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Detection of Gut Microflora and Enzymes Using Metagenomics and Metaproteomics in Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

Ponnusamy Mohanraj[1], Chinnan Velmurugan Karthikeyan[2]*, C Aruchamy Mahalingam[3], Sendha Venkatachary Krishnamoorthy[1], Dananjeyan Balachandar[1], Babu Ramanathan[4]

[1]Department of Sericulture, Forest College and Research Institute, Tamil Nadu Agricultural University, Mettupalayam, Tamil Nadu, India

[2]Department of Biotechnology, MIT College of Agriculture and Technology, Sempatti, Tamil Nadu, India

[3]Department of Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

[4]Department of Biological Sciences, School of Science and Technology, Sunway University, Kuala Lumpur, Malaysia

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***For Correspondence:**
Chinnan Velmurugan Karthikeyan, Department of Biotechnology, MIT College of Agriculture and Technology, Sempatti, Tamil Nadu, India
E-mail: ckskarthik@gmail.com

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ABSTRACT

Microorganisms present in insect guts impart very important role in growth development and adaptation among the hosts. The identification of such microorganism in gut would provide new ways to develop products that would be important in industries and for pest management applications. In our present study, we employed a culture independent metagenomic approach in conjunction with metaproteomic profiling to enumerate the gut microbes in pure races and cross breeds of silkworm, *Bombyx mori* L. The phylogenetic analysis showed that the gut microflora was majorly grouped into four bacterial classes: Gamma proteobacteria, actinobacteria, cocci and bacilli. Two novel proteins in both silkworm races were identified and an additional three proteins were found to be differentially expressed among the races. In conclusion, silkworm undergoes morphological variations upon metamorphosis and studying this gut microflora will provide access to different genomes and the corresponding protein profiles. The results presented in this study would help identify the unknowns of silkworm gut microbiota and their importance in the silkworm.

Keywords: Silkworm; Gut microbes; Metagenomics; Metaproteomics; Genomes; Metamorphosis; Microbiota; Gamma proteobacteria; Actinobacteria; Cocci; Bacilli; Industries; Detoxification; Biochemical tests

INTRODUCTION

Silkworm is important insect for silk industry and the midgut silkworm production primarily depends on the nutrient regulated by enzymes at larval stages. This are regulated by the microbiota system present in the midgut of the insect and it plays a role in adaptation, biomass degradation and compound detoxification [1,2]. The breeds of insect and environmental conditions causes variation in such microbes present in the gut. The disruption of such microbes due to internal and external factors would potentially affect the silkworm health resulting in disease such as Colony Collapse Disease (CCD) [3]. The diversity such gut microbes varies widely according to breeds of insects and their environmental conditions.

The identification of such microbes could be done using carious biochemical tests [4]. But molecular biology techniques had provided better opportunity to understand the diversity without culturing of the live organisms [5]. The most common molecular technique is by using 16S rRNA gene (16S rDNA). The use of 16s rRNA would provide us highly conserved and variable sequences that would be useful in identification of bacterial taxa.

The advances in 'omics' have enabled us to explore microorganism communities in a precised way. The high-throughput metagenome and metaproteome analysis in conjuction with high throughput functional screening and data annotation have helped us in speeding up molecular level

investigations [6-8]. The identification of novel enzymes, pathways and organisms for various applications has been done through these techniques [9,10]. The use of metagenome and metaproteomics has been applied in wood feeding insects and termites to understand biomass degrading mechanism [11] and lower termites [6]. In termites, protozoa and symbiotic bacteria in the hindgut hydrolyses cellulose and hemicelluloses [11-14]. The analysis also resulted in identification of bacterium covering 12 phyla and 216 phylotypes, and also led to identification of more than 100 glycoside hydrolases. The advancements in "meta" approaches have provided the betted understanding of microbial community and lead way to develop them as biocatalysts in industries [15].

In our present study, we used metagenomics and metaproteomics approach to explore the diversity of gut microflora and their potential enzymes in silkworm larvae that would play a crucial role in nutrient uptake, its adaptation, silk quality and production.

MATERIALS AND METHODS

Silkworm breeds and maintenance

Gut microflora of selected pure silkworm races, Pure Mysore (PM) (multivoltine) and CSR-2 (Central Sericulture Research Institute, India) (bivoltine) were enumerated in the present study using a culture free metagenomics approach (Figure S1). Quality mulberry leaves of variety V1 were fed to the silkworm breeds obtained from disease free laying's. The silkworm breeds were grown in rearing room under hygienic conditions with optimum temperature (25ºC-28ºC) and relative humidity (75%-85%). The mulberry leaves were washed thoroughly in running water, shade dried and both sides of leaves were surface sterilized under Ultra Violet (UV) light before giving as feed to silkworms. The leaf feeding was given 3-4 times a day after hatching [16].

Dissection and culturing the silkworm gut microflora

Three days old 5th instar larvae were collected and kept under starvation for 17 hours-19 hours for bivoltine and 20 hours-22 hours for multivoltines. The larvae were surface sterilized with 7% alcohol, anesthetized using 50% alcohol for 1 minute and then dipped in sterile water for softening of skin. Dissection was carried out under aseptic conditions. The extracted guts were homogenized in sterile double distilled water (cells viable for one month) or 6% glycerol (cells viable for one year). For further use, extracts were stored at -4ºC. The total gut microflora was cultured on nutrient agar medium (Sigma-Aldrich, USA).

Isolation of genomic DNA from silkworm gut microbes

The genomic DNA from silkworm gut microbes were extracted using FAST DNA Spin Kit (MP biomedicals, USA) following the manufacturers instruction.

Isolation and cloning of 16S rRNA

The full length 16S rRNA gene was amplified using forward and reverse primers (Forward- 5'AGA GTT TGA TCC TGG CTC AG 3', Reverse-5'ACG GCT ACC TTG TTA CCA CTT 3') by standard PCR procedures with an annealing temperature of 55ºC. The amplified product were purified and cloned onto pTZ57R/T. The construct was transformed into *Escherichia coli* DH5α competent cells and transformants were analysed by blue white screen using X-Gal (20 mg/ml).

Colony PCR and library maintenance

Colony PCR was performed to confirm the insert using M13 forward and reverse primer (Forward- 5'-GCC AGG GTT TTC CCA GTC ACG A-3' and Reverse- 5'-GAG CGG ATA ACA ATT TCA CAC AGG-3') with PCR conditions described earlier. Positive clones were identified by separating amplification products on an agarose gel. All the positive clones were stored as glycerol stock (60% glycerol) in -70ºC for further analysis.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

PCR-amplified 16S rDNA fragments were digested with endonucleases HaeIII (Fermentas, USA) at 37ºC for 3 hours and the fragments were separated on an agarose gel. In order to determine the similarity among clones, a binary matrix was established by recording the presence or absence of bands in ARDRA profile. Pairwise comparisons for similarity were calculated using Jaccard's coefficient and UPGMA algorithm was used for construction of dendrogram in NTSYSpc V2.2 package (Exeter software, USA).

Sequencing and phylogenetic analysis

Sequencing was performed in an applied biosystems automated sequencer (Applied Biosystems, USA) using M13 forward and reverse primers. The sequences were analysed with B2C2 software for chimeras [17]. The homology sequence of 16S rRNA gene sequences was analysed using BLAST tool [18] and phyla-wise grouping was done. The phylogenetic tree was constructed using MEGA V 5.0 software with 16S rRNA gene sequences from different bacteria, obtained from NCBI GenBank database by neighbour-joining method.

Protein profiling of gut microbes

Silkworm total gut was ground in a solution containing 10% Trichloracetic Acid (TCA) (Merck, USA), 0.07% Dithiothreitol (DTT) (Sigma-Aldrich, USA), acetone (Merck, USA) and centrifuged for 15 minutes at 4500 g. The pellets were washed once with ice cold acetone (Merck, USA) consisting 0.07% DTT (Sigma-Aldrich, USA) at -20ºC for 1 hour and centrifuged as above. The final precipitated pellets were lyophilized and used for proteomics analysis.

2D-PAGE

2D-PAGE analysis was performed using the IPG strips (17 cm, pH 4-7) (Bio-Rad, USA) following manufacturers instruction. Briefly, 350 µl of rehydration buffer containing 130 µg of protein were used to rehydrate the strips following isoelectric focusing at 20ºC. The samples were run at 500 V, 1000 V and 3000 V for 1 hour, 1.5 hours and 16 hours respectively. The strips were equilibrated in 10 ml equilibration solution for 15 minutes. The strips were rinsed with electrode buffer and place in acrylamide gel. The gel was electrophoresed at constant current (15 mA) till the dye front reaches bottom of the gel. Gels were fixed and stained by silver staining method. Silver stained gels were scanned using the densitometry scanner and images were analysed using Image Master 2D platinum V.2 Software (GE healthcare, USA).

In-gel digestion of proteins with trypsin and mass spectrometry

The protein spots of interest were excised and were subjected to in-gel digestion with modified trypsin (Roche, USA) to a final concentration of 100 ng/µl. The digested gel pieces were subjected to zip-tip purification following manufacturer's instructions (Sigma-Aldrich, USA). The purified peptides were eluted directly into 1 µl-2 µl matrix consisting 0.1% Trifloro acetic acid and 50% CH_3CN (Fluka, USA). The MS/MS analysis were carried out by using 4700 matrix-assisted laser desorption/ionization (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems, USA).

Database searching

The data was subjected to MASCOT version 2.0 (Matrix Science, UK) for mass analysis and sequence identification. The search was than performed taking other metazoa as taxonomy, which contained 1,54,412 sequences. The data total of 2,464,940 sequence were submitted to the National Center for Biotechnology Information non-redundant (NCBInr) protein database. The protein was identified using monoisotopic and deisotoped masses with fixed modifications of carbamidomethyl (C), variable modifications of oxidation (Met), peptide tolerance of 55 ppm to 370 ppm, peptide charge of 1+ with one maximum number of missed cleavages. The covalent modifications were allowed only for N terminus. Minimum peptide match of 5, matched peptide of ≥ 10% or identification score of ≥ 50% was used for identification of matched peptides. Based on molecular weight or pI proteins, only significant hits, defined by MASCOT probability analysis ($p<0.05$) were accepted.

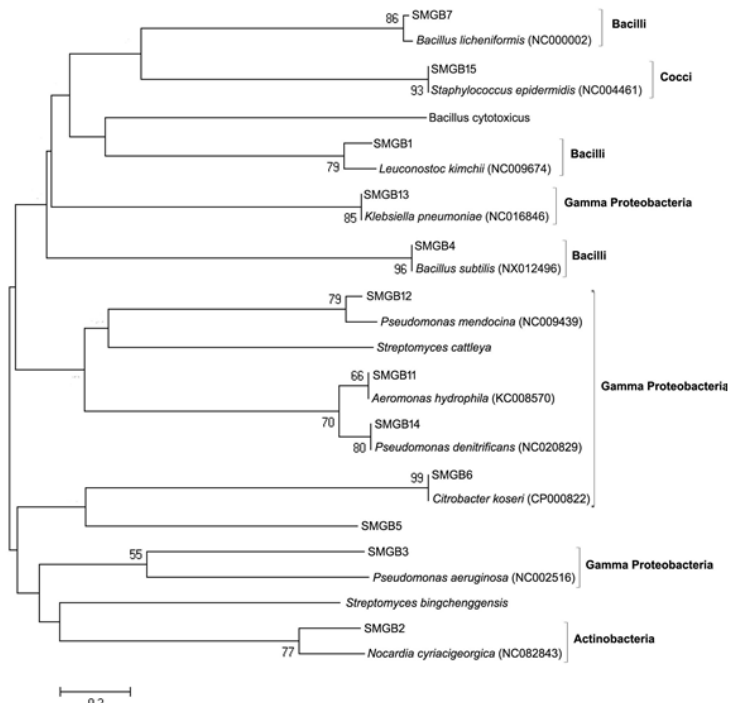
RESULTS

Metagenomic studies on gut microbes of CSR 2 and PM race

PCR amplification was carried out from the genomic DNA isolated from three days old 5th instar larvae gut using universal primers (FD1 and RP2) (Figures S2 and S3). Cloning was carried out onto pTZ57R using HaeIII restriction enzyme. The phylogenetic relationship within the clones to their biodiversity was carried out using B2C2 software (Figure 1). The phylogenetic tree reported in our study with regard to deep branches of distantly related taxa shows only estimation of phylogenetic similarity. 16 clones were identified to belong to major bacterial group. Based on tree, bacteria were classified into four classes including bacilli, gamma proteobacteria, cocci and actinobacteria (Table 1). The cluster containing clone SMGB 5 (silkworm metagenomic gut bacteria) fails to branch within any of the major groups of bacteria. Three clones (SMGB7, SMGB4 and SMGB1) of bacilli showed more than 75% to 96% nucleotide identity with reference organism. Clone SMGB4 showed the highest level of sequence identity (96%) to a bacilli form of bacteria *Bacillus subtilis* and SMGB7 clone 86% nucleotide identity to *Bacillus licheniformis*. The nucleotide identity of gamma proteobacteria with reference organisms were between 55%-99 %. Among the gamma proteobacteria classes, highest percentage of nucleotide similarity was found in SMGB6 clone which has been reported to be closely matched with *Citrobacter koseri*. The low identity was recorded for SMGB3 (55%) that matched with reference organism *Pseudomonas aeruginosa*. The cluster of gamma proteobacteria classes were closely related to each other within same class expect SMGB13 clone, which was closely matched (85% nucleotide identity) with *Klebsiella pneumoniae*.

The clones SMGB15 and SMGB2 were closely related to cocci form of bacteria and actinobacteria. It showed 93% and 97% nucleotide identity to reference organism *Staphylococcus epidermidis* and *Nocardia cyriacigeorgica* respectively. Among the 16 clones, 11 clones matched within each group and 4 clones were out of group. Clones, SMGB1, SMGB7 and SMGB15 formed the maximum cluster in the tree. The bootstrap value between SMGB11 and SMGB14 node were higher (70%) when compared with other nodes. Three clusters were mostly found in the tree including SMGB13, SMGB12, SMGB11, SMGB14 and SMGB3, followed by two clusters which were observed in three clones namely, clones SMGB2, SMGB6 and SMGB5. The clone SMGB5 did not match with any other groups.

Figure 1. Phylogenetic tree based on the 16s rRNA sequence from metagenomic DNA of silkworm gut microbes using neighbour joining method. Boot-steps values of 500 or more are added at the nodes. The scale bar represents 0.2 substitutions/base position.



The results showed all clones to be classified under actinobacteria with match of 100% with reference organisms namely, *Actinoplanes missouriensis* (SMBG5), *Streptomyces griseus* (SMBG8), *Streptomyces* spp., (SMBG9), *Arthrobacter arlaitensis* (SMBG10) and *Bacillus atrophaeus* (SMBG16).

Table 1. Sequence homology of selected 16 S rRNA gene sequences of silkworm, PM and CSR2.

Clone number	Silkworm	16 S rRNA gene sequence homology			
		Species identified	Phylum	NCBI* Accession number	Homology
SMGB7	CSR2	*Bacillus licheniformis*	Bacilli	NC000002	86
SMGB1	CSR2	*Leuconostoc kimchii*	Bacilli	NC009674	79
SMGB4	CSR2	*Bacillus subtilis*	Bacilli	NX012496	96
SMGB15	CSR2	*Staphylococcus epidermidis*	Cocci	NC004461	93
SMGB2	PM	*Nocardia cyriacigeorgica*	Actinobacteria	NC082843	77
SMGB13	PM	*Klebsiella pneumoniae*	Gamma Proteobacteria	NC016846	85
SMGB12	PM	*Pseudomonas mendocina*	Gamma Proteobacteria	NC009439	79
SMGB11	PM	*Aeromonas hydrophila*	Gamma Proteobacteria	KC008570	70
SMGB14	PM	*Pseudomonas denitrificans*	Gamma Proteobacteria	NC020829	80
SMGB6	PM	*Citrobacter koseri*	Gamma Proteobacteria	CP000822	99
SMGB3	PM	*Pseudomonas aeruginosa*	Gamma Proteobacteria	NC002516	55

Metaproteomics analyses of silkworm gut micr nobes

The microbial proteins were extracted from midgut of three-day old fifth-instar silkworm larvae. A total of two extractions with three replications each were separated by 2D gel electrophoresis. The proteins identified varied among the silk worm races and we identified some novel proteins related to abundance and upregulation (Figures S4-S6). Among the proteins identified, protein spots SGP1, SGP2, SGP6 and SGP7 were found only in midgut of PM (Figure 2) and protein spots SGP3, SGP4 and SGP5 were found in CSR2 (Figure 3).

Figure 2. Silver stained 2D gel electrophenogram of proteins extracted from silkworm gut of PM race. **Note:** A- SGp-6 (Upregulated), B-SGP 1 (New protein), C- SGP 2 (New protein), D-SGP 7 (upregulated).

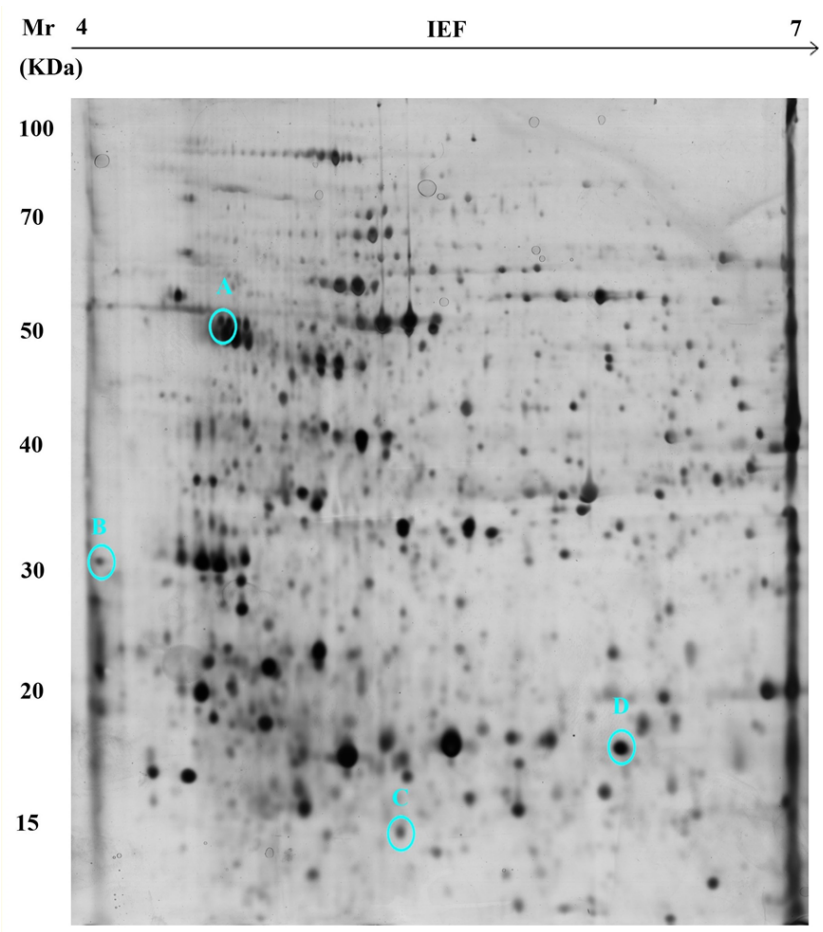
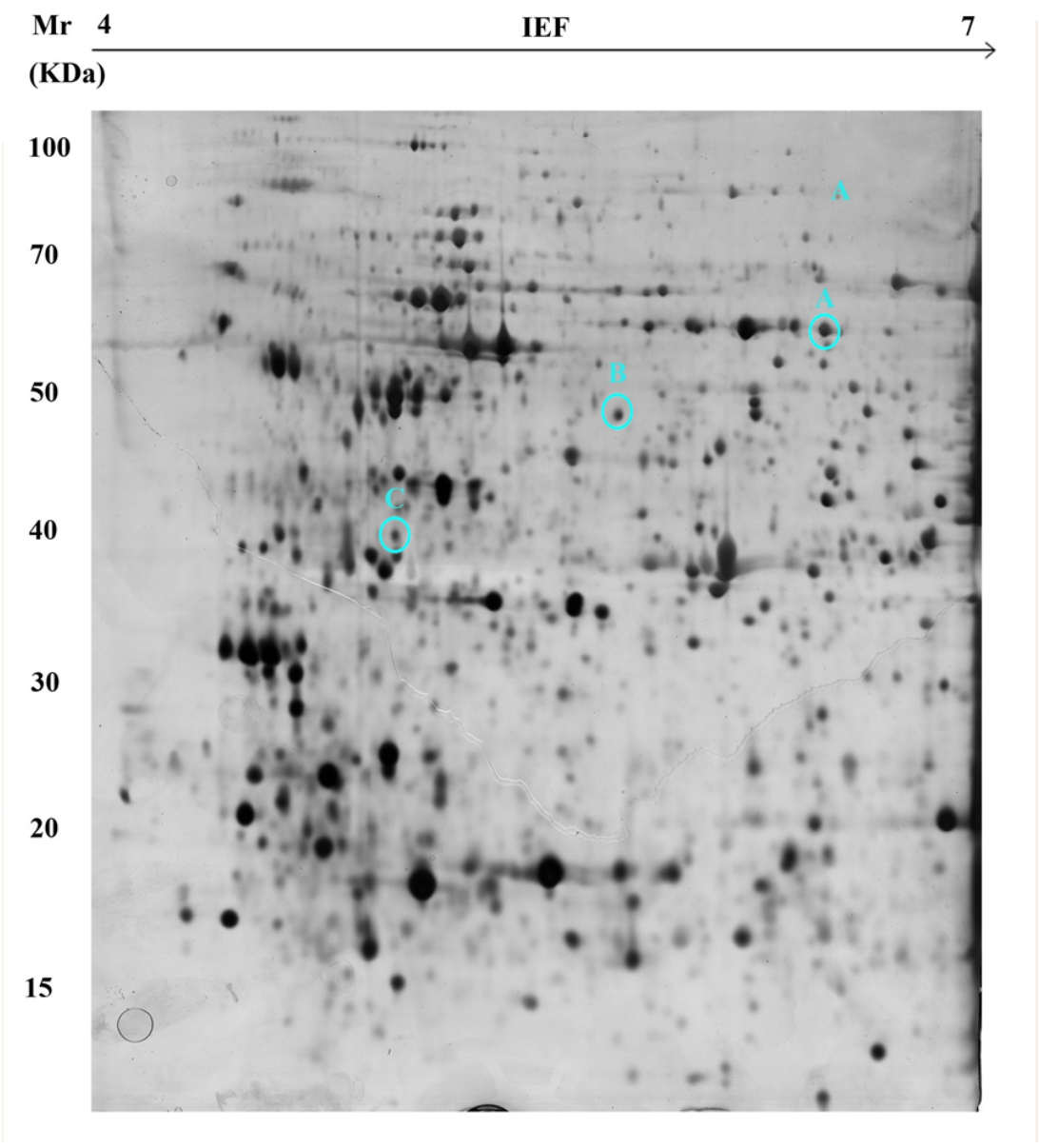


Figure 3. Silver stained 2D gel electrophenogram of proteins extracted from silkworm gut of CSR 2 race. *Note:* A-SGP 5 (Upregulated), B- SGP 3 (New protein), C- SGP 4 (New protein).



The excised protein spots from gel were further analysed by a Voyager DE PRO MALDI-TOF-MS. More than five matched peptides in the MASCOT search with the coverage of larger than 20% were included (Tables 2 and 3). Based on the Gene Ontology (GO) annotation and molecular function, these proteins were classified into three groups.

Table 2. Expression of proteins in the gut microbes identified by MS/MS in pure Mysore.

Spot I.D	Accession no NCBI GI	Protein name	Organism	Protein MW (Da)/pI	Score	Ma	Coverage (%)	Expression pattern
SGP1	498307844	Thioredoxin	*Lactobacillus suebicus*	12341/4.36	61	4	29	New
SGP2	494369769	phenylacetic acid degradation protein	*Hoeflea phototrophica*	15838/6.04	49	7	44	New
SGP6	489787833	RNase HI	*Lactobacillus ruminis*	25933/6.04	71	7	30	Up-regulated
SGP7	His2_clobk	Phosphoribosyl-ATP pyrophosphatase	*Clostridium botulinum*	13048/5.97	56	3	29	Up-regulated

Table 3. Expression of proteins in the gut microbes of identified by MS/MS in CSR2.

Spot I.D	Accession no NCBI GI	Protein name	Organism	Protein MW (Da)/pI	Score	Ma	Coverage (%)	Expression pattern
SGP3	Ure3_chrsd	Urease subunit gamma	*Chromohalobacter salexigens*	11020/4.86	55	4	75	New
SGP4	Sdha_clopr	L-serine dehydratase	*Clostridium propionicum*	3391/4.62	27	1	43	New
SGP5	157165205	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C	*Sphingobacterium spiritivorum*	85115/5.61	70	12	33	Up-regulated

Protein profile of the silkworm PM race gut microbes identified by MS/MS

The mass of the protein spots of gut microflora from PM races were ranging from 10,000 kDa to 30,000 kDa within 4 pI to 6.5 pI region. The two protein spots SGP6 and SGP7 corresponding to RNase HI and Phosphoribosyl-ATP pyrophosphatase were prominent in the silkworm PM gut microflora. The protein spots SGP1 and SGP2 corresponding to thioredoxin and phenylacetic acid degradation protein respectively were found to be new proteins identified in the study. The protein SGP6 matched to a highest MASCOT identical score (score 71) followed by SGP1 protein (score 61), and SGP2 protein (score 49). The proteins thioredoxin and RNase HI found in lactic acid producing bacteria (*Lactobacillus suebicus* and *Lactobacillus ruminis*) had a protein coverage of only 29% and 30% respectively. On the other hand, the proteins produced in *Hoeflea phototrophica* and *Clostridium botulinum* showed a protein coverage of 44% for phenylacetic acid degradation protein and 29% for phosphoribosyl-ATP pyrophosphatase.

Protein profile of the CSR-2 gut microbes identified by MS/MS

The mass of the proteins identified from CSR-2 were ranging from 10,000 kDa to 90,000 kDa with 4 pI to 6 pI region. The most prominent protein identified from CSR-2 gut microflora is the protein corresponding to aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase subunit C protein (spot SGP5). Two additional proteins, SGP3 corresponding to urease subunit gamma and SGP4 corresponding to L-serine dehydratase were also identified in the proteome of CSR-2 gut microflora. The protein SGP5 matched to a highest MASCOT identical score (70) followed by SGP3 protein (score 55) and the lowest score was recorded to SGP4 protein (score 27). The protein aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C produced in *Sphingobacterium spiritivorum* matched to a mass score of 12. Whereas, urease subunit gamma and L-serine dehydratase proteins found in *Chromohalobacter salexigens* and *Clostridium propionicum* showed the mass vales 4 and 1 respectively. The highest protein coverage was recorded to be 75% in urease subunit gamma and 43% in L-serine dehydratase.

Molecular and biological functions of gut microbe proteins identified in PM and CSR2 races

The potential functions of proteins identified in PM and CSR2 gut microflora were described by UniProtKB, Gene Ontology (Tables 4 and 5).

Table 4. Molecular and biological functions of the identified proteins in pure mysore (UniProtKB, Gene Ontology).

Protein name	Molecular function	Biological function
Thioredoxin	Electron carrier activity, protein disulfide oxidoreductase activity	Cell redox homeostasis, electron transport chain, glycerol ether metabolic process
Phenylacetic acid degradation protein	Oxidoreductase activity, transition metal ion binding, thiolester hydrolase activity	May be part of a multicomponent oxygenase involved in phenylacetyl-CoA hydroxylation
RNase HI	Nucleic acid binding, ribonuclease H activity	RNA catabolic process, nucleic acid phosphodiester bond hydrolysis
Phosphoribosyl-ATP pyrophosphatase	ATP binding, phosphoribosyl-ATP diphosphatase activity	Amino-acid biosynthesis, histidine biosynthesis

Table 5. Molecular and biological functions of the identified proteins in CSR2 (UniProtKB. Gene Ontology).

Protein name	Molecular function	Biological function
Urease subunit gamma	Nickel cation binding, urease Activity	Urea catabolic process
L-serine dehydratase, alpha chain	4 iron, 4 sulfur cluster binding, L-serine ammonia-lyase activity, metal ion binding	Gluconeogenesis
Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C	Ligase , transferase	Ligase activity, transferase activity

Based on the molecular functions, the 7 proteins identified in this study were classified under 4 different categories:

1. catalyse (SGP 1).
2. Hydrolase (SGP 1, SGP2, SGP3, SGP6 and SGP7).
3. Lysase (SGP4).
4. Ligase (SGP5).

These are involved in a range of biological functions. Thioredoxin is important for balancing the redox potential which leads to growth inhibition, electron transfer reactions in living cells and metabolic processes. Phenylacetic acid degradation protein is involved in cellular RNA synthesis. RNase H is responsible for the chemical reactions and pathways resulting in the breakdown of RNA. The protein phosphoribosyl-ATP pyrophosphatase is majorly involved in amino acid synthesis. In addition, they are precursors of many molecules such as purines, pyrimidines, histamines, adrenaline, melanin and synthesis of histidine. Urease subunit gamma is responsible for breakdown of urea (the water-soluble compound O=C-(NH2)2). The protein L-serine dehydratase plays a crucial role in formation of glucose from non-carbohydrate precursors, such as pyruvate, amino acids and glycerol. Finally, the protein aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase is an enzyme that acts as catalyst in biochemical reactions.

DISCUSSION

The Phylogenetic analysis of isolated 16S rRNA clones from larval gut consisted of many diverse microbial species and many of them have yet to be characterized. The clones were grouped under four major bacterial classes: Gamma proteobacteria, actinobacteria, cocci, bacilli and the new one proposed to be‘‘Endomicrobia’’ (out groups). The isolates were either strict or facultative anaerobes found to be isolated from animal intestines. Most of the insect gut consists of high population of facultative anaerobe microbes that are anoxic having low redox potential. Similar anaerobes isolated from gut microbiota of the firebug was classified under bacterial phylum actinobacteria and Firmicutes that supplement nutrition required for normal growth [19,20]. Five of the clones were grouped under Gammaproteobacteria that include *Pesudomonas aeruginosa, Citrobacter koseri, Aeromonas hydrophila, Psuedomonas mendocina* and *Klabsiella pneumonia*. The presence of these class of microbes have been recently reported in gut microbes of insects that play a crucial role. Karamipour et al. reported the isolation of gut symbiont microbes belonging to gammaproteobacteria from shield bug *Graphosoma lineatum* [21]. Symbiotic bacteria isolated from oriental fruit fly *Bactrocera dorsalis* showed

enhanced insecticide resistance gammaproteobacteria [22]. Comparative analysis of gut microbiota form various races of mosquitoes showed the presence of microbes belonging to gammaproteobacteria and actinobacteria [23]. The gut contents in millipedes were classified to be bacteria of the gamma subclass of proteobacteria and the actinobacteria. Microbes isolated from silkworm gut microbes in the present study showed similar trend with groups classified under both gammaproteobacteria and actinobacteria. These microbes might have role in either symbiosis or resistance. Nitrogen fixation is also mediated by gut bacteria that is one of the crucial aspects for termite symbiosis as they feed on nitrogen poor wood. Comparative analysis of the gut bacteria of termites and wood-feeding lower termites showed a diverse population of 142 genera, off which nitrogen-fixing bacteria were highly dominant in the wood-feeding termites [24]. Metatranscriptome analysis of subterranean termite gut showed microbes that play direct roles in amino acid biosynthesis, lignocellulose digestion and nitrogen fixation [25]. *Citrobacter freundii* and *Enterobacter agglomerans* nitrogen-fixing bacteria have been previously isolated from several kinds of termites [26,27]. The isolated microbes that are categorized under gammaproteobacterial cluster include *Citrobacter*, *Pseudomonas*, *Klebsiella* and *Aeromonas*. These clones might play a role in nitrogen fixation. Some of the *Nocardia* sp. are also found to be a nitrogen fixer [28] and isolate form. Silkworm gut microbe *Nocardia cyriacigeorgica* might also play a key role as nitrogen fixer. The phylum bacilli takes part in earlier and intermediate steps of polymer degradation. Microbes residing in guts produce different hydrolotic enzymes. Earlier studies showed that *Paenibacillus* ICGEB2008 isolated from the gut of the cotton bollworm produces cellulases and hemicellulases that degrade biomass [29,30]. Similarly, Wenzel et al.[31] has shown to degrade polymeric material under oxygen limitation. In this study, *Bacillus licheniformis* from the silkworm gut could produce cellulolytic enzymes that might aid in the breakdown of mulberry aiding good absorption of nutritional material. On the other hand, *Bacillus subtilis* might produce enzyme lipase that could involve in breakdown of fats to fatty acids and glycerol that are important to both male and female larva. Earlier studies on isolation of lipases from silkworm showed to have antiviral activity against Nucleopolyhedrovirus [32]. While other genera's isolate from the silkworm gut microbes including *Staphylococcus*, *Klebsiella*, *Pseudomonas* and *Aeromonas* are lipase producing bacteria. The lipase-producing bacterial community depends upon type of food materials provided. The results here show that diet has a significant impact on gut microbial community. Cocci, particularly *streptococcus epidermidis* has been previously thought to be causing skin diseases. Recent evidence has been developed involving the word "commensal" meaning one organism benefiting without causing no harm to the other (commensalism) or both find organism beneficial (mutualism and proto-cooperation). *Streptococcus epidermidis* is such an organism that plays an active role in host defense having symbiotic relationship [33]. The cocci obtained from silkworm gut could play a similar role of symbiotic relationship.

The efficient insect immune system allows them to fight against various pathogenic infections. The defense lies in gut microbiota that serves immunity by either producing antimicrobial peptide or by innate immune system. The innate immunity system may be carried out by serious of mechanism [34]. Miyashita et al. showed immune response in silkworm increased by ingested bacteria leading to systemic infection tolerance [35]. This was achieved through the injection of heat-killed microbes, such as *Candida albicans*, marcescens cells, which exhibited tolerance against *Pseudomonas aeruginosa* [36]. The strains isolated from gut microflora could play an important role in governing immune system in silkworm. Particularly with regard to bacilli phylum that has shown studies pertaining to cause immunity in few systems. *Bacillus licheniformis* investigation in animal models particularly mouse showed prevention of asthma development [37]. Similarly, *Bacillus licheniformis* derived bio surfactant showed modulation of immune response *Aeromonas hydrophila* in fish [38]. The other strain *Bacillus subtilis* has also shown immune in various systems. Guo et al. proved probiotic *Bacillus subtilis* strain to stimulate immune system for common infectious disease in elderly period by increasing salivary SIgA and serum IFN-gamma levels [39]. *Pseudomonas mendocina* on other hand has been found to synthesis medium-chain-length polyhydroxyalkanoate (PHAMCL) and Alginate Oligosaccharides (AO). AO has many biological activities including anticoagulation, antioxidation, and immune regulation [40]. Similarly, nano-vaccine developed using membrane protein (OmpW) of *Aeromonas hydrophila* showed a dose dependent Immunity in Rohu fish [41,42]. Recent research enlightened *Pseudomonas aeruginosa* as immune elicitor by secreting type II protease IV functions in *Arabidopsis* [41]. Protease IV activates pathway involving G protein signaling in immune function. Proteins play a major role in bringing about immune system in various insects [42]. In fact, it has been found that silkworm cocoon consists of many proteins that are of immune related. Eventually [43] isolated proteins from *Bombyx mori* cocoon that inhibited the germination of *Beauveria bassiana* spores. Overall, it is important to identify proteins that are expressed in gut microflora by the identified micro-organisms and its role in providing immunity. The protein identification could also enlighten its role on various growth factors, nutrition and immunity. In our experiment, we have identified proteins of gut microbes by 2 electrophoresis combined with MALDI-TOF-MS, and MS spectrum. 5th instar larvae were used to isolate the proteins since most of biochemical metabolism dramatically changes during this period. We identified 7 protein spot, off which highest score was found in SGP6 protein (score 71) and SGP5 protein (score 70). Most of the identified proteins were found to be correlated with innate immunity and metabolism process that are similar with the proteins identified from other insects.

RNase H (SGP6) was the major protein with score of 70. It was found to be highly expressed in gut of silkworm PM when compared with CSR2 race. RNase H is an endoribonuclease that catalyzes cleavage of ribonucleic acid through hydrolytic mechanism. It specifically cleaves the RNA strand within RNA-DNA hybrids. The enzyme plays a huge role in microorganisms to provide immune system against invading pathogens by disrupting their RNA-DNA hybrid. It is found in all organisms ranging from archaea, bacteria and eukaryote. Gupta et al. enlightened RNAse H to inhibit

Mycobacterium infection activity in *Mycobacterium smegmatis* [44]. The enzyme also plays a role in protection of organism against UV and oxidation damage. RNase H enzyme also contributes in growth and development of the organism. They also play role in transcription carrying out metabolism of RNA primers of okazaki fragments formed during lagging strand in DNA replication. An endonuclease R1Bm element was isolated from silkworm *Bombyx mori*, which is widely distributed retrotransposons that had high sequence-specific similarity to human L1 retrotransposon sequence. These endonucleases belong to general class of *Escherichia coli* Exo III having ribonuclease H activity and could in principle be important for retrotransposition [45]. Earlier in silkworm, it was observed that the midgut digestive juice possessed an RNase activity that degrades dsRNA genome of the Cytoplasmic Polyhedrosis Virus (CPV) [46]. The work by Arimatsu et al. [47] also produced similar results wherein 41 kDa RNAse was isolated against polyhedrosis virus from *Bombyx mori* that showed similarity to bovine thymus endoribonuclease H. RNase H isolated from the silkworm in this study might either play a role in immune system or growth and development. Further characterization of this protein could lead us in better understanding of its exact role in the insect gut.

The next highest score was recorded for Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C (SGP5). Higher expression of aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase was found in the gut of bivoltine silkworm larvae pure race CSR2. Aminotransferase in general are enzymes that hydrolyze amino acids particularly glumatine or asparagines to obtain ammonia that are further used by enzyme itself for further catabolism/reactions [48]. Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase catalyses transamidation reaction forming correctly charged Asn-tRNA(Asn) or Gln-tRNA(Gln) in organisms that lack either or both these enzymes. The enzyme utilizes Gln or Asn with ATP for protein synthesis. In *Helicobacter pylori*, the heterotrimeric tRNA-dependent amidotransferase (GatCAB) catalyzes the conversion of misacylated Glu-tRNA(Gln) and Asp-tRNA(Asn) into Gln-tRNA(Gln) and Asn-tRNA(Asn) for protein biosynthesis [49]. Few of heterotrimeric amidotransferase genes (gatA, gatB, and gatC genes) were identified in *Chlamydia trachomatis* genome. This genes play a major role in amino acid synthesis and proper folding of proteins [50]. The enzyme thus isolated could play an important role in protein synthesis or folding in gut microbiota. Raczniak et al. found aspartic proteinases to be widely distributed among plants [51]. The entire role of aspartic proteinase is yet to be established. It is thought to be involved in protein processing or degradation in various stages of plant development. In present experiment, higher expression of aspartyl protein was observed in bivoltine silkworm when compared with multivoltine silkworm. Generally, silkworm larvae were fed on mulberry leaves alone. The aspartyl protein content could be recovered from mulberry leaves since mulberry leaves are rich source of aspartyl protein compared with glutamyl protein.

The other protein identified was thioredoxin (SGP 1) with a score of 61. Thoredoxin have a huge role in this environmental system ranging from plants, bacteria to human system. In plants, it catalyzes reversible disulfide-bond formation to regulate structure and function of many proteins. Under different environmental conditions, it is involved in metabolism, growth, development and gene expression [52]. Thiol-dependent redox enzyme plays role in rapid acclimation of chloroplast metabolism depending on the light availability [53]. In humans, Thioredoxin-1 (TRX1) protein provides anti-oxidant and anti-inflammatory effects by up-regulating inflammatory cytokines [54]. In *Bombyx mori*, the protein BmTrx protects it against oxidate stress due to extreme temperatures and pathogenic infections. The amino acid sequence indicated dithiol/disulfide active site residues (CGPC) to be conserved when compared among insect species [55]. Similar results were confirmed by Holmgren et al. [56] wherein BmTrx acts as protectant against oxidative stress caused by high temperatures, microbial infection and by intracellularly generated reactive oxygen species during metabolism. On the other hand, thioredoxin peroxidase BmTPx play a protective role against oxidative stress caused by temperature and viral infection [57]. Thioredoxin peroxidase (Tpxs) also protects organisms against toxicity caused by Reactive Oxygen Species (ROS) in *Apis cerana* [58]. Many of the antioxidant enzymes including catalases and peroxidases are able to quench oxidants that provide line of defense [59]. This result was consistent with previous observations showing that insect antioxidant enzymes, *Gryllotalpa orientalis* SOD1 [60], *Gryllotalpa orientalis* Prx [60], BmTPx [57], *Bombus ignitus* Txl [60], *Bombus* ignites SOD1 [60], and *Gryllotalpa orientalis* ATX1 [61] showed up-regulation during microbial infection. Our present results suggest that the identified Trx protein might play a major role in protecting organism from various microbial infection and environmental stress.

The next score of protein identified from gut microflora is Phosphoribosyl-ATP pyrophosphotase (PRPP) with 56. PRPP is required for synthesis of purine and pyrimidine nucleotides, for pyridine nucleotide cofactor NAD (P) and for synthesis of amino acids histidine and tryptophan [62]. In *Aspergillus nidulans*, this enzyme is encoded by *PrsA* gene that catalyses reactions of adenine ribonucleotide triphosphate (ATP), ribose-5-phosphate and has central importance in cellular metabolism [63]. In *Mycobacterium smegmatis*, the *PrsA* gene catalyses formation of phosphoribosylpyrophosphate (PRPP) that is used in synthesis of purines, pyrimidines, histidine, tryptophan and pyridine nucleotides. It also produces metabolite decaprenylphosphoryl-arabinose, an essential precursor for the mycobacterial cell wall biosynthesis [64].

In tassar silkworm of first and fifth instar silk glands the analysis of transctipst showed phosphoribosylpyrophosphate gene that act as house-keeping gene to carry out cellular metabolism [65]. Similar results were obtained by identified phosphoribosylpyrophosphate synthetase associated protein in insect skeletal muscle of silkworm. In the present study, higher expression of Phosphoribosyl-ATP pyrophosphatase was observed in PM race when compared to CSR2. The enzyme might govern cellular metabolism and could be involved in increasing survival rate of PM larvae. Earlier studies have shown that PM has higher disease resistant ability compared with other breeds. In our findings, survival rate was significantly

increased in all groups treated with arginine, histidine and their mixtures. Donini et al. have reported oral supplementation of arginine, histidine and their mixtures resulted in stimulatory effect on survival rate of the silkworm *Bombyx mori* [66].

The next identified protein from CSR 2 race was to be urease subunit gamma (SGP3) that scored for 55. Urease hydrolyses urea to form one molecule of carbon dioxide and two molecules of ammonia [67,68]. In bacteria, urease acts as protectant in in acidic environments by neutralizing acids [69]. In animals, it is being used as convenient quantitative measure of nitrogen recycling. Particularly in European hare (*Lepus europaeus*), urease activity has been found to be high in winter periods for proper recycling of nitrogen from diet [70]. In silkworm, urease metabolizes to form ammonia that is assimilated into silk protein. The enzymes are obtained from mulberry leaves and are not synthesized by silkworm itself. Mulberry leaves treated with cowpea seed powder and feeding it to fifth instar larvae of multivoltine cross breed race of silkworm, *Bombyx mori* showed enhanced production of midgut enzymes including protease, amylase, trehalase, sucrase and urease [71]. Transgenic silkworm lines developed through incorporation of artificial gene showed expression of urease which played a major role in nitrogen metabolism [72]. In other bacteria such as *Bacillus* species, urease has been found in the process bio calcification [30]. In order for healthy production of eggs/offsprings, calcium is utilized by the silkworms. Urease involving in calcium accumulation might govern a potential offspring development in silkworms. The calcium crystals produced provides harder shell for silkworm eggs [73]. During formate synthesis, urease is involved in metabolic activity and as protectant in proper metabolic functioning during low pH stress. pH sensitivity is an important factor for the production of silk protein in silkworm. The silk proteins are stored in glands and transported where it undergoes conformational changes in response to pH and converted to beta sheet fibers from alpha helical soluble conformations [74]. Thus, the protein could possibly indirectly participate in formation of silk fibres. Another possible role urease could play in silkworm gut system could be providing immunity. Plant urease has been shown to provide immunity in plants since the past twenty years. In this context, the isoform of urease isolated from jack bean have shown resistance to fungi and insects. On oral administration, ureases are toxic for insects that provide cathepsin-like peptidases (hemipterans) and trypsin-like peptidases (dipterans) for digestion [75]. To counterpart *Helicobacter pylori* infection in humans, a secretory system have been developed that consists of *UreB* gene (urease) constructed along with signal peptide (Bombyxin) from *Bombyx mori*. As an alternate silkworm larvae could be used as insect expression system in production of large scale UreB proteins [76]. In fact, the oral immunization of recombinant UreB with silkworm pupae powder showed therapeutic effects against *Helicobacter pylori* infection when tested in mice [77].

The other protein identified in PM silkworm gut microflora was phenylacetic acid degradation protein (SGP2) which scored for 49. The protein family belong to thioesterase superfamily and are found in phenylacetic acid degradation [78]. They probably play a major role as ring opener in thioesterases. The crystal structure of a phenylacetic acid (PhAc) degradation protein PaaG was derived from thermus thermophiles at 1.85 A and was found to carry out ring opening reaction *via* an isomerase like mechanism [79]. The organisms using aromatic compounds as their growth substrate require energy to breakdown the aromatic ring system. In anaerobic conditions, activation by CoA- thioester formation carries out energy driven reduction of aromatic rings. The exact mechanism of ring opening has been found in *Pseudomonas putida* [80]. The process involves activation of phenylacetyl-CoA and CoA thioesters to ring of 1,2-epoxide by the enzyme exygenase. The non-aromatic epoxide is isomerized to seven-member O-heterocyclic enol ether, an oxepin. The hydrolytic ring is than cleaved and and β-oxidation carries out to form acetyl-CoA and succinyl-CoA. A similar function could be correlated with the identified protein from silkworm gut microflora.

The least score of 27 was identified for L-serine dehydratase (SGP4). It was isolated from the gut of silkworm bivoltine (CSR2) pure race. L-serine ammonia-lyase is a member of β-family of pyridoxal-5′-phosphate (PLP) that catalyzes L-serine (l-threonine) to pyruvate (α-ketobutyrate) and ammonia [81]. It was confirmed while solving its crystal structure from *Rhizomucor miehei* at 1.76 A. The enzyme has been found to be a serine dehydratase and plays similar role as identified above [82]. It also plays an important role in gluconeogenesis during starvation and high-protein diets. In silkworm, knockout of a single gene causes large scale change in metabolic pathway. On knockout, the level of protein remains low and the pathways including pentose phosphate pathway, glycolysis/gluconeogenesis and glycine-serine biosynthetic pathway remains down-regulated [83]. The modification leads to redistribution of nutrients leading to increase in pupal weight. Similarly, the process of gluconeogenesis has also been found in thermal parthenogenesis in domesticated silkworm *Bombyx mori* [84]. Hence the proteins identified could play a role of gluconeogenesis during stress conditions for its survival and production of pupa.

CONCLUSION

Silkworm undergoes morphological variations upon metamorphosis and a key challenge in studying their gut micro flora would provide us access to different genomes and their corresponding protein profiles. The novel proteins identified in the present research might help in further elucidation of their importance in improving nutrient metabolism, stress adaptation, immune system and development of silk cocoon. Further enlightening of these enzymes would give their exact role and function in silkworms.

DECLARATIONS

Ethics approval and informed consent statement

Not applicable.

Consent for publication

Not applicable.

Data availability statement

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Ponnusamy Mohanraj, C Aruchamy Mahalingam conceived and designed the experiments. Ponnusamy Mohanraj performed the experiments. Ponnusamy Mohanraj, Chinnan Velmurugan Karthikeyan, Sendha Venkatachary Krishnamoorthy, Dananjeyan Balachandar, Babu Ramanathan analysed the data. Ponnusamy Mohanraj, Chinnan Velmurugan Karthikeyan, Babu Ramanathan wrote the manuscript.

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