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Gut analysis using 16S rRNA for bacterial identification in the Pure Mysore and FC1xFC2 breeds of silkworm *Bombyx mori*

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Abstract

Silkworm *Bombyx mori*, a typical lepidoptera insect, is extremely important in agriculture with economic importance. Its short life cycle, clear genetic background,
rich genetic resources, and a significant number of genes similar to humans made it famous for many scientific investigations. Two silkworm strains with one Multivoltine (Pure Mysore) and one Bivoltine (FC1xFC2) were considered for gut microbial profiling. The two strains were grouped into two groups namely Healthy and Unhealthy (starved) and were reared. The stage-specific gut tissues were collected for Gut bacteria culturing from two groups of both strains. Gut bacteria were isolated and cultured from all healthy and unhealthy considered groups. Genomic DNA was isolated for further 16S rRNA gene amplification. 16S rRNA gene sequence was determined by Sanger sequencing and the results were queried against the NCBI database. The Blast analysis revealed the predominant bacterial species in healthy and unhealthy groups of Pure Mysore and FC1xFC2. The *Proteus sps*, *Proteus mirabilis, Flavobacterium sps, Pseudomonas*, *Bacillus licheniformis* were disclosed through the 16S rRNA sequencing and few more gut bacteria belonging to *Klebsiella* and *Enterobacter* were revealed through the morphological and a biochemical characterization. The present study unveils the predominant existence of above bacterial species in the intestinal tract of multivoltine and bivoltine breeds of *Bombyx mori*. In recent times, clear knowledge of intestinal microbial diversity and their role in the host metabolism has gained interest for improvement in commercial sericulture. The focus on microbial profiling would pave insights on the insect-gut microbiome interaction and their role in a beneficial way.

Key words. Gut Microbiota; 16S rRNA gene; Multivoltine breeds; Bivoltine breeds; *Bombyx mori;* microbial profiling.

Introduction

The insect silkworm shows its importance in many fields other than Sericulture and life science as they emerged as a promising insect model for their large size, easy rearing and maintenance with rich genetic resources. Domesticated silkworm, despite their rich microbial diversity are poorly characterized indicating the limited study especially on silkworm gut microbiome (Pandiarajan & Krishnan 2018). Insect gut harbors consortium of microorganisms, involved in various significant roles like host nutrition, growth, immunity and reproduction (Brand et al. 1975; Brune 2003; Moran et al. 2005). The host survivability is affected by the loss or alteration of Gut microbes (Fukatsu and Hosokawa 2002).

In insects, only the Gut microbes of termites and desert locusts shows extensive studies to understand their role on the host. (Dillon and Charnley 2002). Many factors like nutritional and climatic factors, rearing practices, races and other factors influences the silkworm growth and development and laterally show influence on the economic characters of cocoon. The silkworm gut acts as a reservoir for countless microorganism and plays vital role in host immunity and survival, nutrition assimilation and reproduction which impacts the growth and development with consequent reflection on the cocoon yield and quality (Barretto D.A et al 2021; Sun, Z et al 2017). The investigation on the silkworm Gut microbiota provides a better perspective on the influence of Gut flora in maintaining the silkworm health thereby reflecting their involvement on the economic characteristics. Therefore, study on the gut population helps in bettering our knowledge and understanding their beneficial or detrimental role in the host silkworm.

Many approaches help in the identification of Gut microbial diversity to unravel the microbial complexity were categorized into culture-dependent approaches and culture independent approach. The classical phenotypic characterization was an inexpensive technique identifies the bacteria based on their morphological and biochemical characterization, several other approaches can also be used for bacterial recognition in which the 16S rRNA sequencing gain interest for its veracity. The advent of 16S rRNA gene sequencing helps in easy and rapid microbial identification with high specificity. The 16S rRNA gene sequencing is widely used phylogenetic tool to study the diversity of microbial variability contributing for species identification. According to Chen et al., (2018) larger studies and information were available on the silkworm *B.mori* biology and physiology, while research on silkworm Gut microbiota is limited and needs more focus to gain better understanding on silkworm-gut association. Therefore, the analysis of gut bacteria in the silkworm breeds considering one multivoltine i.e, Pure Mysore (PM) and one bivoltine i.e,

FC1xFC2 Bivoltine double hybrids (healthy and unhealthy grouping) enabling to comprehend the microbial diversity. Thus, improving the understanding on silkworm and their Gut bacterial association.

16S rRNA gene and its significance

Bacterial profiling by traditional method of phenotypic & biochemical characterization is laborious though they are less expensive. This approach may lead to false representation and fails to produce accurate results in short period of time (Bouvet et al., 2014; Rossi-Tamisier et al.,2015).

The conventional cultural practices limit to the smaller portion of microbial diversity leaving the huge portion of microbes remain uncertain and unculturable (Stewart E J, 2012). A phylogenetic marker gene was identified to elucidate the phylogenetic relation of life forms like bacteria by comparing a stable Genetic code (Woese C 1987). Sequence diversity of hyper variable regions in bacterial 16S rRNA genes are used to identify bacterial population and their taxonomic studies (Choi et al., 1996; Clarridge, 2004; Munson et al., 2004; Petti et al., 2005; Schmalenberger et al., 2001). These regions are broadly used in diversity analysis of microbial population (Xu X et al., 2015; Dehingia M et al., 2015; Methe BA et al.,2012). The advent of 16S rRNA sequencing and its role as a marker gene for bacterial profiling (Tringe S G, Hugenholtz P 2008) had overcome the problems arising from the traditional approach of bacterial identification (Figure 1).

The small ribosomal subunit 16S rRNA gene serves as a very good phylogenetic marker and its sequencing gives information on genetic variability, and contributes higher percentage in species identification than the conventional approach. Universal occurrence of the 16S rRNA gene was found in prokaryotes like Bacteria and Archaea. 16S ribosomal unit is a part of 30S ribosomal subunit with 1500bp length sequence. It has higher consistency to study the phylogenetic relationship with their specific, variable, and highly conserved sequences, which results in the rapid and accurate genetic correlation at genus and species level. (Conlan et al., 2012; Fettweis et al., 2012).

Analysis of 16S ribosomal RNA gene has been considered as the most suitable and commonly accepted tool for bacterial profiling, because of its higher authenticity in classification. The hypervariable regions (V1-V9) of 16S rRNA gene displays the evolutionary divergence. It has been an important phylogenetic tool for microbial classification. These variable regions have unique conserved regions, and universal primers were used for the amplification of these conserved regions. Full-length 16S rRNA gene has

nine highly conserved regions, which forms nine hypervariable regions (Baker GC et al., 2003; Wang Y Qian P-Y. et al., 2009). Gut microbiota assists various physiological mechanisms in concerned host. Microbial assessment has been performed by classical cultural dependent technique and molecular techniques using 16S rRNA gene sequencing. To overcome the problems encountered by the conventional microbial identification technique, the gold standard technique 16S rRNA gene sequencing emerged as novel tool for the bacterial identification.

Approaches for Bacterial Identification

The silkworm is known for its simple gut structures like other lepidopterans, which constitute the major portion of the body cavity with three compartments named Foregut, Midgut (larger part) and Hindgut (Liu H et al., 2016). Symbiotic Gut microbiome in insects plays an important role in its growth, development, pathogenesis etc.

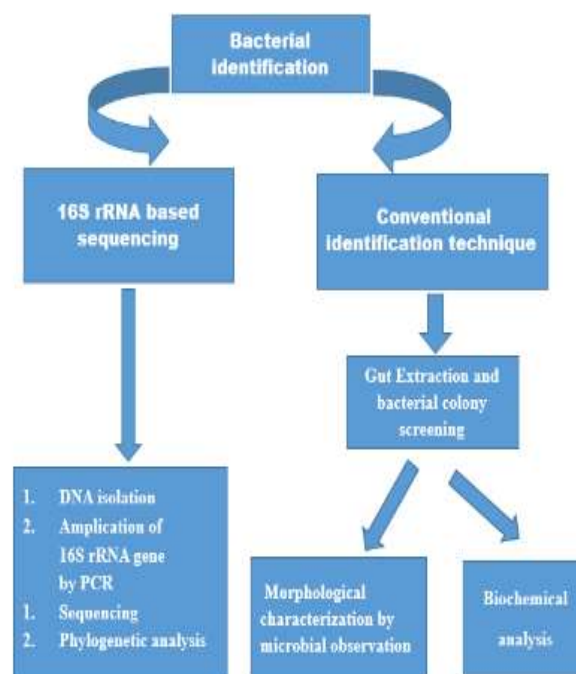

Figure 1. Flowchart of various approaches in Bacterial identification

This study was focused on understanding the current knowledge on the investigation of insect Gut microbiome in selective breeds of silkworms, and their functional role in the insect gut environment. Since Genome analysis has emerged as a major tool to study the composition, function, and evolution of various microbiota, the use of 16S rRNA gene in the profiling the insect Gut microbiota was explored. Silkworm Gut-microbiome study has ample scope to the commercial sericulture. However, there are not as many studies as other lepidopteran insect Gut microbiome, the studies on silkworm gut microbiome are budding in

a faster pace to understand microbial profiling and their contribution to the host in both beneficial and detrimental way. Therefore, knowing the Gut bacterial composition coupled with their role study depicts the effect of microbial association on the host and gives a clear understanding on the silkworm-gut microbe interaction thereby paving a path for improved silk production and survivability.

Hence, profiling the silkworm Gut microbiome helps in a better understanding of the silkworm gut microbiome interaction and their significance. The silkworm breeds namely Pure Mysore Multivoltine and FC1 X FC2, Bivoltine double hybrid breeds were used in the present study to understand the microbial diversity and to profile the predominant bacteria in silkworm gut. Gut microbe analysis of silkworm strains was performed for recognition of different predominant bacterial strains by culturing and sequencing techniques (16S rRNA gene sequencing) thus, the study focuses on unraveling the microbial diversity in the selected silkworm strains of *Bombyx mori.*

Material and Methods

Silkworm Grouping:

Collected Silkworm breeds were grouped into healthy and unhealthy groups. The silkworm subjected to starvation were considered as unhealthy by the internal stress developed by starvation. Each group contained 30 numbers of silkworms. Triplicates of each group were considered. Commercial Bivoltine breeds and high tolerant Multivoltine breeds were taken for the current research study. The groups were named as G1, G2, G3 and G4.

G1 - Group 1: Pure Mysore -Healthy (30 Nos)

G2 - Group 2: Pure Mysore -Unhealthy /starved (30 Nos)

G3 - Group 3: FC1xFC2- Healthy (30 Nos)

G4 - Group 4: FC1xFC2 -Unhealthy/Starved (30 Nos)

The G1 and G3 are considered as healthy groups being regularly fed with best quality of V_1 mulberry variety. The G2 and G4 are considered as unhealthy experimental (starved) batch. They were given one feeding from Day 1 to Day 3 and were kept for starvation on Day 4 and Day 5 until further analysis. These experimental batches are considered as Unhealthy/starved batches and their growth properties like larval weight of healthy and unhealthy groups(starved) of Pure Mysore and FC1xFC2 were recorded till 5th day of the larvae.

Genomic DNA isolation & amplification of 16S rRNA gene

Pure Mysore breeds of Multivoltine collected from Central Silk Board, Bangalore, India and FC1xFC2 Bivoltine double hybrids collected from Andhra Pradesh State

Sericulture Department, Palamaner were used for our experimentation. Collected silkworm breeds were grouped into healthy and unhealthy groups. For the microbial analysis, the gut of 5th day V instar larvae was dissected from four groups. The isolated gut was homogenized in PBS and was subjected to serial dilution to reduce the number of bacterial folds. The dilution factor 10^5 was picked for culture plating.100 µl of bacterial sample were spread on the nutrient agar media and incubated 37°C for 24-72 hrs to observe the bacterial colonies. Later pure colonies were developed from the mixed colonies by streak plating using the subsequent media. The morphological and colony characterization studies like the shape, motility and arrangement of bacteria were studied for the bacterial isolates by microscopic observation. The staining properties were studied by gram staining technique. The biochemical characterization for the classification of bacteria involved various tests like Indole test, Methyl red test, Voges proskauer test, Citrate and Urease test, H_2S production, Catalase and Oxidase test etc., the bacterial identification tests was carried out according to Bergey's manual of systematic bacteriology. The bacterial Genomic DNA was extracted from the 21 pure isolates by using Phenol: Chloroform: Isoamyl alcohol method as represented in Figure 2. 16S rRNA gene was amplified by Polymerase chain reaction by using universal primer 27F 5' GAGTTTGATCCTGGCT CAG-3' & 1495R 5' CGGTTACCTTGTTACGACTT-3'. PCR cycling temperatures were maintained as follows with annealing temperature at 55°C for 45 sec (temperature conditions were shown in Figure 3 & Table 1). All the PCR samples were analyzed by Agarose Gel Electrophoresis and the amplified products were observed under UV Gel documentation system.

Further, the amplified products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's manual to remove the unbound primers and contaminants. Sequencing was performed using the same 27F and 1495R primers. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer and developed 21 sequences for all the 21 bacterial samples of four groups.

Figure 2. Flowchart of genomic DNA isolation from bacterial cultures.

Sequence analysis of 16S rRNA gene

The determined sequences were compared to the sequences available in the GenBank database using BLAST algorithm against NCBI, the sequences are also compared with the sequences available in the Ribosome Database Project (RDP) to identify the sequence homology. The BLAST algorithm listed the similar sequences for all the 21 derived sequence with high similarity score. Twenty-one 16S rRNA gene sequences were submitted to NCBI. The generated accession numbers by NCBI for intestinal bacteria in all four experimental silkworm group were listed below with their assigned accession numbers OP748769, OP748770, OP748771, OP748772, OP748773, OP748774, OP748775, OP748776, OP748777, OP748860, OP748861, OP748862, OP703594, OP703595, OP749865, OP749863, OP749864, OP737801, OP737802, OP749861, OP749862.

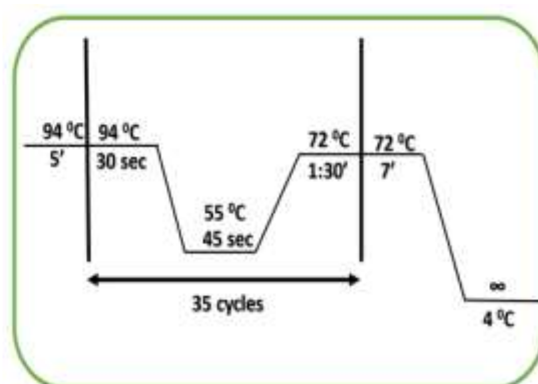

Figure 3. PCR cycling conditions maintained for the 16s rRNA gene.

Table Error! No text of specified style in document.**1 PCR conditions for 16S rRNA gene Amplification**

PCR process	Temperature	Time	Cycle
Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	30 sec	35 cycles
Annealing	55°C	45 sec	35 cycles
Extension/Synthesis	72°C	1:30 min	35 cycles
Final Extension	72°C	7 min	1 cycle
Termination of the process	4°C	∞	-

Results

The isolated genomic DNA was evaluated by Agarose gel electrophoresis where genomic bacterial DNA showed good quality bands for all the 21 samples at 260/280 absorbance ranging between 1.5 to 2.0. The gel image of Genomic DNA was given below in the Figure 4.

Figure 4. Agarose gel Electrophoresis of Genomic DNA (21 bacterial isolates)

The results of the Biochemical characterization of the bacterial isolates were as tabulated in Table 2.

Table Error! No text of specified style in document.**1 Cell morphology and Biochemical characterization test of the bacterial isolates (+ indicates presence and – indicates absence)**

Naname of bacterial isolate	**Gram stain**	**Cell morphology And motility**	**Indole**	**VP**	**Citrate**	**Methyl Red test**	**H_2S production**	**Oxidase**	**Catalase**	**Urease**	**Starch utilization**	**Acid or gas from glucose**	**Identified as**
BI-1	Gram -ve	Rod/non motile	-	-	-	-	-	+	+	+	-	+	Flavobacterium
BI-2	Gram -ve	Rod/Motile	+	+	+	-	-	+	+	-	-	-	Pseudomonas
BI-3	Gram +ve	Rod/ motile	-	+	+	-	-	+	+	+	-	-(G)	Bacillus subtilis
BI-4	Gram -ve	Cocci/Bacilli/ non motile	-	-	-	-	-	-	+	-	-	-	Acinetobacter
BI-5	Gram -ve	Rod/Motile	-	+	+	-	-	+	-	-	-	-	Enterobacter
BI-6	Gram-ve	Rod/Motile	-	+	+	+	+	-	+	+	-	+(G)	Proteus mirabilis
BI-7	Gram -ve	Rod/non motile	-	+	+	-	-	-	+	+	-	+(G)	Klebsiella pneumonia
BI-8	Gram +ve	Rod/motile	-	-	-	-		-	+	+	+	+	Bacillus licheniformis

The standard PCR results expected amplicon size with 1500bp of 16S rRNA gene, which was amplified at 55°C annealing temperature with high integrity in all samples. The resulted amplicon was observed on 1 % Agarose Gel is shown in the following Figure 5.

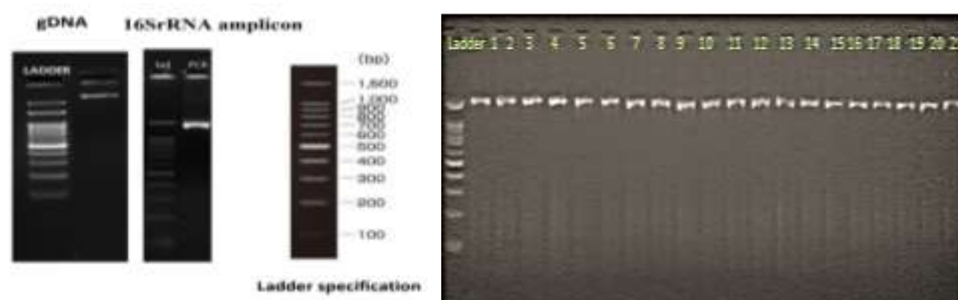

Figure 5. 16S rRNA Gene amplification

Phylogenetic analysis:

Most similar sequences of identified 16S rRNA gene sequences of bacterial species were retrieved by performing BLAST-N tool. Further, multiple sequence alignment was performed to identify conserved regions among them in MEGA software using ClustalW2. Poor and

divergent regions of alignment were eliminated for predicting accurate phylogeny. Neighbor-Joining phylogenetic tree was generated for aligned 16S rRNA sequence using Jukes-Cantor (JC) model using Maximum likelihood substitution (ML) model. Bootstrap tests with 1000 replicates which are associated with clustered taxa together were used to construct the phylogenetic tree. The tree was analyzed to identify the evolutionary relationship between identified bacterial species and their similar species**.** Phylogenetic analysis of 16S rRNA gene sequences of identified bacterial strains in silkworm gut were given in the Fig 6.

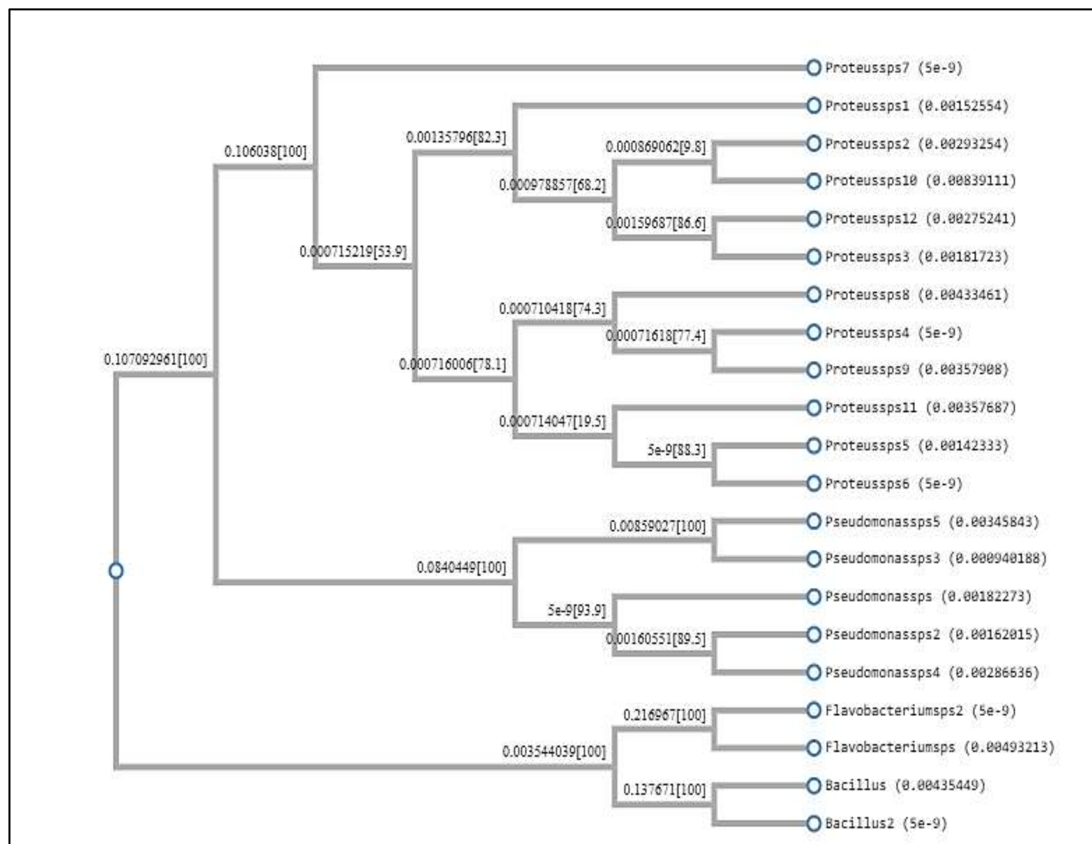

Figure 6 Phylogenetic analysis of identified bacterial strains in silkworm gut (based on 16SrRNA gene)

The gut analysis of identified bacterial strains in the selected silkworm breeds were as tabulated in Table 3.

Table 2 Overview of Gut analysis

Source	Identification by sequence alignment with BLAST algorithm in GenBank	Identification by RDP database (Genus level identification)	Accession numbers assigned
G1- Pure Mysore (Healthy/Control)	*Proteus mirabilis*	*Proteus*	OP748769
	Proteus sp.	*Proteus*	OP748776 OP748772, OP748773
	Pseudomonas sp.	*Pseudomonas*	OP749863

G2 - Pure Mysore (Unhealthy/Starved)	*Proteus sp.* *Proteus mirabilis*	*Proteus*	OP748770 OP748771, OP748774
	Pseudomonas sp.	*Pseudomonas*	OP749862 OP749864
G3 -FC1xFC2 (Healthy/Control)	*Flavobacterium chungangense*	*Flavobacterium*	OP703594
	Bacillus licheniformis *Bacillus licheniformis*	*Bacillus*	OP737801, OP737802
	Proteus sp.	*Proteus*	OP748861, OP748777
	Pseudomonas	*Pseudomonas*	OP749861
G4 -FC1xFC2 (Unhealthy/Starved)	*Pseudomonas sp.*	*Pseudomonas*	OP749865
	Proteus sp.	*Proteus*	OP748775, OP748862 OP748860
	Flavobacterium chungangense	*Flavobacterium*	OP703595

Discussion

The insect- Gut microbiome understanding was made easy with the advent of several modern approaches for bacterial profiling in which the 16S rRNA gene marker sequencing generates rapid and precise data and emerged as a good phylogenetic tool in identifying the genetic diversity. In the current research study, the indigenous native varieties like Pure Mysore - Multivoltine breeds and commercial Foundation cross breeds FC1xFC2 -Bivoltine double hybrids were selected and collected from the authorized silkworm centers like Central Silk Board and Andhra Pradesh State Sericulture Department. The silkworm generations were maintained by rearing practices, the silkworm was grouped into four categories the healthy group, the active feeding worms were considered as healthy whereas for the unhealthy grouping, (starved) were considered as unhealthy groups.

The current study involved the gut analysis of two selected races and breed of silkworms under two groups were reared. The silkworms were grouped and were dissected for gut sample extraction. The gut samples were further processed for bacterial culturing using plating technique for all 4 groups. The colonies were identified to examine the bacterial diversity in gut region of silkworms using 16S rRNA gene amplification and biochemical characterization. The gene amplification was performed using universal primers with PCR method. The obtained 16S rRNA gene amplicon were verified by Agarose Gel Electrophoresis and purified using gel extraction kit. The purified gene amplicon was sequenced using Sanger's Sequencing method and the obtained 21 sequences were aligned and submitted to NCBI. All 21 sequences were compared to the GenBank using BLAST search resulting in homology

sequences. The close sequence relative with higher identity or similarity score were generated. In all four groups the majorly found bacteria belonged to the *Proteus sps* with different species like *Proteus mirabilis, Proteus sp.* etc., followed by *Pseudomonas sp.* and i.e., showing their common existence in both healthy and unhealthy group (G1, G2, G3, G4) of Pure Mysore (PM) Multivoltine and FC1xFC2 double hybrid Bivoltine respectively, whereas the *Bacillus licheniformis* were only found in the FC1xFC2 Bivoltine healthy groups (G3) which show probiotic property and *Flavobacterium* species were found in both healthy and unhealthy group of FC1xFC2 (G3 &G4). All the isolates taken for 16S rRNA sequencing were also confirmed by the phenotypic characterization. In earlier studies, the identified bacterial species were *Enterococcus, Acinetobacter, Bacillus and Enterobacter* and majorly involved in metabolisms of silkworms (Chen *et al.,* 2018). High throughput sequencing studies on silkworms demonstrated the presence of dominant species of bacteria belonging to *Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes* (Chen, B; et al., 2018).

Hui *et al.,* (2010) examined the silkworm larvae and reported 41 bacterial phylotypes through PCR/DGGE technique and 16S rDNA gene library analysis. Enterobacteriaceae species were also identified and reported in silkworm gut (Thangamalar *et al.,* 2009). Cellulolytic gut bacteria help in digestion, absorption of nutrients in silkworms. The taxonomic diversity studies on various silkworm strains such as Dazao (P50) and Qiufeng × Baiyu (QB) by shotgun metagenomics and direct 16S rRNA gene analysis revealed the presence of similar dominant gut bacterial population in all strains. 16S rRNA probes were used to identify *Bacillus subtilis, Pseudomonas fluorescens* and *Streptomyces noursei* in silkworm gut flora (Subramanian, et al., 2009). Gut microbes play an essential role in the digestion of xylan and cellulose rich mulberry leaves by breaking down the nutrients into simpler forms aiding in easy assimilation.

In parallel, the current study identified various bacterial species in gut region emphasizing the presence of various Gamma proteobacteria, the predominant *Proteus* species and *Pseudomonas* in all four groups and the observation of *Bacillus Licheniformis* and *Flavobacterium* species in G3 healthy group of FC1xFC2. *Flavobacterium* observed and reported for the first time in the healthy and unhealthy groups of G3 and G4 of FC1xFC2 Bivoltine breeds. This study signifies the association of gut bacteria with the host system in deciding various underlying mechanisms of silkworms

The current study indicated that a healthy gut bacterial flora tends to improve the silkworm's nutritional effectiveness and economic traits. It also throws light on the interactions between the gut bacteria and the host's natural microbiota that affect the host's

innate immunity, physiology, and capability for proper digestion in silkworms with good yield.

Several bacteria with probiotic qualities have been found in the gut of silkworms, and commercial probiotics can be produced using this beneficial gut flora for increased, high-quality cocoon production. This study has thrown light on a way to evolve mechanisms by which bacteria (probiotics) introduced through mulberry leaves boost the nutritional and economic aspects of the silkworm and in particular, on how probiotic bacteria interact with the host's original microbiota to impact the silkworm's innate immunity, physiology, and ability to assimilate food.

References

Baker GC, Smith JJ, Cowan DA. Review and re-analysis of domain-specific 16S primers. J Microbiol Methods. 2003;55(3):541–55.

Barretto, D.A.; Gadwala, M.; Vootla, S.K. Chapter 1—The silkworm gut microbiota: A potential source for biotechnological applications. Methods Microbiol. 2021, 49, 1–26.

Bergey D,Boone D.2009: Bergey’s Manual of Systematic bacteriology Volume 3, 2nd edition,Springer:127.

Bouvet, P., Ferraris, L., Dauphin, B., Popoff, M., Butel, M. J. and Aires, J. 2014. 16S rRNA gene sequencing, multilocus sequence analysis, and mass spectrometr y identification of the proposed new species “Clostridium neonatale”. Journal of Clinical Microbiology, 52(12): 4129–4136.

Brand JM, Bracke JW, Markovetz AJ, Wood DL, Browne LE. 1975. Production of verbenol pheromone by a bacterium isolated from bark beetles. Nature 254: 136-137.

Brune A. 2003. Symbionts aiding digestion. In: Resh VH, Cardé RT, Editors. Encyclopedia of Insects. pp. 1102-1107. Academic Press.

Chen, B.; Du, K.; Sun, C.; Vimalanathan, A.; Liang, X.; Li, Y.; Wang, B.; Lu, X.; Li, L.; Shao, Y. Gut bacterial and fungal communities of the domesticated silkworm (Bombyx mori) and wild mulberry-feeding relatives. ISME J. 2018, 12, 2252–2262.

Choi, B.K., Wyss, C., Gobel, U.B., 1996. Phylogenetic analysis of pathogen related oral spirochetes. J. Clin. Microbiol. 34, 1922–1925.

Clarridge III, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17, 840–862.

Conlan, S., Kong, H.H. and Segre, J.A. 2012. Species-level analysis of DNA sequence data from the NIH human microbiome project. PLoS One, 7: e47075. doi: 10.1371/journal.pone.0047075

Dehingia M, Devi KT, Talukdar NC, Talukdar R, Reddy N, Mande SS, et al. Gut bacterial diversity of the tribes of India and comparison with the worldwide data. Sci Rep 2015;5:18563.

Dillon R, Charnley K. 2002. Mutualism between the desert locust Schistocerca gregaria and its gut microbiota. Research in Microbiology 153: 503-509.

Fettweis, J.M., Serrano, M.G., Sheth, N.U., Mayer, C.M., Glascock, A.L., Brooks, J.P., Jefferson, K.K., Vaginal Microbiome Consortium (additional members) and Buck G.A. 2012. Species level classification of the vaginal microbiome. BMC Genomics, 13 (8): S17.

Fukatsu T, Hosokawa T. 2002. Capsule transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, Megacopta punctatissima. Applied and Environmental Microbiology 68(1): 389-396.

Hui, X., Muwang, L., Yong, Z., Liping, Z et al (2010). Bacterial community in midguts of the silkworm larvae estimated by PCR/DGGE and 16S rDNA gene library analysis. Acta Entomologica Sinica, 50(3), 222–233

Liu H, Chen B, Hu S, Liang X, Lu X, Shao Y. Quantitative proteomic analysis of germination of Nosema bombycis spores under extremely alkaline conditions. Front Microbiol. 2016;7: 1459.

Methe BA, Nelson KE, Pop M, Creasy HH, et al. A framework for human microbiome research. Nature 2012; 486:215-21.

Moran NA, Russell JA, Koga R, Fukatsu T. 2005. Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. Applied and Environmental Microbiology 71(6): 3302- 3310.

Munson, M.A., Banerjee, A., Watson, T.F., Wade, W.G., 2004. Molecular analysis of the microflora associated with dental caries. J. Clin. Microbiol. 42, 3023–3029.

Pandiarajan, J., & Krishnan, M. (2018). Comparative bacterial survey in the gut of lepidopteran insects with different bionetwork. Microbiology, 87(1), 103–115.

Petti, C.A., Polage, C.R., Schreckenberger, P., 2005. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. J. Clin. Microbiol. 43, 6123–6125

Rossi-Tamisier, M., Benamar, S., Raoult, D. and Fournier, P. 2015. Cautionary tale of using 16S rRNA gene sequence similarity values in identification of human associated bacterial species. International Journal of Systematic and Evolutionary Microbiology, 65, 1929–1934.

Schmalenberger, A., Schwieger, F., Tebbe, C.C., 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small subunit rRNA gene in PCR-based microbial community analyses & genetic profiling. Appl. Environ. Microbiol. 67, 3557–3563.

Stewart EJ. Growing unculturable bacteria. J Bacteriol 2012;194:4151-60.

Subramanian, S., Gadhave, K.R., Mohanraj, P., and Thangamalar, A., Use of 16S rRNA probes for characterization of gut microflora of silkworm (Bombyx mori L.) breeds, Karnataka J. Agric. Sci., 2009, vol. 22, no. 3, pp. 476–478.

Sun Z, Kumar D, Cao G, Zhu L, Liu B, Zhu M, Liang Z, Kuang S, Chen F, Yongjie F, Hu X, Xue R, Gong C (2017) Effects of transient high temperature treatment on the intestinal flora of the silkworm Bombyx mori. Sci Rep 7:3349

Thangamalar, A., GadhaveKiran Ramesh, Subramanian, S., and Mahalingam, C.A., Use of biochemical kits for characterization of Enterobacteriaceae from the gut of silkworm, Bombyx mori L., Karnataka J. Agric. Sci., 2009, vol. 22, no. 3, pp. 514–516.

Tringe SG, Hugenholtz P. A renaissance for the pioneering 16S rRNA gene. Curr Opin Microbiol. 2008;11(5):442–6.

Wang Y, Qian P-Y. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One. 2009;4(10):e7401.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271

Xu X, He J, Xue J, Wang Y, Li K, Zhang K, et al. Oral cavity contains distinct niches with dynamic microbial communities. Environ Microbiol 2015;17:699-710.