Isolation and identification of two bacterial species (Genus: Bacillus) from flacherie-infected larvae of *Samia ricini*

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Abstract
Samia ricini (Eri silkworm) is a commercially exploited, sericigenous lepidopteran species of the northeastern State of India, Assam. The species is often contaminated by various microbial pathogens and as a result, suffers from heavy mortality. Flacherie is one type of microbial disease that causes 30%-100% crop loss annually in the ericulture sector of Assam during summer. However, studies on the identification and pathogenesis of bacteria in Samia ricini are lacking. The present study aims to identify bacterial species involved in the flaccid condition of Samia ricini. For this, hemolymph of infected 5th instar larvae was selected as inoculum and bacteria were cultured and incubated in nutrient agar media at 37^{0}C for 48 hours. Distinct colonies that grew on the culture media were subjected to identification through morphological, biochemical and molecular methods.

The isolated DNA's colony morphology, biochemical tests and 16S rRNA sequencing identified the isolates as Bacillus thuringiensis and Bacillus cereus. The current study gives insight into the bacterial diversity responsible for flaccid conditions in Samia ricini which might help to understand the mechanism of pathogenesis, disease prognosis and colonization of different flacherie-causing bacteria in Samia ricini.

Keywords: Flacherie, Microbial infestation, 16S rRNA sequencing, IMViC test.

Introduction

Commercially exploited sericigenous lepidopterans are exposed to various microbial attacks throughout the world. There are different factors associated with microbial contamination in silkworms. These include fluctuations in temperature and relative humidity, heavy rainfall, unhygienic conditions and improper management of rearing area and supply of decayed, watery and contaminated leaves[6,27,46]. Eri silkworm is an important commercial silk variety of Assam. Being poikilothermic, the species respond quickly to environmental changes that lead the organism towards different pathogenic attacks such as viral, bacterial, fungal and protozoan[46]. Among the different factors that affect eri silkworms, bacterial attack is more prevalent. Flacherie (locally known as mukhloga rog), which is common to eri silkworms, occurred due to different groups of colonizing bacteria[14,28]. Flacherie can be infested either by viruses or bacteria.

Viral flacherie is inflicted by infectious flacherie virus and densovirus[50] whereas bacterial flacherie is caused by different bacteria viz; *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus carboniphilus*, *Bacillus bombysepticus*, *Enterococcus cecorum*, *Enterobacter asburiae*, *Pseudomonas aeruginosa*, *Ornithinibacillus bavariensis*, *Achromobacter xylosoxidans*, *Staphylococcus aureus*, *Serratia marcescens*, *Streptococcus* sp, *Staphylococcus argenteus*, *Paracoccus niistensis and Staphylococcus* sp, etc.[12,22,32,42,46]

There are many causes by which flacherie can be infested in eri silkworms such as the feeding of contaminated, watery foliage, presence of diseased, dead, decayed larvae in and around the rearing area, fecal matters, body fluid of infected larvae, contaminated rearing site and appliances[5,6]. Sluggishness, weakness, loss of appetite, vomiting liquid substances, the release of chain type, semi-solid excreta, loss of clasping power, inability to moult, liquefaction of inner organs, gradual blackening of body and body fluid, unpleasant smell, body softening and rupture etc. are some of the common symptoms of flacherie in silkworms[47,52,62].

In the case of flacherie, during the initial stages of infection, the larva becomes lethargic and stops eating. At an advanced stage of disease, the larva exhibits retarded growth, starts vomiting gut juices and releases semi-solid faeces. Eventually, the larva becomes soft and translucent. Finally, the larvae ferments and the inner content turns into a black-coloured liquid and emits a foul odour[47]. Another important point regarding flacherie infestation is that symptoms remain unnoticed by the rearers during early instar stages which led spreading of disease in the rearing areas. As a result, in the later stages, when larvae grow, symptoms become prominent leading to severe larval mortality and less cocoon production[52]. Proper observation, scientific management, prevention of flacherie infestation and breeding of silkworm variety with high productivity are important issues and concerns in sericulture in terms of its commercial prospects.

Though bacterial disease is common to eri silkworms, proper identification and etiology of bacterial species in eri silkworms is scanty. Earlier reports suggested the presence of bacillus and coccus types of bacteria in the hemolymph of infected eri silkworms[37]. Therefore, the present study aims to identify bacterial species isolated from infected eri silkworms.

Material and Methods

Collection of Eri silkworm larvae and culture of bacteria: Fifth instar infected Eri silkworms were selected for the study and these were collected from Eri-rearing areas of Nayekgaon Pt-I village (26.22/06//N, 90.22/10//E) of Kokrajhar district, Assam. Collected silkworms were kept under sterilized condition, rinsed twice with sterile water and then surface sterilized with ethanol (70% v/v) for 60 seconds followed by final rinsing with sterile distilled water. The hemolymph of the infected silkworms was collected in Eppendorf tubes and one loop-full of hemolymph is streaked on petri plates having nutrient agar media. Petri plates were then incubated at 37^0C for 48 hours[21,57]. Colonies that grew predominantly after 48 hours of incubation were considered for the study and were sub-cultured to attain pure culture. Pure cultures of bacteria were stored in a solution of sterile glycerol (15%) on sterile screw cap microfuge tubes in a ratio of 1:1 and then frozen at -80^0C for further use.

Identification of bacterial species: Bacterial isolates were identified based on colony morphology, microscopic observation through Gram staining and Scanning electron microscopy, biochemical tests and molecular identification methods.

Colony morphology: Different colony characters viz., size, form, margin, elevation and colour, were considered. The works of Ranganathan et al[43], Ammons et al[1], Astuti et al[4], Aneja et al[3], El-Kersh et al[17], Mahdi et al[36], Ghosh et al[19] and Kandi et al[26] were followed to perform colony morphology of the bacterial isolates.

Gram staining method: For Gram staining, the method explained by Ranganathan et al[43] was followed with slight modification. Bacterial samples were smeared onto a slide with a drop of sterile distilled water and fixed. Then, the Gram staining procedure was carried out by staining the slides with crystal violet (1-2 minutes) followed by washing and fixation with Gram's iodine (1-2 minutes). Slides were washed again by ethanol (15-20 seconds) and stained with safranin (30 seconds-1 minute). Slides were washed thereafter and kept for blot dry. After that, slides were observed under the Leica microscope (LEICA DM750) at 100× resolution by adding immersion oil (1-2 drops).

Scanning Electron Microscopy: For SEM, bacterial samples (1.5 ml of bacterial suspension in Eppendorf tube) grown in nutrient broth were first centrifuged at 7000 rpm for 10 minutes. After that supernatant was discarded and the pellets were kept for further processing. To the pellet, 500µl of 1× PBS was added and washed twice (centrifuge at 7000 rpm for 10 minutes). Then 500µl of 2.5% glutaraldehyde was added and kept overnight for cell fixation. After fixation, glutaraldehyde was removed by centrifugation (7000rpm, 10 minutes) followed by washing the pellets with 1× PBS buffer (500µl) (7000rpm, 5min) three times. Pellets were now dehydrated with gradients of ethanol (30%-40%-50%-60%-70%-80%-90%; centrifuge at every step: 700rpm, 5 minutes). Finally, 100% ethanol was added to complete the dehydration process. The samples were on a toothpick and spread in a cover slip under a stereo zoom microscope (Stemi508). Samples were then processed for gold sputter coating and observed under Field emission scanning electron microscope (Sigma VP Fesem, Zeiss) at 10.00KX and 15.00KX magnification.

Biochemical tests: IMViC (Indole, Methyl Red, Voges Proskauer's, Citrate test) and utilization test of 8 different carbohydrates (glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose and sucrose) were performed by using rapid biochemical identification test kits (KB001 HiIMViC™ biochemical test kit, purchased from Hi-Media). At first, kits were opened aseptically and sealing tapes were peeled off. Then, each well of the kits was inoculated with a loopful of inoculum. Kits were then incubated at 35-37^0C for 18-24 hours. After incubation, 1-2 drops of Kovac's reagent (R008), Methyl red reagent (I007) Baritt reagent A (R029) and Baritt reagent B (R030) were added to the good number 1, 2 and 3 to carry out indole, methyl red and Voges Proskauer's tests respectively and results were interpreted as per the standard interpretation chart supplied with the kits. For citrate and carbohydrate utilization tests, after incubation, changes in the color of the wells were also matched with the result interpretation chart supplied with the kits.

Molecular identification method: 16S rRNA gene sequencing and multiple sequence alignment using the NCBI BLAST similarity search tool were used to identify the bacterial isolates. For this, genomic DNA isolation of the bacteria was carried out using a Biobee spin EXpure microbial DNA isolation kit developed by Bogar Bio Bee Stores Pvt. Ltd., Coimbatore, Tamil Nadu, India. For 16S rRNA sequencing, 5µL of the isolated DNA was added in 25µL of PCR reaction solution containing 1.5µL of forward primer (27F: 5' AGAGTTTGATCTGGCTCAG 3') and reverse primer (1492R: 5' TACGGTACCTTGTTAC GACTT 3'), 5µL of deionized water and 12µL of Taq Master Mix (Taq DNA polymerase is supplied in 2X Taq buffer, 0.4mM dNTPs, 3.2mM $MgCl_2$ and 0.02% bromophenol blue). The mixture was then subjected to the PCR (Total number of cycles: 25).

To remove unincorporated PCR primers and dNTPs from PCR products, a Montage PCR Clean-up kit (Millipore) was used. The PCR product was sequenced using the primers. Sequencing reactions were performed using ABI PRISM®BigDyeTM terminator cycle sequencing kits with AmpliTaq® DNA polymerase (FS enzyme; Applied Biosystems). Single-pass sequencing was performed on each template using below 16S rRNA universal primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

The 16S rRNA sequence was blast using the NCBI blast similarity search tool. The phylogeny analysis of the query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences[16]. The resulting aligned sequences were cured using the program Gblocks 0.91b. Gblocks eliminate poorly aligned positions and divergent regions to remove alignment noise[55]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as a substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster. Tree Dyn 198.3 was used for tree rendering[15]. All the identified sequences of bacteria were submitted to the NCBI nucleotide database to get the GenBank accession numbers.

Results and Discussion

In the present study, infected larvae of *S. ricini* were collected from the aforementioned collection sites to identify the causative microbial agent. Two distinct colonies of bacteria were isolated from the hemolymph of the collected silkworm larvae. The purity of these cultures was maintained by repetitive streaking (sub-culture) and then identified by following the above-mentioned approaches.

Colony morphology of the isolates: All the isolated bacterial cultures appeared rounded with the entire margin. They formed large colonies in the culture media. The S1 colony was slightly raised, while the S2 colony had convex elevation. Both colonies were displayed in nutrient agar media after 48 hours of incubation. Gram staining and observation through a Leica microscope revealed a Gram-positive (purple color), rod-shaped bacillus, which was later confirmed by Field emission scanning electron microscopy (Table 1 and fig. 1 to 8).

Biochemical tests: IMViC test (Indole test, Methyl Red Test, Voges- Proskauer Test and Citrate test) and utilization of 8 different carbohydrates viz., glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose and sucrose were carried out to differentiate and to identify bacterial colonies based on different biochemical activities such as presence of enzymes, metabolism of protein, fat and carbohydrate, compound utilization etc.

In the present study, all the isolates showed negative results for the methyl red test and positive results for Voges Proskauer's and citrate utilization test. Isolate S1 was found indole positive, whereas S1 showed negative results during the IMViC test (Table 1). In the case of the carbohydrate utilization test, both the isolates showed the inability to ferment adonitol, arabinose, lactose, sorbitol, mannitol and rhamnose after 18-24 hours of incubation. However, both the isolates were able to ferment glucose and sucrose after incubation (Table 1).

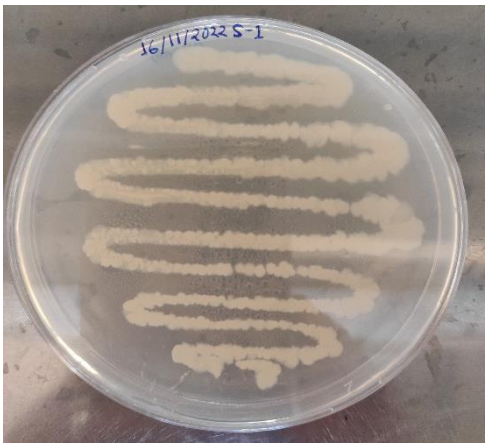

Fig. 1: S1 on Nutrient Agar Media

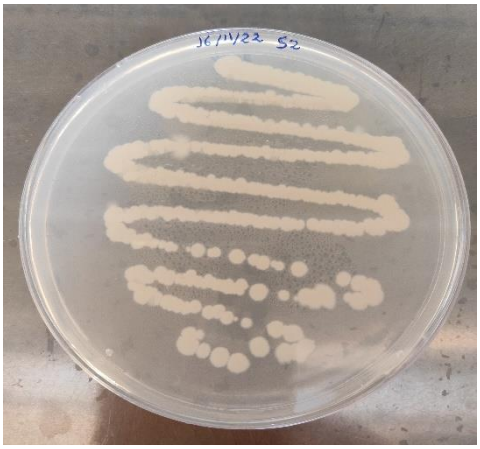

Fig. 2: S2 on Nutrient Agar Media

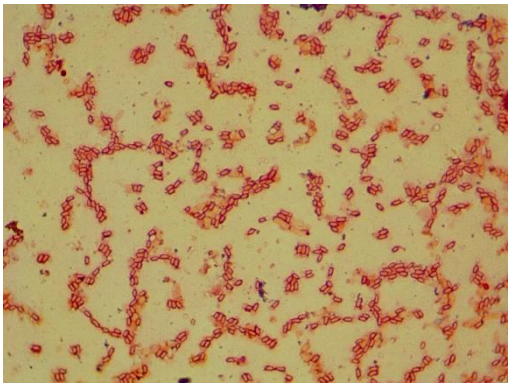

Fig. 3: Gram-staining of S1 isolate

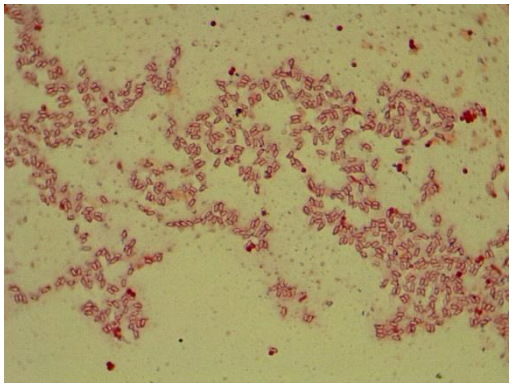

Fig. 4: Gram-staining of S2 isolate

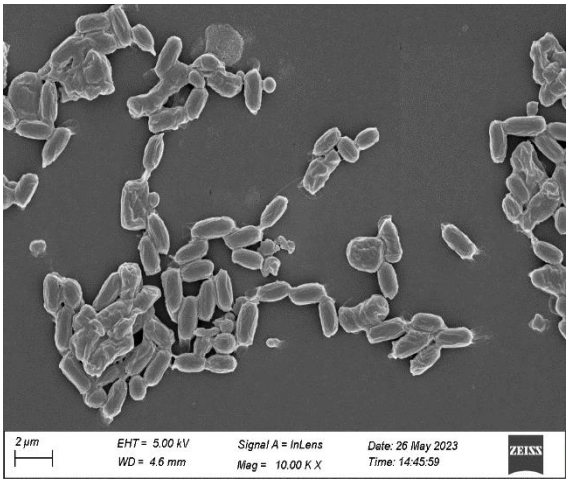

Fig. 5: FESEM of S1 isolate at 10.00 KX

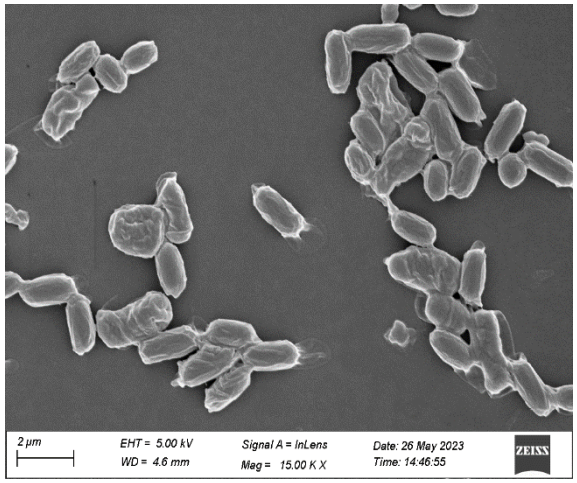

Fig. 6: FESEM of S1 isolate at 15.00 KX

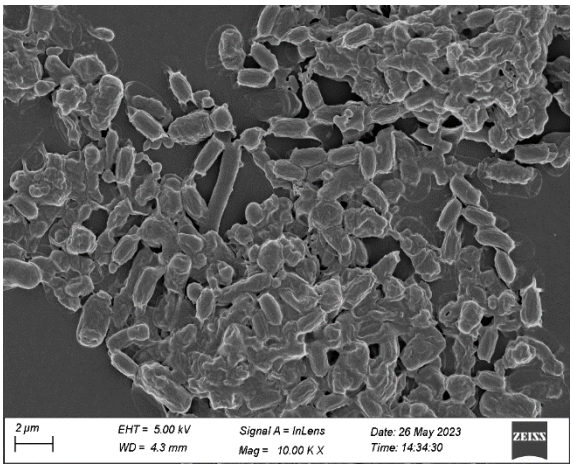

Fig. 7: FESEM of S2 isolate at 10.00 KX

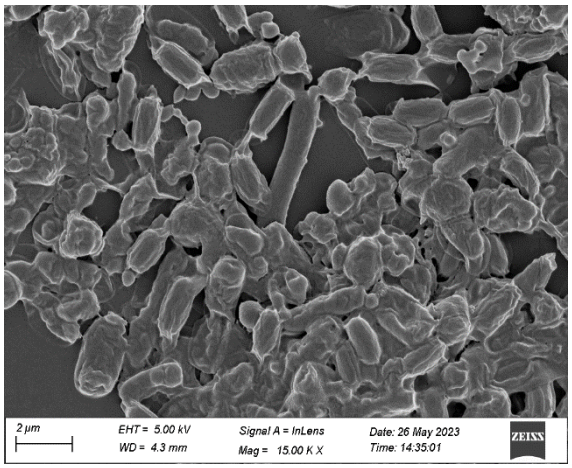

Fig. 8: FESEM of S2 isolate at 15.00 KX

Table 1
Morphological and biochemical identification tests of the bacterial isolates (S1 and S2)

Identification Tests Performed	S1	S2
Morphological Identification Tests		
1. Size	Large	Large
2. Form	Circular	Circular
3. Margin	Entire	Entire
4. Elevation	Slightly raised	Convex
5. Color	White	White
6. Gram Staining	Purple, Gram Positive	Purple, Gram Positive
7. Shape	Rod-Shaped, Bacillus	Rod-Shaped, Bacillus
Biochemical Identification Tests		
1. Indole	Positive	Negative
2. Methyl Red	Negative	Negative
3. Voges Proskauer	Positive	Positive
4. Citrate Utilization	Positive	Positive
5. Glucose	Positive	Positive
6. Adonitol	Negative	Negative
7. Arabinose	Negative	Negative
8. Lactose	Negative	Negative
9. Sorbitol	Negative	Negative
10. Mannitol	Negative	Negative
11. Rhamnose	Negative	Negative
12. Sucrose	Positive	Positive

Molecular identification method: Results of 16S rRNA sequence through NCBI-blast similarity search tool and multiple sequence alignment of the gene sequences identified S1 and S2 as *Bacillus thuringiensis* and *Bacillus cereus* respectively. 16S rRNA gene sequences of the identified bacteria showed various degrees of similarity with the nearest strain type (Table 2, Table 3).

Sequences were submitted to the NCBI nucleotide database. Submitted sequences of these identified species were accepted and available under GenBank accession numbers OQ220313 against *Bacillus thuringiensis* and OQ220314 against *Bacillus cereus.*

Outbreak of diseases due to different biotic and abiotic factors is one of the noxious problems in sericulture. It hinders total productivity and deteriorates the quality of silk. Being multivoltine, eri silkworm is exposed to different environmental conditions such as temperature, humidity, rainfall etc. Fluctuations in these parameters nurture a wide range of microbial species that eventually create hazards in the rearing areas of eri silkworms.

Table 2
Accession numbers and the percentage identified of the sequences producing significant alignments with the query sequence of the S1 isolate

Accession Numbers	Percentage identified	Accession Numbers	Percentage identified
OP703396.1	100%	ON351573.1	100%
CP106947.1	100%	ON351572.1	100%
ON045795.1	100%	ON351571.1	100%
OP209990.1	100%	ON351569.1	100%
OP209989.1	100%	CP094624.1	100%
OP209988.1	100%	CP045585.1	100%
OP204093.1	100%	CP074714.1	100%
OP160529.1	100%	OM280115.1	100%
OP160528.1	100%	OP474008.1	99.92%
CP072691.1	100%	ON738723.1	99.92%
ON763233.1	100%	ON350771.1	99.92%
ON629769.1	100%	ON697194.1	98.85%
ON598626.1	100%	ON351574.1	100%

Table 3
Accession numbers and the percentage identified of the sequences producing significant alignments with the query sequence of the S2 isolate

Accession Numbers	Percentage identified	Accession Numbers	Percentage identified
MH737743.1	98.24%	MN197732.1	98.04%
ON138985.1	98.24%	LC483989.1	98.04%
MK615862.1	98.24%	MK956956.1	98.04%
KY820906.1	98.05%	MK694749.1	98.05%
MT102922.1	98.14%	MK578213.1	98.04%
MN128034.1	98.14%	MK074711.1	98.05%
KX023349.1	98.05%	MK468700.1	98.05%
MW492396.1	98.05%	MH595932.1	98.05%
MT872665.1	98.14%	MH552993.1	98.04%
KY750690.1	98.05%	ON510003.1	98.04%
MN309946.1	98.14%	OM291371.1	98.04%
MK480518.1	97.96%	OM258613.1	98.04%
LC208134.1	98.14%	OK632091.1	98.04%
LC189361.1	98.14%	OK632089.1	98.04%
ON631071.1	97.96%	OK148146.1	98.05%
MW559313.1	98.14%	OK036743.1	98.04%
MW559267.1	98.14%	LC647359.1	98.04%
MW559242.1	98.14%	MZ558048.1	98.05%
MW559235.1	98.14%	LR535764.1	98.04%
JN377787.1	98.05%	MW405765.1	98.05%
MK598808.1	98.04%	MW341456.1	98.05%
MK418365.1	98.04%	MT783975.1	98.04%

In addition, the supply of contaminated, watery and decayed leaves with an unhygienic condition in and around the rearing beds also favours microbial growth; thus, imparting a negative impact on eri silkworms. Microbial growth and its outbreak in rearing areas due to different biotic and abiotic factors have already been reported by different researchers[22,27,44,46,58].

Another remarkable point related to silkworm pathogenicity is adaptability. For a long time, commercial saturniids have been domesticated under a captive environment, making a significant difference between domesticated and wild silkworm varieties in terms of combating environmental stresses. The correlation between environmental stresses and the sensitivity of domesticated silkworms was well explained by Kant et al[27] in the case of *Bombyx mori*. The report justified the inability of mulberry silkworms to withstand extreme natural fluctuation. This had led microbial communities to infect silkworms and made them susceptible to flacherie. The present work can be correlated with the observation and explanation of Kant et al[27] as both *S. ricini* and *B. mori* are completely reared in indoor conditions. However, field observation also resonates with the current explanation.

During sample collection; unhygienic conditions, limitations in scientific approaches, fluctuating temperature and relative humidity, rainfall, leaves scarcity and frequent alteration of food plant leaves were observed within the sample collection sites.

In the present study, consequences of disease outbreaks with heavy mortality were observed. Symptoms such as loss of clasping power, vomiting, loss of appetite, release of chain type excreta, laziness, foul smell, blackening of body and body fluid were observed in the rearing sites frequently. These were found similar to the symptoms of flacherie as explained by Samson[47], Sivaprasad et al[52] and Zhang et al[62]. Therefore, these larvae were collected from the rearing sites as a source of inocula to carry out the present study.

Morphological, biochemical and molecular approaches identified the isolated bacterial colonies as *Bacillus thuringiensis* and *Bacillus cereus*. Determining causative agents related to microbial infestation is necessary to understand the course of infection. The first step in this regard is the observation of different colony characteristics. Colony characteristics help in differentiating bacterial colonies into taxonomic groups. The contrasting characteristics such as coloration, size, shape, form and retention of stains can be easily observed which helps in the efficient and easy differentiation of cultures, especially when cultures are clumped together in one culture media. Similar to the morphological approach of identification, biochemical tests also differentiate and help in identifying bacterial species based on different biochemical activities such as carbohydrate metabolism, fat metabolism, protein metabolism, utilization of compounds etc. IMViC test (Indole test, Methyl red test, Voges-Proskauer test and Citrate test) is one such kind of test that helps in identifying bacteria, especially coliform. The indole test detects the presence of the enzyme tryptophanase that breaks down amino acid tryptophane, Methyl red test detects the presence of mixed-acid fermentation, the Voges-Proskauer test detects the presence of acetylmethylcarbinol acetylase which produces acetone and citrate test detects the ability of a bacteria to utilize citrate as a sole source of carbon. Similarly, tests for different carbohydrates also represent the fermentation capacity of carbohydrate compounds by the bacterium. The trend of biochemical tests however has more impression, as in recent years many rapid biochemical test kits were developed by various agencies. These kits have shortened time, costs and labor and ensured identification accuracy[63].

Another reliable method of bacterial identification is 16S rRNA gene sequence and homology searching through multiple sequence alignment. This method eases the process of identification and enhances accuracy as compared to conventional methods. The superiority of 16S rRNA sequencing over conventional phenotypic methods was explained by Bosshard et al[9] and Tang et al[56]. Another advantage of performing 16S rRNA sequencing is that it can identify an unknown bacterium, apart from prior characterization within a short period (2-3 days) as compared to biochemical tests[13]. Most of the recent publications, however, applied this molecular approach to identify their isolated bacterial colonies[13,22,32,42,54,56,62]. The present study provides the evidence of bacterial species responsible for flacherie in *S. ricini*.

A wide range of bacterial species are responsible for flacherie in different commercial lepidopterans. It includes *Aeromonas caviae*, *Bacillus thuringiensis*, *Bacillus bombysepticus*, *Bacillus carboniphilus*, *Paracoccus niistensis*, *Staphylococcus argenteus*, *Enterococcus cecorum*, *Enterobacter asburiae*, *Bacillus cereus*, *Serratia marcescens*, *Streptococcus* sp and *Staphylococcus* sp.[12,32,42,46,48,51] Bacteria are cosmopolitan organisms and their infliction can be through different media. Toxicosis of bacteria in rearing areas can occur through supplied leaves, cadavers, faeces, dampness, unhygienic conditions, human interference, flies, insects, or alterations in temperature and humidity[24].

The present study emphasized the pathogenesis of *S. ricini* larvae by two different species of bacteria. Results of the present study isolated and identified *B. thuringiensis* and *B. cereus* from flacherie-infected silkworms. These two species are known for their toxic effects on different species of commercial and non-commercial lepidopterans viz., *Bombyx mori*, *Antheraea assama*, *Antheraea mylitta*, *Prodenia litura*, *Papilio demoleus*, *Plusia orichalcea*, *Leucinodes orbonalis* and *Chilo partellus*[7,21,22,46,59]. Toxins produced by these two species are highly infective for the organisms within the orders Lepidoptera, Diptera and Coleoptera. *B.*

thuringiensis, a member of the *B. cereus* group[23], is well known for its entomopathogenic properties as it produces Cry and Cyt toxins[10,40,41].

The species can be distinguished from other members of the *B. cereus* group based on the ability to produce parasporal crystalline inclusions, the crystal proteins (Cry Proteins), or δ-endotoxin which are plasmid-encoded[18,60]. When any insect ingests *B. thuringiensis*, crystal proteins mainly interact with different proteins of the insect's midgut and form a prepore oligomer structure which subsequently inserts the cellular lining of the midgut membrane. As a result, the insect stops feeding and finally dies by osmotic shock. *B. thuringiensis* also produces another toxin, thuringiensin or β-exotoxin, a thermostable secondary metabolite (small-molecule oligosaccharide) which encompasses insecticidal activity against different insect groups viz., Diptera, Coleoptera, Lepidoptera, Hymenoptera, Orthoptera and Isoptera as well as several nematode species[49].

On the other hand, the pathogenic effect of *B. cereus* was due to the synthesis of enterotoxin and emetic toxin or cereulide[61]. Enterotoxins induce gastrointestinal disease diarrhoea and emetic toxin causes emetic syndromes including vomiting[53,61]. Emesis occurs 1-6 hours after ingestion of the toxins, while diarrhoea occurs 8-24 hours after ingestion[11]. Enterotoxins that elicit diarrhoea disrupt the integrity of the plasma membrane of epithelial cells in the small intestine[53]. There are other pore-forming cytotoxins produced by *B. cereus* that cause cell lysis and tissue damage. Hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe) and cytotoxin K (Cytk) are three types of pore-forming cytotoxin that cause cell lysis and are part of the virulence regulatory system, activated by the transcriptional regulator, PlcR[8,20,31,33,34].

Over the years, the effects of these two bacterial species on the larvae of commercial Indian silkworms were assessed. However, there is very limited study regarding the pathogenic implication of these bacterial species on the larvae of *S. ricini*. Toxic effects of different toxin proteins purified from *B. thuringiensis* were tested against larvae of *S. ricini* which resulted reduction of larval weight as well as moderate mortality (23%-28%)[30]. Likewise, the pathogenicity of *B. cereus* SW7-1 strain was assessed on *B. mori* which showed dose-dependent (1.91×10^3-1.91×10^7 concentration of bacterial suspension) pathogenic implication on the 5th instar larvae[32].

It is a well-known fact that silkworms are prone to bacterial infection due to their poor immunity and high sensitivity. In addition, handling and rearing techniques can also facilitate bacterial contamination. The identified bacterial species of the present study occur in diverse natural sources. They are found in soil and sediments, decaying organic matters, vegetables, cereals, pulses, spices, fresh and marine waters, raw and powdered milk, plants and leaf surfaces, gut and intestinal tract of caterpillars as well as diseased insects[2,18,20,25,35,38,45]. Both the species are spore-forming bacteria so their transmission may be possible through spores that contaminate foods and different rearing equipment as well as human interference. These species and their spores can easily be transmitted by dust[39]. The present study describes the pathogenesis of *B. thuringiensis* and *B. cereus* in *S. ricini* and indicates the genus Bacillus's versatility as pathogenic to *S. ricini*. However, further analysis and exploration are necessary in terms of the pathogenesis of these isolated bacterial species in *S. ricini*.

Conclusion

The present study addresses the isolation and identification of bacterial species responsible for flacherie in *S. ricini*. The bacterial attacks can bring drastic physiological changes to the life cycle of silkworms. In the case of eri silkworms, studies regarding bacterial attack are limited which obstruct the understanding of host-pathogen interaction and the body's defense mechanism against the bacteria responsible. Through the present study, two bacterial species were identified that are responsible for promoting flaccid conditions in *S. ricini*. The synergistic interaction of pathogens is enough to alter innate immunity and accelerate lethality.

The combined presence of *Bacillus thuringiensis* and *Bacillus cereus* in the larvae of *S. ricini* is enough to provoke pathogenesis and mortality even after utmost care and proper maintenance of the rearing areas. So, the study needs further investigation to combat flacherie in *S. ricini* and to understand the pathogenic mechanism caused by these bacterial species.

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