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Identification and pathogenicity assessment of bacterial strain P1 isolated from the gut of diseased Eri silkworm, *Samia ricini* Donovan

Dimpi Moni Kalita[1*], Sunayan Bardoloi[2], Sanghamitra Saharia[1], Shibani Kalita[1], Sanjana Sharmin[1], Dhirunabh Swargiary[3], Tapashi Sutradhar[1], Kakali Deka[1]

[1]Department of Zoology, Gauhati University, Guwahati, India.
[2]Department of Zoology, Girijananda Chowdhury University, Guwahati, India.
[3]Advanced Level Institutional Biotech Hub, B. Borooah College, Guwahati, India.

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ABSTRACT

Pathogenicity assessment studies in economically important species like silkworms are very important for the development of sericulture-based industries. The present study is a sincere attempt in this regard. The objective of this study is to isolate and identify a pathogenic bacterial strain from the gut of diseased Eri silkworms (*Samia ricini*) and assess its pathogenicity. Hence, gut bacterial colonies from *S. ricini* were isolated, and out of them, one strain (P1) was used for this study, which was identified as *Bacillus cereus* (PP505527) by 16S rRNA sequencing. Different concentrations of the strain were prepared to study the lethality of the bacterium. The Lethal concentration (LC_{50}) value of *B. cereus* was recorded to be 8.90×10^7, 2.55×10^6, 1.83×10^5, and 4.67×10^2 CFU/ml at 24, 48, 72, and 96 hours, respectively. The current study might provide valuable insights into understanding the disease prognosis of Eri silkworms and potential disease management strategies for these economically important silkworms.

1. INTRODUCTION

Pathogens are a varied group of organisms that can cause numerous health ailments to living organisms. All species existing in this biosphere are exposed to such pathogens which are responsible for various diseases. Like all other organisms, insects too are highly prone to various pathogens, many of which are the causes of various diseases. Insect diseases are very specific and can vary from species to species. The gut of insects harbors numerous symbiotic bacteria, but due to contamination of food or other sources, pathogens of various kinds often make their way into the insect gut and lead to alteration of the gut environment, thereby causing various pathogenic conditions [1].

So pathogenic assessment studies in insects abound in the biological literature. But among all insects, pathogenicity assessment of gut microbes is largely done in silkworm varieties due to their economic importance. Silkworms are affected by a number of diseases among which, nuclear polyhedrosis, bacterial and viral flacherie, muscardine, and pebrine are most common. Silkworms are exposed to these pathogens during their rearing process and as such understanding the microbes associated with such diseases in silkworm have proved to be a major achievement in the production of better breeds of silkworms resulting in rapid advancement in the sericulture industry as a whole. As sericulture is one of the most profitable cash crops and it involves different sections of people such as farmers, reelers, twisters, weavers, and traders in the production and marketing operations, such a study is very pertinent under the existing scenario.

Bacterial diseases are common in silkworms and occur frequently, mainly in summer and autumn rearing seasons when hot and humid conditions prevail [2]. The symptoms of diseases vary from species to species which may include sluggishness, retarded growth, flaccidity, dysentery, reddish black body color, deformed cocoon, and so on [2,3].

The Eri silkworm (*Samia ricini*) is a completely domesticated and commercially exploited variety of silkworms in the Indian subcontinent. The production of Eri silk is traditional in the north-eastern states of India and particularly carried out by the tribal people of Assam, Bihar, Orissa, U.P., West Bengal, and some other states of India. But crop loss caused by bacterial infections is a huge concern for the silkworm rearers, hence identifying the pathogens is a major step towards preventing the incidence of diseases. Although in earlier studies various pathogens have been isolated and identified from the gut of *Bombyx mori* and *Antheraea assamensis*, no such

*Corresponding Author
Dimpi Moni Kalita, Department of Zoology, Gauhati University, Guwahati, India. E-mail: dimpimkalita @ gmail,com

studies have been carried out in *S. ricini*. Also, the molecular characterization of gut microbiota associated with this economically significant silkworm remains less explored. To address these gaps, this study aims to isolate and identify a bacterial strain P1 from the gut of diseased Eri silkworms (*S. ricini*) and assess its pathogenicity. This is the first attempt at identifying disease-causing bacteria of Eri silkworm. The current study will provide valuable insights into understanding the disease prognosis of Eri silkworms and possible approaches for managing diseases in these economically important silkworms.

2. MATERIALS AND METHODS

2.1. Experimental Silkworm

Diseased fifth instar Eri silkworms were collected from Government Eri Silkworm grainage, Barduar, Assam (25.938879ºN and 91.468233ºE). The gut of these silkworms was extracted and used for the isolation of pathogenic bacteria [4].

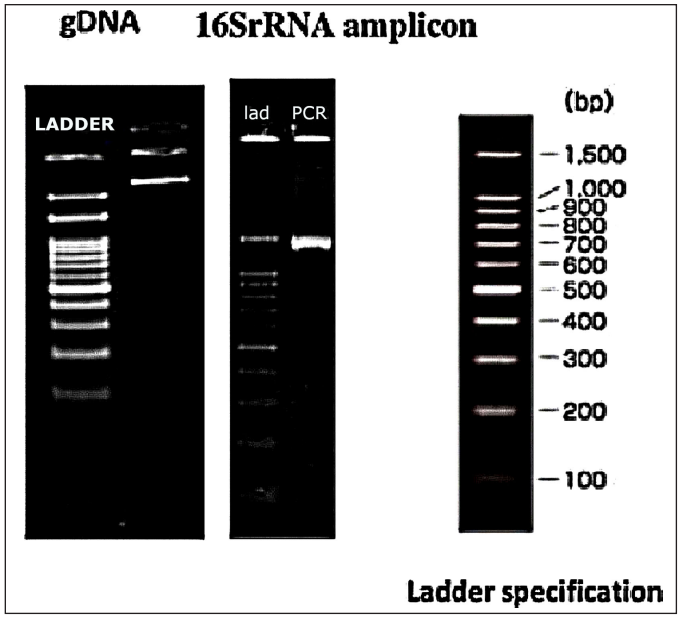

Figure 1. P1 Genomic DNA and amplification QC.

2.2. Gut Homogenization

The extracted gut was then homogenized with a mortar and pestle in 10 ml of sterile saline water. The stock was used for streaking purposes in nutrient agar (NA) plates [5].

2.3. Isolation and Culture

Streaks were made on nutrient agar plates from the gut stock using a sterile inoculating loop, and the plate was incubated for 24 hours at 37°C. After the growth of bacterial colonies, pure culture was performed by selecting distinct morphological colonies and restreaking them on the fresh nutrient agar plates. They were then kept in the incubator for bacterial growth. The process was repeated until individual colonies were obtained. Among the isolated bacteria, P1 was selected for this study.

2.4. DNA Extraction and 16S rRNA Sequencing

The DNA of the bacterial isolates was extracted by following the method described by Moore *et al.* [6]. The bacterial isolate was inoculated into 5 ml of LB culture broth and incubated at 37°C overnight. After transferring the culture to a tube, the cells were centrifuged at 10,000 rpm for 5 minutes. The resulting pellet was resuspended in a solution containing 567 µl of Tris-EDTA buffer, 5 µl of RNAse A, 4 µl of proteinase K (18 mg/ml), and 15 µl of SDS (10%) and incubated for 1 hour at 37°C. Following this, 100 µl of 5M Sodium chloride and 80 µl of 10% of 0.7M cetyltrimethylammonium bromide/NaCl solution were added, thoroughly mixed, and incubated at 65°C for 10 minutes. Next, 0.7 ml of a 24:1 chloroform/isoamyl alcohol solution was incorporated into the mixture, which was then centrifuged for 5 minutes at 10,000 rpm. The aqueous phase was collected to which a mixture of 25:24:1 of phenol/chloroform/isoamyl alcohol was added. It was then centrifuged at 15,000 rpm for 10 minutes. An equal volume of isopropanol was added to the supernatant and centrifuged for 20 minutes at 15,000 rpm. The DNA pellet was washed with 70% ethanol and centrifuged for 10 minutes at 12,000 rpm. The pellet was dissolved in 50 µl of Tris-EDTA buffer [5].

A single band of high-molecular-weight DNA was revealed upon evaluating the quality of the DNA using 1.0% agarose gel. The 16S rRNA was amplified with 16SrRNA-F and 16SrRNA-R primers, using the BDT v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyzer [7]. A distinct 1,500 bp PCR amplicon band was observed on the agarose gel which was then purified to eliminate contaminants.

Table 1. Top 10 BLAST hits against the standard(nr/nt) database used to construct the phylogenetic tree.

Description	Max score	Total score	Query cover	*E* value	Per. identity	Accession
Bacillus cereus strain BAB-6967	2,747	2,747	100.00%	0	100.00%	MF351827.1
Bacillus cereus strain ABW-16	2,743	2,743	99.00%	0	100.00%	ON763255.1
Bacillus cereus strain SNW-26	2,743	2,743	99.00%	0	100.00%	ON763249.1
Bacillus sp. strain VMF2	2,743	2,743	99.00%	0	100.00%	OL872285.1
Bacillus cereus strain SK-37	2,743	2,743	99.00%	0	100.00%	OL839988.1
Bacillus sp. BAB-5792	2,741	2,741	99.00%	0	100.00%	KX181401.1
Bacillus paramycoides strain HIGB13	2,739	2,739	99.00%	0	99.93%	OR414055.1
Bacillus paramycoides strain DS13	2,739	2,739	100.00%	0	99.93%	MN391011.1
Bacillus cereus strain AFS099958	2,737	2,737	99.00%	0	100.00%	OP986960.1
Bacillus cereus strain AFS087345	2,737	2,737	99.00%	0	100.00%	OP986906.1
Bacillus anthracis strain A168	2,673	29,390	99.00%	0	100.00%	CP076170.1

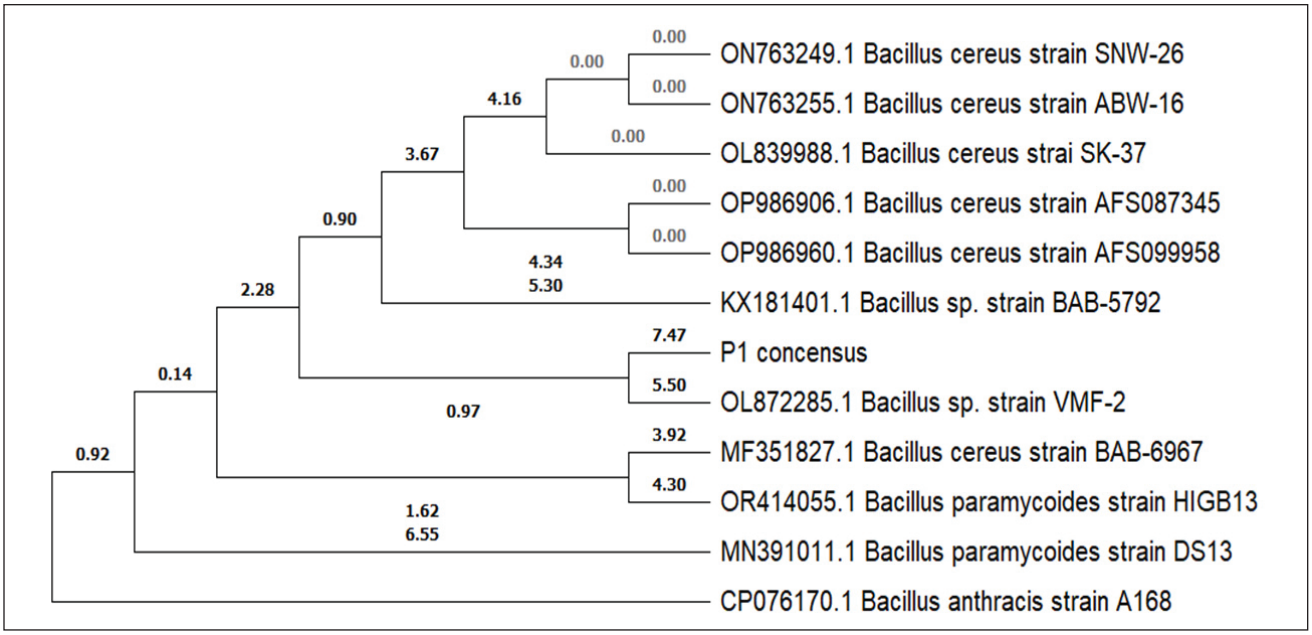

Figure 2. Phylogenetic tree of P1 (neighbour joining method) with its closely related species (downloaded from NCBI) based on 16s rRNA sequencing.

Table 2. Data on probit value against test concentrations of *B. cereus* on fifth instar Eri silkworm. Mortality at 24 hours was presented as mean of three replicates of silkworm larvae.

CFU/ml	log10 of concentration	Total exposed	Portality at 24 hrs	% mortality	Probit value	Probability
0	control	10	0	0	0	0
1×10^1	1	10	0	0	0	0.003
1×10^2	2	10	0	0	0	0.010
1×10^3	3	10	0	0	0	0.027
1×10^4	4	10	1	10	3.72	0.062
1×10^5	5	10	1.3	13	3.87	0.126
1×10^6	6	10	3	30	4.48	0.224
1×10^7	7	10	3.3	33	4.56	0.356
1×10^8	8	10	4.6	46	4.9	0.508
1×10^9	9	10	6.6	66	5.41	0.659

Table 3. Data on probit value against test concentrations of *B. cereus* on fifth instar Eri silkworm. Mortality at 48 hours was presented as mean of three replicates of silkworm larvae.

CFU/ml	log10 of concentration	Total exposed	Mortality at 48 hours	% mortality	Probit value	Probability
0	Control	10	0	0	0	0
1×10^1	1	10	0	0	0	0.003
1×10^2	2	10	0	0	0	0.01
1×10^3	3	10	0.66	6.6	3.45	0.027
1×10^4	4	10	1.3	13	3.87	0.062
1×10^5	5	10	3.3	33	4.56	0.126
1×10^6	6	10	4.6	46	4.9	0.224
1×10^7	7	10	6	60	5.25	0.356
1×10^8	8	10	7.33	73.3	5.61	0.508
1×10^9	9	10	8.66	86.6	6.08	0.659

Table 4. Data on probit value against test concentrations of *B. cereus* on fifth instar Eri silkworm. Mortality at 72 hours was presented as mean of three replicates of silkworm larvae.

CFU/ml	log10 of concentration	Total exposed	Mortality at 72 hours	% mortality	Probit value	Probability
0	Control	10	0	0	0	0
1×10^1	1	10	0	0	0	0.020
1×10^2	2	10	0.66	6.6	3.45	0.057
1×10^3	3	10	1.33	13.3	3.87	0.137
1×10^4	4	10	3.33	33.3	4.56	0.271
1×10^5	5	10	4.6	46	4.9	0.449
1×10^6	6	10	6	60	5.25	0.639
1×10^7	7	10	7.6	76	5.71	0.799
1×10^8	8	10	9.3	93	6.48	0.907
1×10^9	9	10	9.66	96.6	6.75	0.965

Table 5. Data on probit value against test concentrations of *B. cereus* on fifth instar Eri silkworm. Mortality at 96 hours was presented as mean of three replicates of silkworm larvae.

CFU/ml	log10 of concentration	Total exposed	Mortality at 96 hours	% mortality	Probit value	Probability
0	Control	10	0	0	0	0
1×10^1	1	10	3.3	33	4.56	0.320
1×10^2	2	10	4.6	46	4.9	0.426
1×10^3	3	10	5.3	53	5.08	0.537
1×10^4	4	10	6.3	63	5.31	0.645
1×10^5	5	10	7.3	73	5.61	0.743
1×10^6	6	10	8	80	5.84	0.824
1×10^7	7	10	8.6	86	6.08	0.887
1×10^8	8	10	9.3	93	6.48	0.932
1×10^9	9	10	10	100	8.95	0.962

Using aligner software, a consensus sequence of the 16S rRNA gene was generated by aligning the forward and reverse sequences.

2.5. Phylogenetic Analysis

A BLAST search of the 16s rRNA gene was carried out with the NCBI GenBank database [8]. The top 10 sequences with the highest identity scores were selected and aligned using the Clustal W multiple alignment software. Using the neighbor-joining method, a phylogenetic tree was constructed [9]. All evolutionary analyses were performed using MEGA11 software [10].

2.6. Assessment of Lethal Concentration

2.6.1. Preparation of bacterial suspension

Various concentrations (ranging from 10^9 to 10^1 CFU/ml) of the stock culture were made by serial dilution method. 10 ml of each culture was centrifuged for 10 minutes at 5,000 rpm and the obtained residues were resuspended in PBS [5].

2.6.2. Determination of lethal concentration (LC_{50})

The concentration at which half of the silkworms die within a specified time interval is known as lethal concentration. LC_{50} was calculated using the method followed by Saharia *et al.* 2024 [7]. Different concentrations of the identified strain ranging from 10^9 to 10^1 CFU/ml were prepared. To calculate the LC_{50} value, three replicates with 10 freshly molted fifth-instar larvae each for each concentration were taken along with one control group. Each group of silkworms was injected with different concentrations of bacteria as mentioned above. The control group was injected with PBS. Frequent monitoring was conducted to observe mortality for up to 96 hours. The number of silkworms that died at various concentrations and time intervals (24–96 hours) was recorded for all groups, and the mean mortality was then calculated (Tables 2–5). Using IBM SPSS version 25.0, LC_{50} values were determined for a 24–96 hours period through probit analysis following Finney's method [11]. Linear regression curves were drawn with probit mortality on the Y-axis and log concentration on the X-axis in MS Excel 2010 (Figs. 3–6).

3. RESULTS

3.1. Identification of the Species

Upon pure culture, the P1 colony was observed macroscopically to be dull greyish, opaque, and fuzzy with a rough matted surface. DNA was isolated from this culture and a fragment of 16S rRNA was amplified. A prominent 1500 bp PCR amplicon band was detected when separated on an agarose gel (Fig. 1). A consensus sequence of 1487 bp of the 16S rRNA gene was generated. To identify the isolated P1 strain, BLAST search analysis was done which revealed

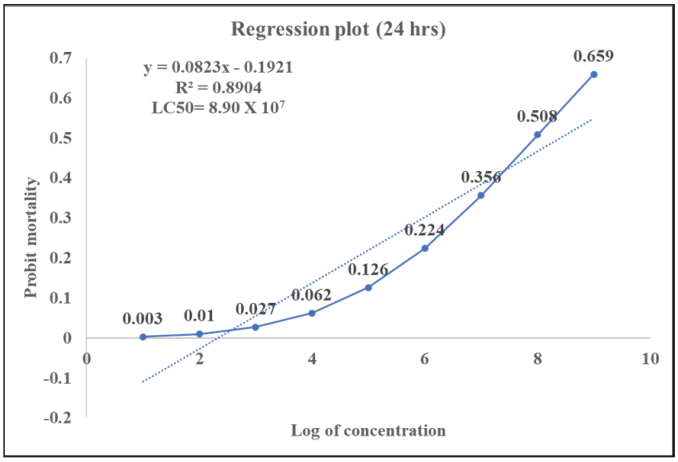

Figure 3. Regression plot of probit mortality and log concentration for *B. cereus* to *S. ricini* fifth instar larvae for 24 hours.

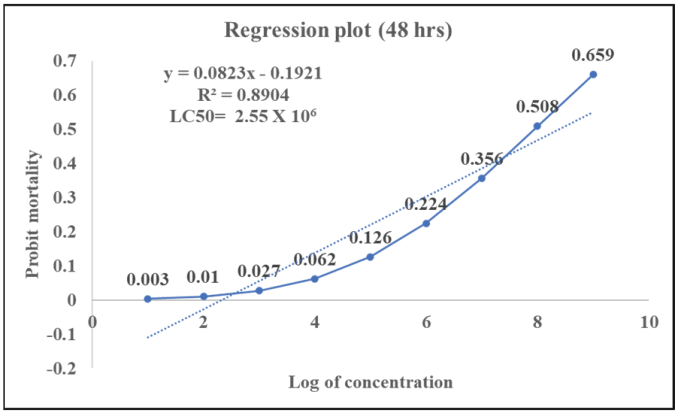

Figure 4. Regression plot of probit mortality and log concentration for *B. cereus* to *S. ricini* fifth instar larvae for 48 hours.

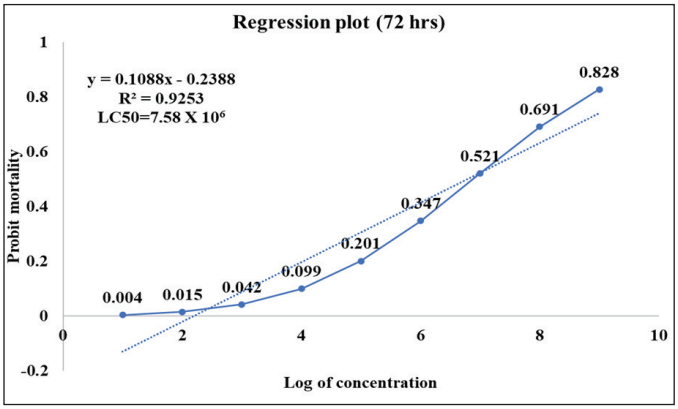

Figure 5. Regression plot of probit mortality and log concentration for *B. cereus* to *S. ricini* fifth instar larvae for 72 hours.

a 100% similarity between the P1 strain and previously documented *Bacillus cereus* strains confirming it as *B. cereus* (Table 1). The obtained sequence was submitted to NCBI GenBank and assigned the accession number PP505527. To demonstrate the evolutionary relationship, phylogenetic analysis was conducted based on 16S rRNA sequence homology which is presented in Figure 2. The

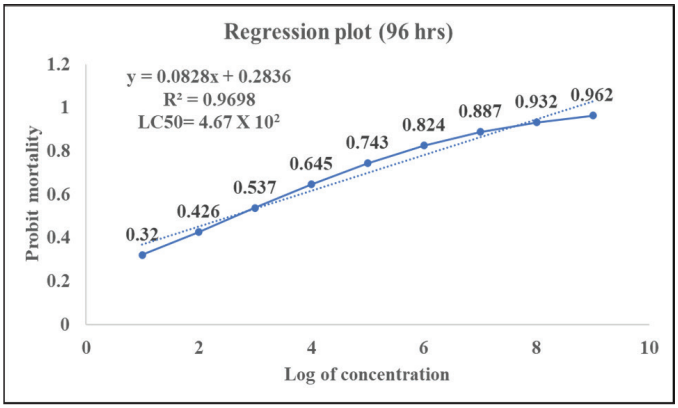

Figure 6. Regression plot of probit mortality and log concentration for *B. cereus* to *S. ricini* fifth instar larvae for 96 hours.

homology search was performed using BLAST analysis with 10 other closely related bacterial species from NCBI GenBank, utilizing 1,000 bootstrap runs.

3.2. Determination of Lethal Concentration

The silkworms fed with P1 exhibited similar symptoms to those of the diseased silkworms selected for the experiments. It was noted that as the duration and concentration of *Bacillus cereus* increased, the mortality rate of Eri silkworm larvae also rose, while the control groups showed no mortality. In this research, the average mortality rates of the Eri silkworms were assessed at various intervals from 24 to 96 hours, as detailed in Tables 2–5. Using the gathered data, LC_{50} values were determined through Probit analysis with IBM SPSS 25.0. Linear regression curves were then created in MS Excel 2010, plotting probit mortality on the Y-axis and log concentration on the X-axis (Figs. 3–6). The LC_{50} value of *Bacillus cereus* was recorded to be 8.90×10^7, 2.55×10^6, 1.83×10^5, and 4.67×10^2 CFU/ml at 24, 48, 72, and 96 hours, respectively.

4. DISCUSSION

The present study aimed at isolating and identifying a disease-causing strain of bacteria from the gut of Eri silkworm. Through 16S rRNA sequencing, the bacterial strain of interest was identified as *Bacillus cereus*. A similar method of identification has been adopted by other researchers as well [12–14] and so on, for the identification of bacterial strains in *A. assamensis and B. mori*. In the study conducted by Choudhury *et al.* [12], 19 strains of bacteria were isolated from diseased *A. assamensis* out of which strain AC-3 was found to be highly pathogenic and hence further processed for identification. AC-3 was identified as *Pseudomonas aeruginosa* which caused 100% mortality upon 72 hours of injection. Furthermore, in our study, 16S rRNA gene homology of identified bacterial strains was followed by phylogenetic analysis of the bacteria, which reveals the systematic position of the identified bacteria. This is an important step in the identification process and adopted by various researchers [7,15] and so on. to establish evolutionary relationships between the strain in question and the already-known species.

Similar to the results found in this study, *B. cereus* was also identified as being pathogenic to *B. mori* in a few other studies [16,17,3]. Different species of *Bacillus* were found to be the abundant bacterial genera in diseased *Antheraea proylei* J in the study conducted by Devi *et al.* [15]. However, the incidence of

B. cereus has not yet been reported in the silkworm *S. ricini*. In a study [18], various species of *Bacillus* were isolated from silkworm *B. mori* which were pathogenic to the host. Along with six other bacterial species, *B. cereus* was also identified to cause flacherie in silkworm *B. mori* in a study conducted by Sakthivel *et al.* [2]. Li *et al.* [17] as mentioned earlier, went further and carried out the pathogenicity assessment of *B. cereus* in *B. mori*. The bacterium could reproduce septicemia-like symptoms with a high mortality rate when re-fed to healthy silkworms which was parallel to the findings in our study. They reported the LC_{50} value to be 5.45×10^4 CFU/ml after 48 hours of inoculation which is lesser than the value reported in this study which is 2.55×10^6 CFU/ml. This difference in toxicity might be due to the involvement of different strains of *B. cereus* and host specificity. This study is a preliminary investigation and aims mainly at identifying the causal organism for bacterial infection. Therefore, further detailed study in this regard is very pertinent for enhancing our knowledge of the diversity and composition of gut bacteria in eri silkworms, as well as for improving their disease management.

5. CONCLUSION

Bacterial diseases frequently affect silkworms, causing significant losses in sericulture. In this study, a bacterial strain labeled P1 was isolated from the gut of the Eri silkworm, *S. ricini*, and identified as *B. cereus*. This bacterium was found to be highly pathogenic to the silkworms. This study represents one of the initial efforts to genetically characterize the gut microbiota of *S. ricini*. Detailed studies, however, are necessary to understand how this bacterium causes disease in silkworm larvae. Therefore, investigating this bacterium could have important implications for sericulture. A more thorough analysis of gut bacteria is required to gain a deeper understanding of the role microbiota play in various physiological processes of this significant silkworm.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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There is no funding to report.

9. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

13. PUBLISHER'S NOTE

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