**Characterization of Gut Microbiota in Eri Silkworm (Samia ricini) using 16S rRNA Gene Sequencing**

**Abstract**

The gut microbiota of Samia ricini (Eri silkworm) plays a crucial role in host physiology, particularly in digestion, immunity, and silk production. This study employed 16S rRNA gene sequencing to investigate the bacterial diversity within the midgut of S. ricini. Four representative sequences (PPAI01, PPAI02, PETAI01, and PETAI02) were analyzed using BLASTn comparison against the NCBI GenBank database. The sequences exhibited high similarity (96.53%–98.29%) to bacterial species within the Bacillaceae family, including Bacillus licheniformis, Lysinibacillus fusiformis, Bacillus subtilis, and Bacillus amyloliquefaciens. These bacteria are known for producing digestive enzymes, antimicrobial compounds, and phytohormones, indicating a potential symbiotic relationship with the host. The findings suggest that these microbial taxa may enhance nutrient assimilation, promote gut health, and contribute to silk biosynthesis. This study provides a foundation for future probiotic strategies aimed at improving larval health and sustainable sericulture.

Key words: Samia ricini, Gut microbiota, Bacillus species, Probiotics, 16S rRNA sequencing, gut microbiota.

**Introduction**

Insects harbor a diverse and dynamic community of microorganisms within their digestive tracts, collectively known as the gut microbiota. These microbial communities play essential roles in host physiology, including digestion, nutrient absorption, immune modulation, and defence against pathogens (Douglas, 2015). In lepidopterans, such as the Eri silkworm (Samia ricini), gut microbiota contribute significantly to host development and adaptation to dietary and environmental changes. Samia ricini is a multivoltine, polyphagous silkworm species cultivated primarily in Northeast India for its coarse, durable silk known as eri or endi. Unlike the domesticated Bombyx mori, S. ricini is reared on castor (Ricinus communis) and thrives under semi-domesticated conditions. As such, its gut microbiome may reflect a more diverse microbial ecosystem, shaped by natural interactions with the environment and host plant. Despite its economic significance in rural sericulture, relatively little is known about the microbial ecology of the Eri silkworm gut, especially in comparison to the extensively studied B. mori. Previous studies in insect microbiology have shown that gut-associated bacteria can influence host growth, silk yield, disease resistance, and even tolerance to environmental stressors (Engel & Moran, 2013). These interactions are often species-specific and mediated by core microbiota, many of which belong to the genera Bacillus, Enterococcus, Lactobacillus, and Pseudomonas. In silkworms, bacteria like Bacillus subtilis and Bacillus licheniformis have been reported to produce enzymes that aid in the digestion of plant material, as well as metabolites that suppress the growth of pathogenic organisms (Ranjan et al., 2009). Advances in molecular biology, particularly the amplification and sequencing of the 16S ribosomal RNA (rRNA) gene, have enabled precise identification and phylogenetic classification of gut microbiota. The 16S rRNA gene contains conserved and variable regions, making it an ideal target for characterizing bacterial diversity and establishing evolutionary relationships. Sequence-based methods, such as Sanger sequencing, remain robust and reliable tools for taxonomic identification of culturable and unculturable microbial taxa. In this study, we aimed to investigate the bacterial composition of the gut microbiota in Samia ricini by amplifying and analyzing partial 16S rRNA gene sequences. Using sequence alignment and phylogenetic comparison with reference strains in the NCBI database, we identified dominant bacterial taxa associated with the silkworm gut. This work contributes to the growing field of insect microbiome research and offers a foundation for exploring probiotic and microbial enhancement strategies in sustainable sericulture.

**Methodology**

**Sample Collection and Bacterial DNA Extraction**

Healthy fifth instar larvae of the Eri silkworm (Samia ricini) were selected for gut microbial analysis. Under aseptic conditions, larvae were surface sterilized with 70% ethanol and dissected to isolate the midgut region. The gut contents were carefully collected and suspended in sterile phosphate-buffered saline (PBS). Samples were homogenized and subjected to centrifugation to pellet bacterial cells.

Genomic DNA was extracted from the bacterial pellet using the Cetyltrimethylammonium bromide (CTAB) method, with modifications suitable for bacterial isolates. The pellet was resuspended in 500 µL of CTAB extraction buffer, vortexed, and incubated at 60°C for 30 minutes. Following incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added to the lysate, mixed briefly, and centrifuged at 14,000 × g for 5 minutes. The aqueous phase was collected and mixed with 0.7 volume of cold isopropanol to precipitate DNA. The resulting pellet was washed with 70% ethanol, dried, and resuspended in 30 µL of TE buffer. RNase A was added to remove RNA contamination, and DNA quality and concentration were measured using a spectrophotometer.

**PCR Amplification of 16S rRNA Gene**

The 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) as described by Weisburg et al. (1991). PCR reactions were carried out in a total volume of 25 µL containing: 2 µL of 10X PCR buffer, 0.5 µL of MgCl₂ (25 mM), 1 µL of dNTP mix (10 mM), 1 µL each of forward and reverse primers (10 pM), 0.5 µL of Taq DNA polymerase (5 U/µL), 1 µL of template DNA (25–40 ng), and the remaining volume adjusted with nuclease-free water.

The thermal cycling conditions for PCR amplification were as follows: an initial denaturation at 95°C for 2 minutes, followed by 30 cycles comprising denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes, with the reactions subsequently held at 4°C for storage or further analysis. PCR products were verified through 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

**Purification and Sequencing**

Amplified PCR products showing clear bands were excised from the gel and purified using a silica column-based gel extraction kit, following the manufacturer’s protocol. DNA was eluted in 20 µL of elution buffer. The purified amplicons were then subjected to Sanger sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and sequencing was performed in both forward and reverse directions on an ABI 3500 Genetic Analyzer. The sequencing reactions were carried out under the following thermal cycling conditions: an initial denaturation at 95°C for 2 minutes, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and termination at 60°C for 4 minutes. Upon completion of the cycles, the reactions were held at 4°C until further processing. Post-sequencing purification was performed using EDTA and ethanol precipitation to remove unincorporated dyes, followed by resuspension in Hi-Di™ formamide, denaturation at 95°C, and immediate loading onto the sequencer.

**Sequence Analysis and Phylogenetic Identification**

Raw sequence data were manually curated and assembled using BioEdit software to obtain high-quality consensus sequences. These sequences were subjected to BLASTn analysis against the NCBI GenBank database to identify homologous bacterial sequences. The top hits were selected based on maximum percentage identity, alignment coverage, and E-value. Taxonomic classification was determined based on the closest phylogenetic matches, and sequences with ≥96% identity were considered to represent the same or closely related species.

The taxonomic relevance of identified strains was interpreted with respect to their known roles in host-microbe interactions, digestion, and health-promoting activities within insect guts. Sequences were further aligned for phylogenetic comparison using ClustalW and MEGA software (v11) to construct a neighbor-joining phylogenetic tree and confirm the evolutionary relationships of the isolates.

**Results and Discussion**

The sequence analysis of gut microbiota from Samia ricini (Eri silkworm) provided insights into the taxonomic diversity and functional potential of bacterial communities residing in the insect gut. Using 16S rRNA gene sequencing and BLASTn comparison against the NCBI GenBank database, four representative sequences (PPAI01, PPAI02, PETAI01, and PETAI02) were identified, each showing high similarity to known bacterial strains. The percentage identities ranged from 96.53% to 98.29%, indicating close phylogenetic relationships with beneficial bacterial species. These findings support the presence of a functionally rich and possibly symbiotic gut microbiota in S. ricini, with potential implications for host digestion, immunity, and silk production (Table 1).

**Table 1. Identification of Bacterial Strains from Query Sequences Based on 16S rRNA Gene Similarity**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sequence ID** | **Nucleotide Sequence (partial)** | **BLAST Hit Description** | **Percentage Identity** |
| PPAI01\_query | CCAGTATCAGTATCAGACGTGATAGTCTGAGCGGACCGACTGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAAGCCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTATGTGGCGAGCGTTGTCCGGAATTATCTGGCGCGTAAAGCGCGCGCAGCGGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACCTGTAGCAGTGGAAATGCGAGAGATGTGAGGAACACCAGTATCGTA | *Bacillus licheniformis* strain TB212 16S rRNA gene, partial sequence | 97.94% |
| PPAI02\_query | TGTTTACTTTCGTATCATCGTGACAGTCGAGCGAACAGATCTGGGAGCTTGCTCCTTTGACGTTAGCGGCGCGACGGGTGAGTAACACGTGGGCAACCTACCCTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAATCTCTTTTGCTTCATGGTGAAAGACTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGGGGGGGGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAAAGAGGAAAGTGGAATTCCAAGTGTAGAGCAT | *Lysinibacillus fusiformis* strain OSR6 16S rRNA gene, partial sequence | 98.29% |
| PETAI01\_query | GGTTGTTGGTGCTACCGCTATCATCGTGATAGTCGAGCGGAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAAGAGGAGAGTGGAAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAAGGAGCGAAGCGTGGCGAAGCGAACAGGATCACATACCCGTGTTACTCCAACGCCGTCAACGATGATCTGCTACAGTCGTTAAGGAGGTTTCCGTCTCCTTAGTTGGTGACAGCTAGACGCACTGAAGTTCATCACGACTGGGGCGACGGACTGTAGCGGGAGTTGTTATACCTCTATAGGAAATTAGAGCAAAGCGCGCCTGGCAGACCCGTGAACCCCTAGTGAGTTTCTAGTACAAGGCATAGCACATAACGCATTACCGGCGTCTTGAACTGTCACTGTAGACTATCTAGGAATATCGGATCAGCATCGGCGAGCTACGACAAGTGCGCCTAGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAAGGGGGTTTCCGCCCCCTTAGTTGACTGCAGCTAACAGG | *Bacillus subtilis* strain MDR12 16S rRNA gene, partial sequence | 96.91% |
| PETAI02\_query | TTACGTCTCTCTCTGCGCTACCTTGTTACGACTTACGTGACCTCGGGGACTCTCTGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACATCAGTATATGTCGGTGAGTGTAACTAAGTCGTAACAAGGTAGCCTATCTCTAGGATTGTCACAGGATGTCAAGACCCTGCTAAGGTTTCTTCGCGTGTACTTGGAATAAACCCCCCCCTCCCCTCCCA | *Bacillus amyloliquefaciens* strain BCd4 16S rRNA gene, partial sequence | 96.53% |

The BLASTn-based taxonomic identification of partial 16S rRNA gene sequences from the gut of *Samia ricini* revealed the presence of four closely related bacterial strains:

|  |  |  |
| --- | --- | --- |
| **Sequence ID** | **BLAST Hit Description** | **Percentage Identity** |
| PPAI01 | *Bacillus licheniformis* strain TB212 | 97.94% |
| PPAI02 | *Lysinibacillus fusiformis* strain OSR6 | 98.29% |
| PETAI01 | *Bacillus subtilis* strain MDR12 | 96.91% |
| PETAI02 | *Bacillus amyloliquefaciens* strain BCd4 | 96.53% |

All identified strains belonged to the phylum *Firmicutes*, family *Bacillaceae*, indicating a taxonomically coherent and functionally specialized gut bacterial community.

**i. PPAI01\_query: Bacillus licheniformis strain TB212**

The sequence PPAI01 demonstrated 97.94% identity with Bacillus licheniformis strain TB212, a well-characterized gram-positive bacterium. B. licheniformis is widely recognized for its industrial utility, particularly in the production of enzymes such as proteases and amylases, which facilitate the breakdown of proteins and starches, respectively (Schallmey et al., 2004). These enzymes could play a vital role in the digestion of complex macromolecules within the silkworm gut. Moreover, B. licheniformis has been reported to exhibit antimicrobial activity, contributing to gut microbial balance and defense against pathogens (Priest, 1977). Its identification in the Eri silkworm gut suggests a dual role—enhancing digestive capacity and supporting gut health through competitive exclusion and bioactive metabolite production (Table 1).

**ii. PPAI02\_query: Lysinibacillus fusiformis strain OSR6**

The second sequence, PPAI02, showed 98.29% identity with Lysinibacillus fusiformis strain OSR6. L. fusiformis is a rod-shaped, spore-forming bacterium frequently found in soil environments. It is known for its ability to degrade organic matter and contribute to nitrogen cycling, processes essential for maintaining ecological balance and nutrient availability (Ahmed et al., 2007). Additionally, L. fusiformis is involved in the production of phytohormones and biocontrol agents, suggesting its potential to enhance host nutrient assimilation and immunity. The presence of this bacterium in the gut of S. ricini may point to its involvement in metabolic support functions, including nitrogen fixation and breakdown of plant-derived compounds, which are crucial for larval growth and silk protein biosynthesis (Table 1).

**iii. PETAI01\_query: Bacillus subtilis strain MDR12**

Sequence PETAI01 was closely related (96.91% identity) to Bacillus subtilis strain MDR12. B. subtilis is a prominent model organism in microbiology and biotechnology, known for its endospore formation, genetic tractability, and ability to produce a wide array of extracellular enzymes and antimicrobial peptides (Earl et al., 2008). Its probiotic potential has been extensively documented in both vertebrates and invertebrates, where it contributes to intestinal health by modulating the gut microbiota and enhancing host immunity. The detection of B. subtilis in the Eri silkworm gut underscores its likely involvement in improving digestive efficiency, suppressing pathogenic microbes, and maintaining gut microbial homeostasis. This bacterium may also influence silk quality through improved nutrient bioavailability.

**iv. PETAI02\_query: Bacillus amyloliquefaciens strain BCd4**

The final sequence, PETAI02, shared 96.53% identity with Bacillus amyloliquefaciens strain BCd4. This species is known for its role in producing secondary metabolites such as lipopeptides (iturin, fengycin) and antibiotics like bacilysin, which have antifungal and antibacterial properties (Chen et al., 2007). In agricultural settings, B. amyloliquefaciens is used as a biocontrol agent and plant growth promoter due to its phosphate solubilization and nitrogen-fixing abilities. Its presence in the gut microbiota of S. ricini suggests a possible symbiotic role in enhancing nutrient cycling, promoting gut health, and protecting against microbial infections. Such bacteria could be harnessed as part of a probiotic strategy to improve silkworm health and sericultural productivity.

The identification of these bacterial species highlights the functional diversity of the gut microbiota in Samia ricini. All four identified strains belong to the Firmicutes phylum, particularly the Bacillaceae family, which is commonly associated with nutrient processing and antimicrobial activity in insect guts. These bacteria likely contribute to essential physiological processes such as digestion of complex carbohydrates, protection from pathogens, and modulation of the immune response. Given their known roles in promoting health and growth in other hosts, the presence of Bacillus and Lysinibacillus species in the Eri silkworm supports the idea that manipulating gut microbiota through dietary intervention or probiotic supplementation could enhance silk production and larval health. Moreover, these findings open avenues for future research into the functional genomics and metabolic contributions of gut bacteria in non-model lepidopterans, thereby advancing sustainable practices in sericulture. The four sequences analyzed revealed strong phylogenetic affiliation with well-documented bacterial species within the genera *Bacillus* and *Lysinibacillus*. These bacteria are known for their functional contributions to host biology, including digestion, immunity, and growth promotion. The high percentage identity (96–98%) validates the accuracy of identification and suggests that these taxa are integral components of the gut microbiota in *Samia ricini*. Their presence reinforces the hypothesis that gut microbes play an important symbiotic role in enhancing the silkworm's physiological functions, with potential applications in probiotic development and sustainable sericulture practices.

The sequence PPAI01 showed a 97.94% identity with Bacillus licheniformis, a bacterium widely known for its production of digestive enzymes such as proteases and amylases. These enzymes likely support the breakdown of complex macromolecules in the silkworm gut, aiding nutrient absorption. Furthermore, B. licheniformis is known for its antimicrobial properties, contributing to gut microbial homeostasis and pathogen resistance. Sequence PPAI02 was closely related (98.29%) to Lysinibacillus fusiformis, a soil bacterium associated with organic matter decomposition and nitrogen cycling. Its potential ability to produce phytohormones and biocontrol agents suggests a supportive role in enhancing larval nutrition and immunity. These attributes could promote larval growth and positively influence silk protein biosynthesis. Sequence PETAI01, matched to Bacillus subtilis (96.91% identity), highlights the presence of a bacterium extensively used in biotechnological and probiotic applications. B. subtilis is known for producing extracellular enzymes, antimicrobial peptides, and forming endospores. Its presence in S. ricini gut microbiota likely enhances digestive efficiency, suppresses pathogenic organisms, and maintains microbial balance.

The final sequence, PETAI02, aligned with Bacillus amyloliquefaciens (96.53% identity), a bacterium valued for its production of bioactive secondary metabolites like iturin and fengycin. It also contributes to nutrient cycling and disease suppression in agricultural systems. In the silkworm gut, it may play a vital role in promoting gut health and protecting against microbial pathogens. Collectively, the identified bacterial strains demonstrate the functional diversity and symbiotic potential of the gut microbiota in S. ricini. Their known capabilities—ranging from enzymatic digestion and nitrogen cycling to antimicrobial activity—underscore their importance in supporting host physiology. The dominance of Bacillus and Lysinibacillus species aligns with reports from other insect gut microbiome studies, suggesting evolutionary adaptation to mutualistic interactions. These findings support the hypothesis that gut microbiota contributes significantly to the overall health and productivity of S. ricini. By manipulating these microbial communities through targeted probiotic supplementation, it may be possible to enhance silk yield, improve larval resilience, and reduce dependence on chemical inputs in sericulture. Further research is warranted to explore the functional genomics and metabolic pathways of these gut microbes to unlock their full potential in sustainable silk farming.

**Conclusion**

This study reveals a diverse and functionally rich gut microbiota in Samia ricini, dominated by bacterial species from the genera Bacillus and Lysinibacillus. The identified strains are closely related to bacteria known for their digestive enzyme production, antimicrobial activity, and nutrient cycling capabilities. These gut microbes likely play essential symbiotic roles that enhance digestion, immunity, and silk production in the Eri silkworm. The high sequence similarity (96–98%) confirms the presence of these integral bacterial taxa in the silkworm gut. Understanding their functional contributions opens promising avenues for probiotic development and microbial management strategies to improve larval health and sustainable silk farming. Future research should focus on the functional genomics and metabolic pathways of these gut bacteria to better harness their benefits for sericulture.

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