied anterior to the crop to release the gut which was homogenized in sterilized water.

The stock solution was prepared by taking 1 mL of the suspension and was mixed with 9.0 mL sterile water. From the stock solution 1mL was taken and mixed with 9mL of sterile water and it was considered as sample A. There by using serial dilution method 7 samples were prepared. 1mL of first four samples was added in four separate petriplate. From 5th sample onwards triplets were made for each sample. 15mL of nutrient agar medium was added in each petriplate which were incubated for 24 hours at 37° C. After 48 h, colony forming units (cfu) were determined by visual counting. Purified colonies were grouped according to their morphology and cell characteristics (Huang 1999).

Identification and characterization of bacteria

During investigation of unknown species of microbes, many methods are used. Petriplates were observed after 24h of inoculation for the bacterial colonies. 30 colonies with various morphological features were selected for morphological studies. Besides gram staining, molecular technique and biochemical tests were carried out for ammonia (Collins 1989) and activities of various enzymes such as amylase (Hols et al. 1994), catalase (Mac Faddin 2000), cellulose (Delalibera et al. 2005 as modified from Teather and Wood 1982) and lipase (Bergey 1974) following standard methods.

Growth

To study bacterial growth, a loop full of bacteria from the each culture plates were inoculated in to sterile broth respectively and incubated under optical growth conditions. The degree of turbidity of the broth indicates growth rate of bacteria in it. Using spectrophotometer the optical density of the broth was read under 600 nm, for 24 hours, 48 hours and 72 hours.

16S rRNA gene sequence

Preparation of template DNA – Pure cultured bacterium was used for gene sequencing. Colonies were picked up with a sterilized toothpick, and suspended in 0.5 mL of sterilized saline in a 1.5 mL centrifuge tube and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 mL of Insta Gene Matrix (Bio-Rad, USA), incubated at 56°C for 30 min and then heated 100°C for 10 min. After heating, supernatant was used for PCR.

PCR - 1µl of template DNA was added in 20 µl of PCR reaction mix. 518F/800R primers were used and then performed 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragments were amplified about 1,400bp in the case of bacteria. Include a positive control (*E. coli* genomic DNA) and a negative control in the PCR.

518F 5' CCAGCAGCCGCGGTAATACG 3'

800R 5' TACCAGGGTATCTAATCC 3'

Purification - Purification of PCR products done by Montage PCR clean up kit (Millipore) according to the manufacturer's instructions.

Sequencing - The purified PCR products of approximately 1,400 bp were sequenced by using the primers (785F 5' GGATTAGATACCCTGGTA 3' and 907R 5' CCGTCAATTCCT TTRAGTTT 3'). Both these primers amplify the V5- V6 region of the 16S r RNA. Sequencing was performed by using big dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied BioSystems model 3730XXL automated DNA sequencing system (Applied Bio Systems, USA) (Weisburg et al. 1991).

Phylogenetic tree construction

The culture sequences obtained were subjected to BLAST analysis and phylogenetically similar strains sequence and other phylogenetically related sequence were selected from the GeneBank and they were subjected to multiple sequence alignment and then align sequences were trimmed to similar length in nucleotides and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6. In the tree the numbers at the nodes indicates the levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support] based on a neighbour joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated.

RESULTS AND DISCUSSION

Chrysocoris stolli Wolf is a polyphagous phytosuccivorus bug which infests *Cassia occidentalies, Croton sparisiflorum* and *Croton bonplandianum*. *Croton bonplandianum* has been reported to contain secondary metabolites such as alkaloids, terpenoids and toxic

component like phorbol esters (Rizk 1987, Phillipson 1995), norcinoacutine and new alkaloid 3-methoxy-4, 6-dihydroxymorphinandien-7- one were isolated (Tiwari et al. 1981). The insect which feed on these plants containing alkaloids and toxic components might have the bacteria in the gut region to digest these chemical components.

Of the total 30 isolates, 23 isolates were gram negative and 7 were gram positive (Table 1). Bacterial strains were different in shape and colour (Table 1). Out of 30 bacterial isolates 6 colonies were filamentous (white), 3 colonies were rhizoid (white), 7 colonies were rhizoid (cream), 2 colonies were round with radiating margins and cream in colour, 5 colonies were round in shape and cream in colour, 1 colony was white round and another 2 round colonies were yellow in colour, 3 colonies were yellow spindle, 1 colony was round with rough surface which was cream in colour and the morphology of this colony was different from other colonies. From these 30 colonies, 2 colonies differing in their morphology were selected and sent for 16s rDNA sequencing.

Table 1. Characterization of bacterial colonies

Colony	Shape	Colour	Gram's test
CSI01	Filamentous	White	Negative
CSI02	Rhizoid	White	Positive
CSI03	Rhizoid	White	Positive
CSI04	Rhizoid	Cream	Positive
CSI05	Round with radiating margins	Cream	Negative
CSI06	Round	Cream	Negative
CSI07	Round with radiating margin	Cream	Negative
CSI08	Rhizoid	White	Negative
CSI09	Filamentous	White	Negative
CSI10	Filamentous	White	Negative
CSI11	Rhizoid	Cream	Negative
CSI12	Rhizoid	Cream	Negative
CSI13	Spindle	Yellow	Negative
CSI14	Filamentous	White	Negative
CSI15	Filamentous	White	Negative
CSI16	Rhizoid	Cream	Negative
CSI17	Filamentous	White	Negative
CSI18	Round	Cream	Negative
CSI19	Round	Cream	Negative
CSI20	Rhizoid	Cream	Negative
CSI21	Round	Cream	Negative
CSI22	Rhizoid	Cream	Negative
CSI23	Round	Cream	Positive
CSI24	Rhizoid	Cream	Positive
CSI25	Round	Yellow	Positive
CSI26	Spindle	Yellow	Negative
CSI27	Round with rough surface	Cream	Negative
CSI28	Round	White	Negative
CSI29	Spindle	Yellow	Negative
CSI30	Round	Yellow	Positive

Growth rate

At 24 hours, out of 30 isolates, the turbidity of the 27 broths increased slightly, but CSI13, CSI22, CSI06 showed high turbidity and high growth rate (Table 2). At 48 hours, CSI13 continued to maintain high growth, CSI09, CSI18 started to grow after 24 hours and attain peak in this 48 hours incubation. At 72 hours CSI09 showed peak growth and CSI18 also

Table 2. Growth rate of isolates

	Optical density at 600 nm at time in hours		
Colony	24 hrs	48 hrs	72 hrs
CSI01	0.043	0.067	0.103
CSI02	0.046	0.072	0.09
CSI03	0.033	0.059	0.102
CSI04	0.025	0.059	0.075
CSI05	0.031	0.077	0.125
CSI06	0.056	0.071	0.141
CSI07	0.036	0.1	0.169
CSI08	0.041	0.103	0.211
CSI09	0.054	0.136	0.38
CSI10	0.056	0.116	0.252
CSI11	0.047	0.125	0.275
CSI12	0.041	0.086	0.26
CSI13	0.072	0.148	0.239
CSI14	0.04	0.1	0.174
CSI15	0.034	0.084	0.211
CSI16	0.033	0.081	0.175
CSI17	0.036	0.108	0.271
CSI18	0.043	0.136	0.311
CSI19	0.037	0.115	0.234
CSI20	0.044	0.101	0.226
CSI21	0.041	0.101	0.249
CSI22	0.061	0.114	0.25
CSI23	0.044	0.083	0.179
CSI24	0.052	0.1	0.218
CSI25	0.046	0.072	0.211
CSI26	0.022	0.076	0.157
CSI27	0.036	0.054	0.09
CSI28	0.028	0.081	0.21
CSI29	0.04	0.077	0.172
CSI30	0.049	0.083	0.197

showed high growth rate. From this, it is clearly understood that growth rate of each isolates is different, and they need specific period to grow. Some isolates have shown peak growth at 24 hours and after 24 hours the growth rate was not much increased and some other isolates which were showing slow growth at 24 hours, the growth rate increased later and at 72 hours they showed maximum rate of growth and optical density.

Biochemical characterization of bacterial colonies

Insects display a wide range in degree of dependence on gut bacteria for basic functions. Some insects may lack the enzymes naturally to digest the food eaten; the symbionts present in the gut of the insect may help in the digestion of that food. Out of 30 colonies, 22 isolates showed positive result for the amylase test and 8 were negative (Table 3). In the cellulolytic test, 12 isolates were positive and 18 showed negative result. In the catalase activity, 16 isolates showed positive result and 14 showed negative results. In the lipolytic test, only 3 isolates were positive and remaining 27 isolates failed to secrete lipase enzyme; the bacteria present in the gut of this insect might not have actively involved in the lipolytic process. CSI11 was the only isolate which showed the positive result for all biochemical tests.

The isolates which showed the positive results was able to secrete the respective enzymes of these biochemical tests. The enzymatic activity was noted by calculating enzymatic index of the isolates. If the enzymatic index of the isolate was more or equal to 1, enzymatic activity of that isolate was relatively more. In 30 isolates, all amylase and cellulase positive isolates were having enzymatic index of more than 1 hence and therefore, they have more enzymatic activity (Table 3).

Phylotype distribution in 16S rRNA Gene Libraries

The isolate CSI27 was selected based on their different morphological character and sequenced partially up to genera level. 16S rRNA shows the sequence CSI27 is similar to members of the *Citrobacter europaeus* (Plate 1).

Table 3. Biochemical characterization of bacterial colonies

Colony	Catalase Activity	Amylase Activity	Enzymatic Index	Cellulase Activity	Enzymatic Index	Lipase Activity	Ammonia Test
CSI01	+	-	Nil	-	Nil	-	+
CSI02	-	-	Nil	+	2.5	-	+
CSI03	+	+	1.86	-	Nil	-	+
CSI04	+	+	2	-	Nil	-	+
CSI05	+	+	2.10	-	Nil	-	+
CSI06	-	-	Nil	+	2.3	-	+
CSI07	+	-	Nil	-	Nil	-	+
CSI08	+	+	1.90	-	Nil	-	+
CSI09	-	+	2.91	+	1.9	-	+
CSI10	-	+	2.85	+	2.35	-	+
CSI11	+	+	2.66	+	2.4	+	+
CSI12	-	+	1.31	+	2.05	-	+
CSI13	+	-	Nil	+	2.18	-	+
CSI14	+	+	1.55	-	Nil	-	+
CSI15	+	-	Nil	-	Nil	-	+
CSI16	+	-	Nil	-	Nil	-	+
CSI17	-	+	1.36	+	1.52	-	+
CSI18	-	+	2.30	+	2.17	-	+
CSI19	-	+	2.33	-	Nil	-	+
CSI20	-	+	2	-	Nil	-	+
CSI21	-	+	1.56	+	2.33	-	+
CSI22	-	+	2.36	+	2.5	+	+
CSI23	-	-	Nil	-	Nil	-	+
CSI24	-	+	2.14	+	1.78	-	+
CSI25	-	+	1.65	-	Nil	-	+
CSI26	+	+	3.5	-	Nil	-	+
CSI27	+	+	2.66	-	Nil	-	+
CSI28	+	+	3	-	Nil	-	+
CSI29	+	+	3	-	Nil	-	+
CSI30	+	+	1.9	-	Nil	+	+

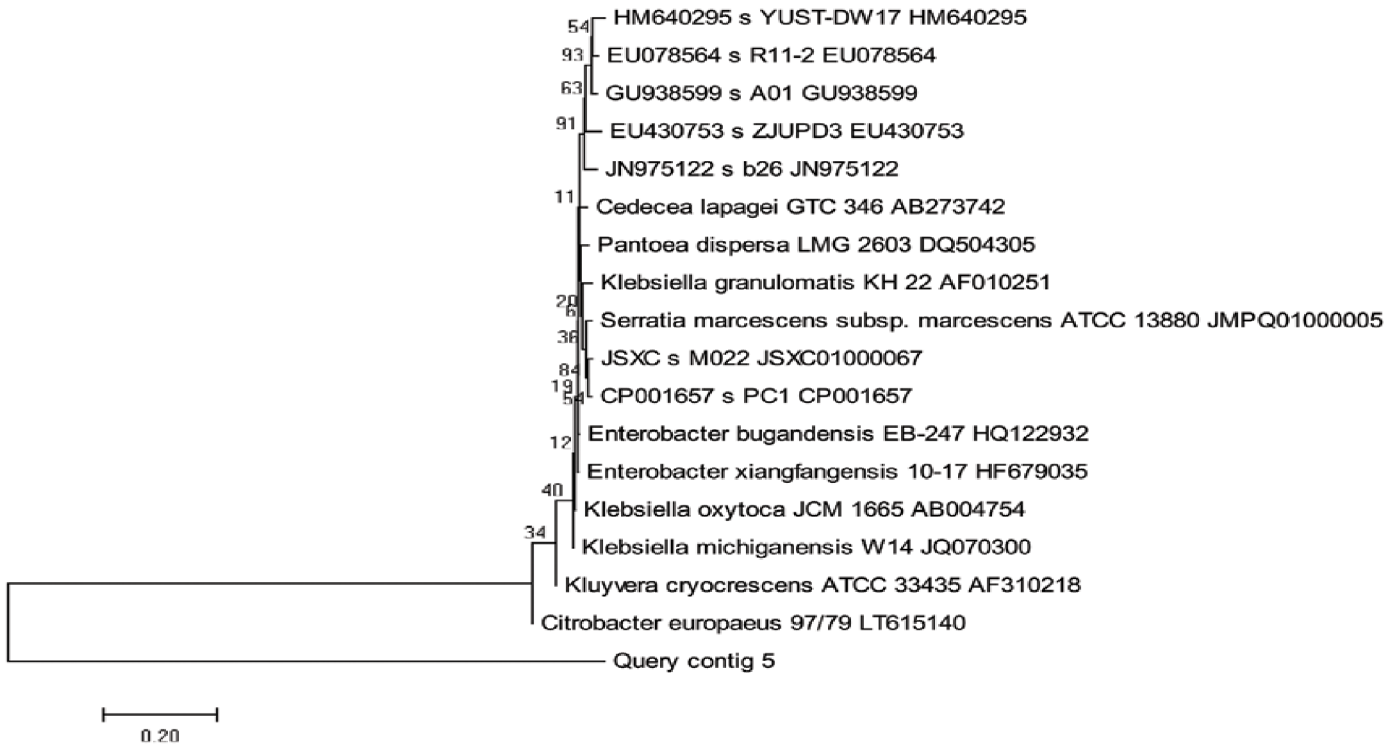

Plate 1. Phylogenetic tree of CSI 27.

GGCCGTGATGTCGACTTGCCAGTGTGGTGTCCTTGAGATCGTGAGCTGTGCGCGAGCTAACGCGATAAGTCGAC
CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGATGCAACGCGAAGAACCTTACCTGCTCTTGACATCCTGAGAACTTTCCAGAGATGCTTTGGTGC
CTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGTCCTTTGTTGCCAGCGGCTCGGCCGGGAACTCTAAGGAGACTGCCAGTGATAAACTGG
AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACTAGGGCTACACACGTGCTACAATGGCCCATAC
AAAGAGATGCGACCTCGCGAGATCAAGCTGATCTCATAAAGTGCATCGTAGTCCGGATTGCAGTCTGCAACTCG
ACTGCATGAAGTCGGAATCGCTAGTAATCGTAAATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACAC
ACTCGCCCGTCACACCATGGCGAGTCGACTTACTCGCAGATAGCTAGCGGCACGCCTACGACCTTCAGGAGGAC
ACACAACGCACATAGTCGATTCATCGTTTACTGGCGTGAATCTACCAGGGGAGGCCAAAACTGTTTGCTCCCCA
CGATCTCGCACGCGGAGCGGGGGCAGTAGTTGTCCAGGGGGCCGCCTCCCCCACCGGGGTTTTCCCCCCAAACT
CTCTACGCATTTCACCGCGTACACCGGGAATTCTACCCCCCTCTACCAAAAACTCTAGCCTGCCACGTTTTCGA
ACGCAGCCCCCACGTTGAGTCCCCGGGGATTTCACATTCCGACTTTTGACAAACCCCCCCTGCGTGCGCTTTCA
CGCCCAATAATTCCAAATAACGCCCGCACCCCCTCGTATTAACCGCGCGCTGCGGGCACGAAAAATACGCCGGT
GCTTCTTCTCGCGGGAAAAATCAATCAGAGGAGGGTAGTAACCCCACCACCCCCCCCCCCGCAAAAAAATGCTT
TACAACCCGAACACCTTCTTCATACACGCGGCACTGGCGGCATCAGGCTTGCTGCCCACTTGTGAAAAAACCCC
CACTGCCCCCCCCCCCGGAAAAGAGAGGCTGGACCGTGTCTCAGTCCCAGTGGGACGGAGCACCCCCTCAGACC
AGCTACGAGATCGGTCGGCCGATGGTGAGCACCGCCACCCCCCACACTACTACCTAGATCCGCAGCTAGGCACA
TCATGAGTGAGCAACACCCCCCAACATCCCCCCCGTTATGGTCTTGCGAATGTTATGCGGTATTAGCTACCGTT
TCCAGAAGTTATGCCCCCTCTATCAGGCAAGAATCCCAGACATCACGCACCCGTACCGCCGCTCAACCCTCGGA
CAAAGCAGCCCAGTGTCCGCTCGATCTACATGTGTGAGTCGATGCCTCCAGCGTTCGATCTGACAGATCAAAAA
AAAAAAAAAAAAAAAAAAAGGCACACAACTACACAGGCCTCCCCCCCACCAACCCCCCCCCCACCCCCACCCCCC
CCCCCCCCCCCCCCCCCACCCCCCCCCCCCCCCCCCCCCACAAAAACCCACTTTCTTTTTTTCCTGTCCCCCTCCCC
CCCGACTCTTCCTTTCTTTCTTCTCTCCCCCCCCCTTTCCCTTCACTTAGTTTTTTTTTTACCCCCCCCCCTTT
TCTCCTATCTCCTCTCCTCTCTGTGCTCTTCCCCCCACCCCCCCGCCCGTCCTCCCCCCTCTTTCACCCCCTTC
CACCTTCGCTTTCTCCCCCCTCCCCCTGTT

Plate 2. Fasta format of isolate CSI 27

Two totally different isolates CSI27 and CSI30 based on their morphological features were sent for rRNA sequencing, In that CSI27 was partially sequenced up to genera level. The genomic analysis of CSI27 showed close similarity with *Citrobacter europaeus* belonging to the family of enterobacteriaceae.

Citrobacter is a genus of gram negative coliform bacteria in the enterobacteriaceae family. *Citrobacter* species are differentiated by their ability to convert tryptophan to indole. In that *Citrobacter koseri* is the only *Citrobacter* to be commonly indole positive. *Citrobacter* shows the ability to accumulate uranium by building phosphate complexes (Macaskie et al. 1992).

Citrobacter species are considered to be harmless inhabitants in intestinal tracts of man and animals. They are commonly distributed in soil, sewage, water and food (Nayar et al. 2014). Many reports published reports regarding bacterial flora in the digestive tract of insects has the *Citrobacter* species, *Citrobacter freundii* was isolated from the digestive tract of the ground beetle, *Poecilus chalcites* (Lehman et al. 2008).

Citrobacter europaeus is the newly identified bacteria isolated from the human faeces and well water (Ribeiro et al. 2017). No work has been done on this species. Further studies are required to understand the characteristics of this new species and also to study the effects of these bacteria in the biodegradation because some *Citrobacter* species are able to degrade many organic and inorganic compounds. *Citrobacter freundii* has been listed and documented as a potential organism for remediation of copper. This potential organism can be used for bioremediation of heavy metals to clean up the environment (Macaskie et al. 2006).

Taxonomic Hierarchy of CSI27 (*Citrobacter europaeus)*

Domain - Bacteria
Phylum - Proteobacteria
Class - Gammaproteobacteria
Order - Enterobacteriales
Family - Enterobacteriaceace
Genus - *Citrobacter*
Species - *europaeus* (Ribeiro et al. 2017).

CONCLUSION

Studies on insect-microbiota interaction are growing subject in many laboratories. Understanding the role and interactions of symbiotic bacteria in insect gut is very important. This microbiota may interact with the host development, nutrition, and immunity. The present study described the isolation and characterization of 30 bacterial strains from the gut of *Chyrosocoris stolli.* In the 30 colonies, the presence of gram negative bacteria is more. CSI27 rRNA sequencing shows that, it is closely related to *Citrobacter europaeus,* which is newly isolated bacteria and need many more investigations to understand the role of this bacteria in the gut of insects as well as in the environment.

REFERENCES

Aronson, A.I., W. Beckman and P. Dunn. 1986. *Bacillus thuringiensis* and related insect pathogens. Microbiology Review 50: 1-24.

Bergey, D. H., R.E. Buchanan and N.E.Gibbons. 1974. Bergey's Manual of Determinative Bacteriology. Baltimore, Williams & Wilkins.Co. pp.3-29.

Bridges, J.R. 1981. Nitrogen fixing bacteria associated with bark beetles. Microbial Ecology 7: 131-137.

Chellaram, C. and G. Priya. 2012. Effect of textile effluent on biochemical parameters in some vegetable crops. Indian Journal Innovations and Developments 1:S8, 18-20.

Collins, C.H., P. M. Lyne and J.M. Grandge. 1989. Microbiological Methods. Sixth Edition, Butterworths & Co. Publishers Ltd. P-99.

Dillon, R. J. and V.M. Dillon. 2006. The gut bacteria of insects: Nonpathogenic Interactions. Microbial Ecology International Symposium, pp. 375-380.

Douglas, A. E. 2009. The microbial dimension in insect nutritional ecology. Functional Ecology 23: 38-47.

Hols, P., T. Ferain, D. Garmyn, N. Bernard and J. Delcour. 1994. Use of homologous expression secretion signals and vector free stable chromosomal integration in engineering of *Lactobacillus plantarum* for alpha amylase and levanase expression. Applied and Environmental Microbiology 60(5):1401–1413.

Huang, X.L. 1999. Guidance of Microbiology Experiment. China Higher Education Press, pp.114-116.

Kehinde, O. A. and B. Adewunmildowu. 2011. Occurance and distribution of microflora in the gut regions of the variegated grasshopper, *Zonocerus variegates*, during development. Zoological Studies 50(4): 409-415.

Lehman, R.M., J.G. Lundgren and L.M. Petzke. 2008. Bacterial communities associated with the digestive tract of the predatory ground beetle, *Poecilus chalcites,* and their modification by laboratory rearing and antibiotic treatment. Microbial Ecology 57(2):349-358.

Lu, W. J., H.T. Wang, Y.F. Nie, Z.C.Wang, D.Y. Huang and J.C Chen. 2004. Effect of inoculating flower stalks and vegetable waste with ligno-cellulolytic microorganisms on the composting process. Journal of Environmental Science and Health, Part B. 39(5-6): 871–887.

Macaskie, L.E., K.M. Bonthrone and D.A. Rouch. 2006. Phosphate- mediated heavy metal accumulation by a *Citrobacter* sp. and related enterobacteria. Federation of European Microbiological Societies Microbiology Letters 121:2,141-146.

Macaskie, L.E., R.M. Empson, A.K. Cheetham, C.P. Grey and A.J. Skarnulis. 1992. Uranium bioaccumulation by a *Citrobacter* sp. as a result of enzymically mediated growth of polycrystalline HUO2PO4. Science 257(5071): 782-784.

MacFaddin, J.F. 2000. Biochemical Tests for Identification of Medical Bacteria. 3rd edition. Pp. 527.

Moran, NA and P. Baumann. 2000. Bacterial endosymbionts in animals. Current Opinion Microbiology Journal 3: 270-275.

Moran, NA. 2001. Bacterial menagerie inside insects. Proceedings of the National. Academy of Sciences USA 98: 1338-1340.

Moran, NA. 2006. Symbiosis. Current Biology 16: R866-R871.

Phillipson, J.D. 1995. A matter of some sensitivity. Phytochemistry 38(6): 1319-1343.

Ribeiro, T.G., D.Clermont, R. Branquinho and S. Brisse. 2017. *Citrobacter europaeus* sp, isolated from water and human faecal samples. International Journal of Systematic and Evolutionary Microbiology 67 (1):170-173.

Nayar,R., I. Shukla and A. Sultan. 2014. Epidemiology, Prevalence and identification of *Citrobacter* species in clinical specimens in a tertiary care hospital in India, International Journal of Scientific and Research Publications 4:2250-3153.

Rizk, A.M. 1987. The chemical constituents and economic plants of the Euphorbiaceae. Botanical Journal of the Linnean Society 94(1-2): 293-326.

Sapp, J. 1994. Evolution by Association: A History of Symbiosis. New York: Oxford University press.Pp.94-6321.

Smith, J.M. 1989. Generating novelty by symbiosis. Nature 341: 284-285.

Tiwari, K.P., R.N. Choudhary and G.D. Pandey. 1981. The alkaloids. Photochemistry 20(4): 863-864.

Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173(2):697-703.