Detection of gut microflora and enzymes using metagenomics and metaproteomics in silkworm, Bombyx mori

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Abstract

Background

Microorganisms living in insect gut plays a crucial role in the adaptation, growth and development of the insect hosts. The identification and molecular analysis of insect gut microbes will enable us to develop novel strategies for industrial product development, effective utilization of by products and facilitate us with the best pest management practices. 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.

Results

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 between the races.

Conclusions

Silkworm undergoes radical morphological variations upon metamorphosis and a key challenge in studying gut microflora is the ability to access different genomes and the corresponding protein profiles. The results presented in this study may help to bridge the gap between the unknowns of silkworm gut microbiota and their importance in the silkworm gut ecosystem.

Background

Silkworm, *Bombyx mori* is the principal beneficial insect to silk industry. Silk production is primarily dependent on silkworm larval nutrition that is regulated by the midgut enzymes in food digestion. Gut microbes of the insects play an essential role to the adaptation, biomass degradation, nutrient production and compound detoxification [1],[2]. Influence or interference to these gut microbes due to internal and external factors could potentially affect the silkworm health including cause of serious diseases such as Colony Collapse Disease (CCD) [3]. The diversity of symbiotic gut bacteria varies widely according to different breeds of insects and environmental conditions.

The bacterial cultures can be identified using a variety of biochemical tests like specific enzymes are secreted by different genus of bacteria [4]. Molecular techniques provide an opportunity to describe the microbial diversity independent of culturing live bacteria, which is an important adjunct to the culture - dependent approach [5]. The most common molecular approach to explore microbial diversity and to identify uncultured bacteria is by using 16S rRNA gene (16S rDNA). The gene is approximately 1500 bp long and codes for the smaller subunit of ribosomal RNA of prokaryotes. The 16S rRNA gene combines highly conserved and variable regions as a useful tool in identification of bacterial taxa by their sequences.

The recent advances in ‘omics’ technologies have enabled us to explore microorganism communities in an unprecedented way. The high-throughput metagenome and metaproteome analysis have helped us in speeding up molecular level investigations in conjunction with use of complementary data annotation and high-throughput functional screening [6], [7], [8]. Efforts have been dedicated in discovery of novel enzymes, pathways and organisms for various applications [9],[10]. Metagenome and metaproteomics sequencing have also become an important approaches for exploring biomass degrading mechanisms in other insects such as wood-feeding insects [11] and lower termites [6]. Symbiotic bacteria and protozoa in the hindgut of termite play an important role in hydrolysis of cellulose and hemicelluloses [12], [13], [11], [14], [15]. These analyses not only revealed a diverse group of bacteria covering 12 phyla and 216 phylotypes, but also led to identification of more than 100 candidate glycoside hydrolases. The advancements in “meta” approaches aided us to better understand the microbial diversity and has been driven by increasing demands for biocatalysts for industrial applications [16].

In our present study, we used the metagenomics and metaproteomics tools to study the diversity of gut microflora in silkworm larvae and identification of potential enzymes that could play a role in contributing towards silkworm midgut ecosystem,

silkworm adaptation, nutritional values, silk production and quality.

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. 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 (25–28**°**C) 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 [17].

Dissection and culturing the silkworm gut microflora

Three days old 5th instar larvae were collected and kept under starvation for 17–19 hour for bivoltine and 20–22 hour for multivoltines. The larvae were surface sterilized with 7 per cent alcohol, anesthetized using 50 per cent alcohol for 1 min 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 per cent glycerol (cells viable for one year). The extracts were stored at − 4°C until further use. The total gut microflora was cultured on Nutrient Agar medium (Sigma-Aldrich, USA).

Isolation of genomic DNA from silkworm gut microbes

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

Isolation and cloning of 16S rRNA

Full-length 16S rRNA gene of each silkworm pure race was amplified using forward primer (5'AGA GTT TGA TCC TGG CTC AG 3') and reverse primer RP2 (5'ACG GCT ACC TTG TTA CCA CTT 3') using the following amplification conditions: initial denaturation at 95ºC for 1 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55ºC for 1 min and extension at 72°C for 1 min, and a final elongation at 72°C for 1 min. The PCR product was purified using the PCR clean up kit (Qiagen, USA). The purified PCR products were cloned into pTZ57R/T (T/A cloning kit, Fermentas, USA) following manufacturer's instruction. The ligated products were cloned into *E. coli* DH5α competent cells (Thermofisher scientific, USA). Briefly, 10 µl of ligation mixture was gently mixed with 100 µl of competent cells and incubated at 42ºC for

45 sec using dry block heater. The mixture was added to a 500 µl of LB (Luria Bertani) broth (Sigma-Aldrich, USA) and incubated in a shaker at 37 ºC with 200 rpm for 1 hour. 100 µl of cell suspension was spread on the surface of a LB agar plate containing ampicillin (100 µg/mL) (Invitrogen, USA); X-Gal (20mg/mL) (Sigma-Aldrich, USA) and IPTG (20%) (Sigma-Aldrich, US). After 18 hours of incubation at 37 ºC, the plates were stored at 4ºC for 1–2 h to differentiate clear blue and white colonies.

Colony PCR and Library maintenance

Colony PCR was performed to confirm the insert using M13 forward sequencing primer (5'-GCC AGG GTT TTC CCA GTC ACG A-3') and M13 reverse sequencing primer (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 transferred to 96-well microtiter plates containing 100 µl of LB broth with ampicillin to which 50 µl of 60% sterilized glycerol (Merck, USA) was added. The clone libraries were stored at -70 ºC until further analysis.

Amplified ribosomal DNA restriction analysis (ARDRA)

PCR-amplified 16S rDNA fragments were digested with endonucleases *Hae*III (Fermentas, USA) at 37 ºC 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 for chimeras with B2C2 software [18]. The identity of 16S rRNA gene sequences was verified by similarity search using BLAST tool [19]. The phyla-wise grouping was done using BLAST analysis of 16S rRNA gene sequences and number of clones with similar ARDRA profile. The phylogenetic tree was constructed with existing 16S rRNA gene sequences from different bacteria, obtained from NCBI GenBank database by neighbour-joining method using MEGA V 5.0 software. Data related to the study is deposited in the publicly available repository and can be accessed through the following link: http://purl.org/phylo/treebase/phylows/study/TB2:S22446.

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) and acetone (Merck, USA). After centrifugation for 15 min at 4500 g, the pellets were washed once with ice cold acetone (Merck, USA) containing 0.07% DTT (Sigma-Aldrich, USA) at -20ºC for 1 hr and centrifuged again for 15 min at 4500 g. The final precipitated pellets were lyophilized and used for proteomics analysis.

2D-PAGE

2D-PAGE analysis was performed using the 17 cm IPG strips (pH 4–7) (Bio-Rad, USA) following manufacturers instruction. Briefly, the strips were rehydrated overnight with 350 µl of rehydration buffer containing 130 µg of protein at room temperature. Isoelectric focusing (IEF) was carried out at 20ºC and the running conditions were as follows: 500V for 1 hour followed by 1000V for 1 hour and finally 3000V for 16 hours. The focused strips were equilibrated twice for 15 minutes in 10 ml equilibration solution. Equilibrated IPG strips were rinsed with electrode buffer and placed on the top of a polyacrylamide gel (Bio-Rad, USA). The strips were covered with 2 ml of agarose solution and 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 from the gel into 1 mm-cubes and were subjected to in-gel digestion. Sequencing grade, modified trypsin (Roche, USA) to a final concentration of 100 ng/µl was used for digestion. The digested gel pieces were subjected to Zip-Tip purification following manufacturer's instructions (Sigma-Aldrich, USA). The purified peptides were eluted from the ZipTip directly into 1–2 µl matrix containing 50% CH_3CN and 0.1% Trifloro acetic acid (Fluka, USA). The peptides were subjected to MS/MS analysis on a 4700 matrix-assisted laser desorption/ionization (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems, USA). Briefly, the samples were analyzed using a delayed ion extraction, and positive ion reflection mode, at 20 kV of accelerating voltage, 60–65% grid voltage, and 100 ns delay time. The spectra were acquired from m/z 800 to 2500 and were calibrated using trypsin auto-digestion ion peak m/z (842.510 and 2211.1046) as internal standards. A 0.3 µl of protein mix was analyzed for external calibration of the mass spectrometer.

Database searching

The identification of protein was carried out using the MASCOT version 2.0 (Matrix Science, UK). The data were sent to the National Center for Biotechnology Information non-redundant (NCBInr) protein database, which contained a total of 2,464,940 sequences at the time of the study. The search was performed taking Other Metazoa as taxonomy, which contained 1,54,412

sequences. The following parameters were used in all searches: maximum number of missed cleavages allowed was 1, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (Met), peptide tolerance of 55 to 370 ppm, peptide charge of 1+, monoisotopic and deisotoped masses of peaks were used for protein identification. Possible covalent modification considered in search procedure was acetylation of the N-terminus. Identification criteria were based on the number and coverage of matched peptides: minimum peptides required to match was 5, coverage of matched peptides ≥ 10 per cent or the identification scores from software ≥ 50. In addition, the consistency of theoretical molecular weight or pI of proteins with the observed molecular weight or pI from 2D map was an important reference for mass identification. 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). Cloning was carried out onto pTZ57R using *Hae*III restriction enzyme. The phylogenetic relationship within the clones to their biodiversity was carried out using B2C2 software (Fig. 1). The branching order of phylogenetic tree reported in this study with regard to deep branches for distantly related taxa represent only an estimation of phylogenetic similarity. A total of 16 clones were sequenced and shown to belong in major division of bacteria. 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 *B. subtilis* and SMGB7 clone 86% nucleotide identity to *B. licheniformis.* The nucleotide identity of gamma proteobacteria with reference organisms were between 55 to 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 pneumonia.*

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

		16 S rRNA gene sequence homology			
Clone Number	**Silkworm**	**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

The clones SMGB15 and SMGB2 were closely related to cocci form of bacteria and actinobacteria. It showed 93 and 97% nucleotide identity to reference organims *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.

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

Metaproteomics analyses of silkworm gut microbes

The silkworm midgut microbial proteins were extracted from the three-day old fifth-instar larvae. A total of two extractions with three replications each were separated by 2D gel electrophoresis. The number of protein spots found was varied according to silk worm race. Among the proteins identified, protein spots SGP1, SGP2, SGP6 and SGP7 were found only in midgut of PM (Fig. 2) and protein spots SGP3, SGP4 and SGP5 were found in CSR2 (Fig. 3). The excised protein spots from gel were further analyzed 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 molecular function and gene ontology (GO) annotation, these proteins were classified into three groups based on cellular component, molecular functions including binding, catalytic, transporter, electron carrier and antioxidant and biological processes involved in metabolism, cellular process and biological regulation.

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 to 30,000 kDa within 4 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 (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 to 90,000 kDa with 4 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). 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) and 4. Ligase (SGP5). These proteins 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 mainly involved in the synthesis of amino acids. In addition, they are the 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 subunit C is an enzyme that acts as catalyst in biochemical reactions.

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

Discussion

Phylogenetic analysis of 16S rRNA isolated clones from microflora of larval gut microbes 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). All of the known species among these isolates were either strict or facultative anaerobes that have been found and frequently 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 [20], [21]. The presence of strict and facultative anaerobic organisms in silkworm gut is a reasonable expectation. Five of the clones were grouped under Gammaproteobacteria that include *Pesudomonas Auriginosa, 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. [22] Reported the isolation of gut symbiont microbes belonging to gammaproteobacteria from shield bug *Graphosoma lineatum*. Symbiotic bacteria isolated from oriental fruit fly *Bactrocera dorsalis* showed enhanced insecticide resistance gammaproteobacteria [23]. Comparative analysis of gut microbiota form various races of mosquitoes showed the presence of microbes belonging to gammaproteobacteria and Actinobacteria [24]. 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 and resistance. Nitrogen fixation mediated by gut bacteria is one of the crucial aspects for termite symbiosis since termites 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 [25]. Metatranscriptome analysis of subterranean termite gut showed microbes that play direct roles in nitrogen fixation, amino acid biosynthesis, and lignocellulose digestion [26]. *Citrobacter freundii* and *Enterobacter agglomerans* nitrogen-fixing bacteria have been previously isolated from several kinds of termites [27], [28]. 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 [29] 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 the guts of produce a variety of hydrolytic enzymes. Earlier studies have showed that *Paenibacillus* ICGEB2008 isolated from the gut of the cotton bollworm produced several biomass-degrading enzymes, including cellulases and hemicellulases [30], [31]. *Bacillus* are predominant with titres of up to 10^{7} CFU/ml gut contents [32], and play a major role in the guts of invertebrates in the first and second step of the degradation of 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 [33]. 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 provide an evidence that diet has a significant impact on gut microbial community. Cocci, particularly *streptococcus epidermis* 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 epidermis* is such an organism that plays an active role in host defense having symbiotic relationship [34]. The cocci obtained from silkworm gut could play a similar role of symbiotic relationship.

Insects possess an efficient immune system that allows them to deal with pathogenic infections. The defense system 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 [35]. [36]showed primed immune response in silkworm

triggered by ingested bacteria leading to systemic infection tolerance. This was carried out by injecting heat killed microbes including *P. aeruginosa*, heat-killed *S. marcescens* cells and heat-killed *C. albicans* cells that showed tolerance against *P. 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, *B. 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. [39] 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. *Pseudomonas mendocina* on other hand has been found to synthesis medium-chain-length polyhydroxyalkanoate (PHAMCL) and alginate oligosaccharides (AO). Alginate oligosaccharide has many biological activities, such as antioxidation, anticoagulation, and immune regulation [40]. Similarly nano-vaccine developed using outer membrane protein (OmpW) of *Aeromonas hydrophila* showed a dose dependent Immunity in Rohu fish(*Labeo rohita*) [41]. Recent research enlightened *Pseudomonas aeruginosa* as immune elicitor by secreting type II protease IV functions in Arabidopsis [42]. Protease IV activates pathway involving G protein signaling in immune function. Proteins play a major role in bringing about immune system in various insects [43]. In fact, it has been found that silkworm cocoon consists of many proteins that are of immune related. Eventually [44] isolated proteins from *B. 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 two-dimensional 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 proteins identified were found to be related with metabolism process and innate immunity, which were 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 degrades the RNA strand in 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. In a recent research by [45] in *M. smegmatis* enlightened RNAse H to inhibit mycobacterium infection activity. 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 carry out metabolism of the RNA primers of Okazaki fragments formed during lagging strand in DNA replication during transcription. 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 *E. coli* Exo III having ribonuclease H activity and could in principle be important for retrotransposition [46]. 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) [47]. The work by [48] also produced similar results wherein 41 kDa RNAse was isolated against polyhedrosis virus from *B. 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 charecterization 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 [49]. Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase catalyses formation of correctly charged Asn-tRNA(Asn) or Gln-tRNA(Gln) through the transamidation reaction in organisms that lack either or both of asparaginyl-tRNA or glutaminyl-tRNA synthetases. The reaction utilizes glutamine or Asparagine along with ATP for protein synthesis. In *Helicobacter pylori*, the heterotrimeric tRNA-dependent amidotransferase (GatCAB) is essential for protein biosynthesis because it catalyzes the conversion of misacylated Glu-

tRNA(Gln) and Asp-tRNA(Asn) into Gln-tRNA(Gln) and Asn-tRNA(Asn) [50]. Few of heterotrimeric amidotransferase genes encoded by *gatA*, *gatB*, and *gatC* genes were identified in *Chlamydia trachomatis* genome. This genes were found to play an important role in amino acid synthesis and proper folding of proteins [51]. The enzyme thus isolated could play an important role of protein synthesis in gut microbiota. [52] found aspartic proteinases to be widely distributed among plants. 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. It involves in metabolism, gene expression, growth, and development under different environmental conditions [53]. Thiol-dependent redox enzyme plays a central role in rapid acclimation of chloroplast metabolism based on light availability [54]. In humans, Thioredoxin-1 (TRX1) protein provides anti-oxidant and anti-inflammatory effects by up-regulation of pro-inflammatory cytokines [55]. In *Bombyx mori*, BmTrx has been shown to protect against oxidative stress caused by extreme temperatures and microbial infection. The amino acid sequence indicated dithiol/disulfide active site residues (CGPC) to be conserved when compared among insect species [56]. Similar results were confirmed by [57] wherein BmTrx protects against oxidative stress caused by extreme temperatures and microbial infection as well as 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 [58]. Thioredoxin peroxidases (Tpxs) play important role in protecting organisms against toxicity of reactive oxygen species (ROS) and regulating intracellular signal transduction in *Apis cerana cerana* [59]. Many of the antioxidant enzymes including catalases and peroxidases are able to quench oxidants that provide line of defense [60]. This result was consistent with previous observations indicating that of insect antioxidant enzymes, *Gryllotalpa orientalis* SOD1 [61], *G. orientalis* Prx [61], BmTPx [58], *Bombus ignitus* Txl [62], *B. ignites* SOD1 [62], and *G. orientalis* ATX1 [63] were up-regulated during microbial infection. Our present results suggest that the new protein of Trx could play a key role in protecting from 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 [64]. In *Aspergillus nidulans*, this enzyme is encoded by Prs gene catalyses the reaction of ribose-5-phosphate and adenine ribonucleotide triphosphate (ATP) and has central importance in cellular metabolism [65]. In *Mycobacterium smegmatis*, the gene PrsA encoding for the synthesis phosphoribosylpyrophosphate (PRPP) was found as a key metabolite for several biosynthetic pathways including those for histidine, tryptophan, nucleotides and decaprenylphosphoryl-arabinose, an essential precursor for the mycobacterial cell wall biosynthesis [66]. In silkworm especially tassar silk worm *Antheraea mylitta*, analysis of transcripts expressed in one and fifth instar silk glands showed phosphoribosylpyrophosphate gene as house-keeping gene to carry out cellular metabolism [67]. 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. [68] have reported oral supplementation of arginine, histidine and their mixtures resulted in stimulatory effect on survival rate of the silkworm *B. mori*.

The next identified protein from CSR 2 race was to be Urease subunit gamma (SGP3) that scored for 55. Urease catalyzes hydrolysis of urea to form two molecules of ammonia and one molecule of carbon dioxide [69] and [70]. In general, urease protects bacteria in acidic environments by neutralizing acids [71]. 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 [72]. In silkworm, urease is important for the nitrogen metabolism of silkworms because the formation of ammonia 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 [73]. Transgenic silkworm lines developed through incorporation of artificial gene showed expression of urease which played a major role in nitrogen metabolism [74]. In other bacteria such as *Bacillus* species, urease has been found in the process bio calcification [75]. 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 [76]. Urease genes and genes encoding proteins are also involved in formate synthesis and also protects the cells by counteracting low pH resulting from formate metabolism. 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 [77]. 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, urease isoforms have been isolated from seed of *Canavalia ensiformis* (Jack Bean) that provide resistance to insects and fungi. When administered orally, ureases are toxic for insects that provide cathepsin-like peptidases (hemipterans) and trypsin-like peptidases (dipterans) for digestion [78]. To counterpart *H. pylori* infection in humans, an secretory system have been developed that consists of Ure B gene (Urease) constructed along with signal peptide bombyxin from *B. mori*. The expression system was found to be higher in terms of production and would aid in the large-scale expression, yield of UreB in silkworm larvae [79]. In fact, the oral immunization of silkworm pupae powder containing recombinant UreB of *Helicobacter pylori* provided therapeutic effects against *Helicobacter pylori* infection when tested in mice [80].

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 [81]. They have been thought to play a major role as thioesterases in ring opening. 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 [82]. 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 biochemical mechanism of exact ring opening has been elucidated from *Pseudomonas putida* [83]. Intermediates are processed as CoA thioesters and the aromatic ring of phenylacetyl-CoA becomes activated to a ring 1,2-epoxide by oxygenase. The reactive non- aromatic epoxide is isomerized to a seven-member O-heterocyclic enol ether, an oxepin followed by hydrolytic ring cleavage and β-oxidation steps leading to 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) dependent enzymes that catalyze conversion of $_{L}$-serine (l-threonine) to pyruvate (α-ketobutyrate) and ammonia [84]. 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 [85]. It 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. The levels of proteins involved in glycolysis/gluconeogenesis, pentose phosphate pathway, and glycine-serine biosynthetic pathway remains down-regulated [86]. 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* [87]. Hence the protein identified could play a role of gluconeogenesis during stress conditions for its survival and production of pupa.

Conclusion

Silkworm undergoes radical morphological variations upon metamorphosis and a key challenge in studying gut microflora is the ability to access different genomes and the corresponding protein profiles. The novel proteins identified in the present study may play an important role in silkworm 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.

Abbreviations

CCD - Colony Collapse Disease

PM - Pure Mysore

CSR-2 - Central Sericulture Research Institute, India

ARDRA - Amplified ribosomal DNA restriction analysis

IEF - Isoelectric focusing

DTT - dithiothreitol

NCBI - National Center for Biotechnology Information

TCA - Trichloracetic acid

MALDI - Matrix-assisted laser desorption/ionization

UV - Ultra violet

LB - Luria Bertani

ToF - Time of Flight

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Authors' contributions: PM, CAM conceived and designed the experiments. PM performed the experiments. PM, CVK, SVK, DB, BR analysed the data. PM, CVK, BR wrote the manuscript.

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Figures

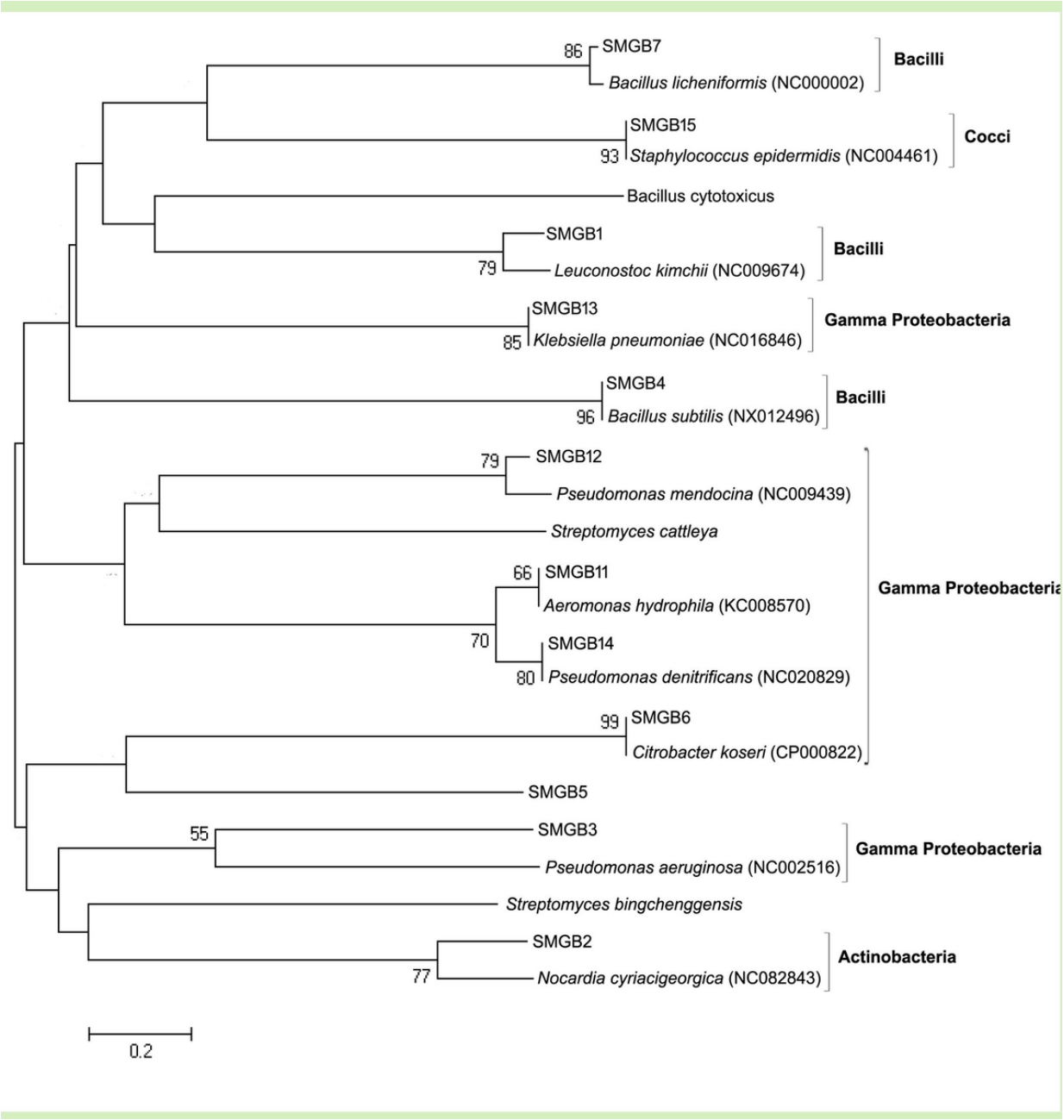

Figure 1

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

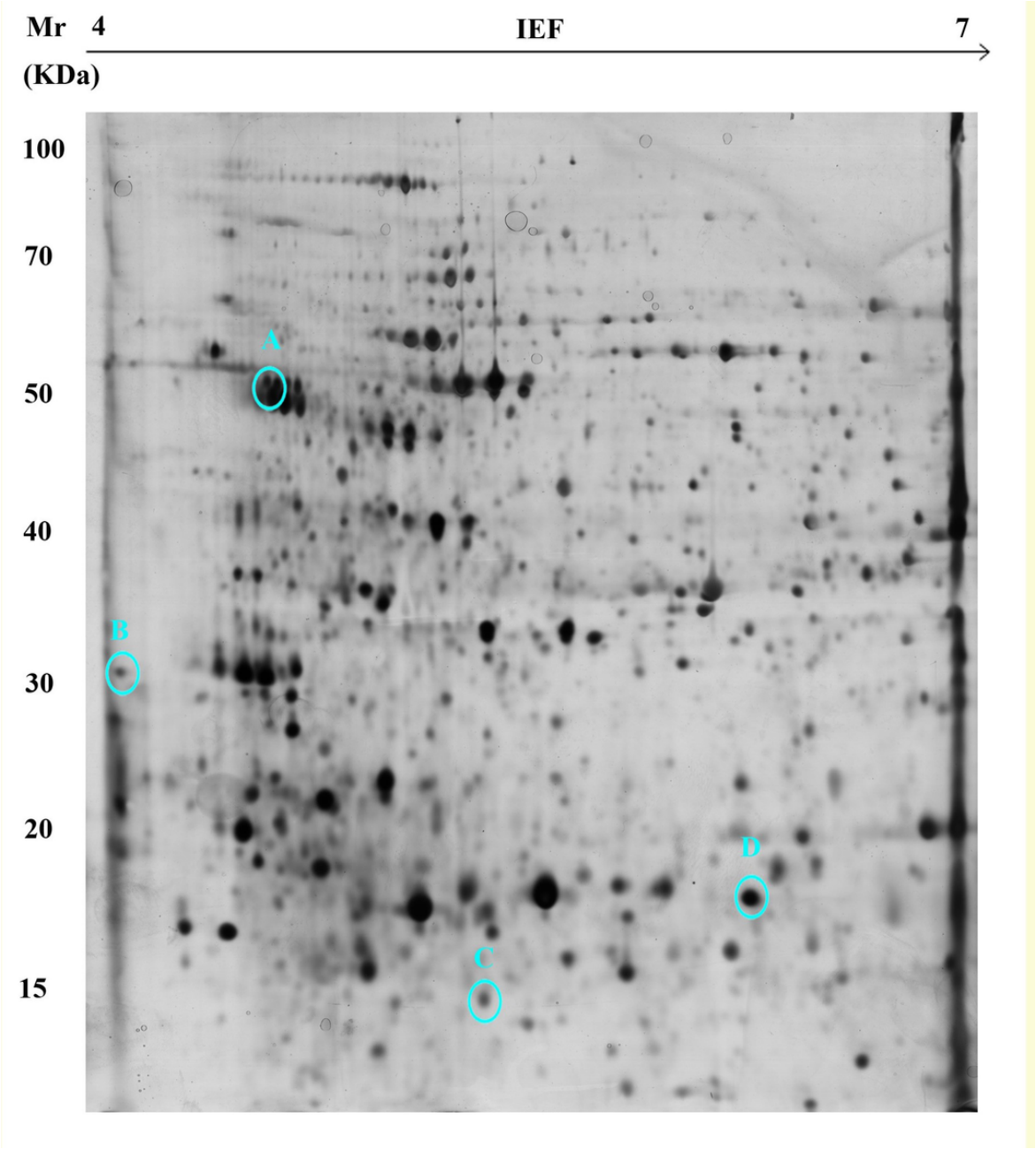

Figure 2

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

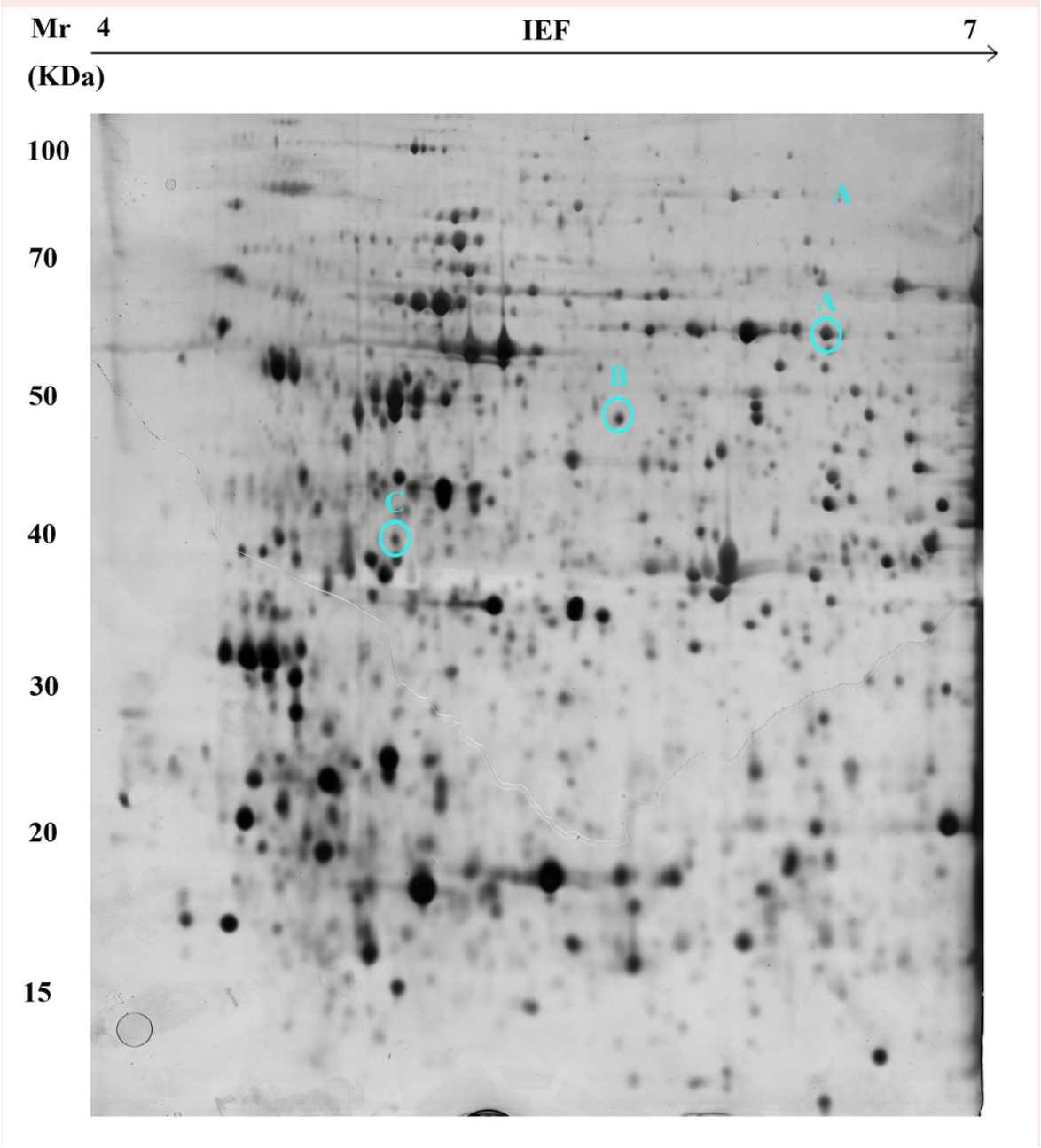

Figure 3

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

Supplementary Files

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