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RESEARCH ARTICLE OPEN ACCESS

Pathogenic Roles of *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila*, and *Kocuria rhizophila* in Bacterial Flacherie of *Samia ricini*

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Abstract

In the realm of sericulture, ericulture stands as a beacon of hope for rural communities, fostering economic growth and livelihood prospects. However, the influence of disease, particularly flacherie, threatens to undermine this progress. To combat this menace, researchers embarked on a quest to unmask the causative agents behind bacterial flacherie in *Samia ricini*. For this, flacherie-infected larvae were collected, and hemolymph was isolated and streaked onto Petri plates having nutrient agar media. Yellowish colonies developed within 48-72 hours at 37 °C were subjected to identification through colony morphology study, biochemical tests, and 16S rRNA gene sequencing. The colonies were identified as *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila,* and *Kocuria rhizophila*. The study reveals that the *S. rhizophila,* once deemed harmless, has the potential to cause bacterial flacherie in *S. ricini* and highlights their associated risks. This research documented the infestation of *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila*, and *Kocuria rhizophila* in *Samia ricini* for the first time. This knowledge may further empower the sericulture industry to craft tailored solutions to mitigate flacherie, ensuring the resilience of rural livelihoods and the sustained growth of sericulture.

Keywords: Pathogenicity, Coccus, *Bacillus*, NCBI BLAST, FESEM

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INTRODUCTION

Eri silkworm rearing is a vital economic activity in northeast India, contributing 21.10% to the nation's silk production.[1] However, the silkworms are susceptible to microbial contaminants like viruses, bacteria, fungi, and protozoa,[2,3] which can significantly impact their growth, development, and productivity. Flacherie, a bacterial and viral disease, is a significant threat to eri silkworms, causing detrimental changes to their physiology and metabolism.[4] This disease is characterized by symptoms such as sluggishness, vomiting of gut juices, loss of appetite, semi-solid, chain-type excreta, alteration of body color, and a foul smell.[5-7]

Various pathogenic bacteria, including *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus bombysepticus*, and *Pseudomonas aeruginosa*, have been identified as causative agents of flacherie in silkworms.[2,8-11] The disease can lead to significant economic losses for sericulture farmers, emphasizing the need for effective management strategies. Factors such as overcrowding, malnutrition, insufficient cleaning, fluctuations in temperature and humidity, and contaminated or decayed leaves can encourage microbial growth and infection.

While research has primarily focused on *Bombyx mori*, the Indian mulberry silk moth, this study aims to identify infectious bacterial colonies isolated from *Samia ricini*, the eri silkworm. By understanding the potentially harmful impacts of bacteria on *S. ricini*'s health, we can develop targeted interventions to improve disease management and promote sustainable sericulture practices. This study will contribute to the growing body of research on eri silkworm health and provide valuable insights for the sericulture industry.

MATERIALS AND METHODS

Collection of infected larvae and culture of bacteria

Under aseptic conditions, flaccid fifth instar larvae of eri silkworms (n = 30) were collected from three eri silkworm rearing sites located in Nayekgaon, Harinaguri, and Gendrabil village of Kokrajhar district, where sericulture is a prominent livelihood for the inhabitants. These infected silkworms, carried in sterilized collecting jars, were further rinsed twice with sterile distilled water and surface sterilized using 70% ethanol for 60 seconds under an aseptic environment. A final rinse with sterile distilled water followed. The hemolymph of infected silkworms was collected in Eppendorf tubes, and a loopful of hemolymph was streaked onto nutrient agar plates. These plates were incubated at a range of 30-37 °C for 48 hours.[12,13] Dominant bacterial colonies grown on the plates were identified, subcultured for purity, and stored in sterile glycerol (15%) in screw-cap microfuge tubes (1:1 ratio) at -80 °C for further use.[14]

Identification of bacterial species

Colony morphology

Colony characteristics such as size, form, margin, elevation, and colour were studied following different methods.[15-21]

Gram staining method

Bacterial smears were prepared on slides, heat-fixed, and stained sequentially with crystal violet (1-2 minutes), Gram's iodine (1-2 minutes), and ethanol for decolorization (15-20 seconds). Safranin (30 seconds-1 minute) was used as the counterstain. Slides were observed under a Leica DM750 microscope using immersion oil at 100 × magnification.[22]

Field Emission Scanning Electron Microscopy (FESEM)

For SEM, bacterial cultures in the nutrient broth were centrifuged at 7000 rpm for 10 minutes to collect pellets. The pellets were washed with 1 × PBS buffer, fixed with 2.5% glutaraldehyde overnight, and washed again with 1 × PBS buffer. Dehydration was carried out using graded ethanol concentrations (30%-100%), with centrifugation at 7000 rpm for 5 minutes at each step. Samples were spread on coverslips under a Stereo Zoom microscope (Stemi 508), gold-sputtered, and observed using a SIGMA VP FESEM (ZEISS) at 15.00 KX and 20.00 KX magnification.

Biochemical tests

IMViC tests (Indole, Methyl Red, Voges–Proskauer, Citrate) and carbohydrate utilization

tests (glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose, sucrose) were conducted using HiIMViC™ Biochemical Test Kits (HiMedia). Wells were aseptically inoculated with bacterial samples and incubated at 35-37 °C for 18-24 hours. Post-incubation, reagents such as Kovac's, Methyl Red, and Barritt's were added to respective wells for specific tests. Colour changes in wells were interpreted using the standard chart provided with the kits.

Molecular identification method

The 16S rRNA gene sequencing and the NCBI BLAST similarity search tool were used to identify the bacterial species. Genomic DNA was extracted using the Biobee Spin EXpure Microbial DNA Isolation Kit (Bogar Bio Bee Stores Pvt. Ltd., Tamil Nadu, India). PCR amplification of the 16S rRNA gene was performed using primers 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGTACCTTGTTACGACTT-3') in a reaction mixture containing Taq Master Mix, deionized

Table 1. Colony morphology of the isolates

Isolates	Size	Shape	Type	Colour	Elevation	Margin	Gram-Staining
S1	Small (2-3 mm)	Circular	*Bacillus*	Yellow	Convex	Entire	Negative
S2	Small (2-3 mm)	Circular	*Bacillus*	Yellow	Convex	Entire	Negative
S3	Small (2-3 mm)	Circular	*Bacillus*	Yellow	Slightly convex	Entire	Positive

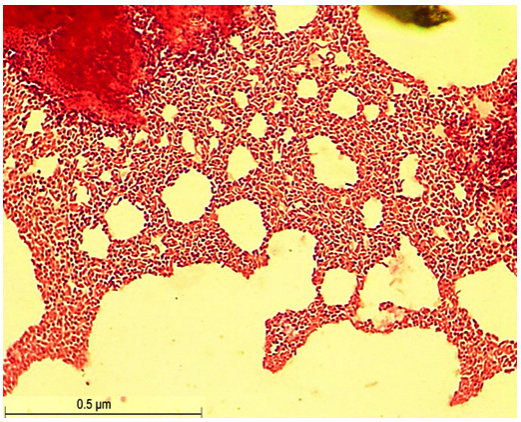

Figure 1. Gram Staining of S1 isolate

Table 2. Biochemical tests of the isolates

Biochemical tests	S1	S2	S3
Indole	Negative	Negative	Negative
Methyl Red	Negative	Negative	Negative
Voges Proskauer's	Negative	Negative	Positive
Citrate	Positive	Positive	Positive
Sucrose	Positive	Positive	Negative
Glucose	Positive	Positive	Positive
Lactose	Negative	Negative	Negative
Mannitol	Negative	Negative	Negative
Sorbitol	Negative	Negative	Negative
Rhamnose	Negative	Negative	Negative
Adonitol	Negative	Negative	Positive
Arabinose	Negative	Negative	Positive

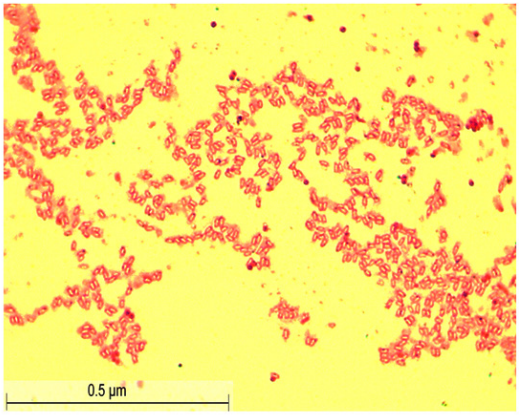

Figure 2. Gram Staining of S2 isolate

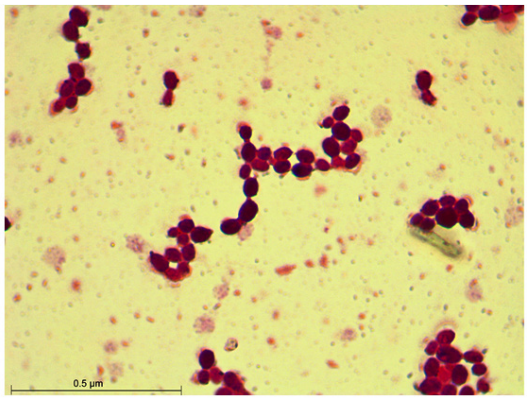

Figure 3. Gram Staining of S3 isolate

water, and primers. Unincorporated primers and dNTPs were removed using a Montage PCR Clean-up Kit (Millipore). Sequencing was performed with an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit using an ABI 3730xl sequencer. Sequences were aligned using MUSCLE 3.7,[23] and curated with Gblocks 0.91b[24] to remove alignment noises. Phylogenetic analysis was conducted using PhyML 3.0 with the HKY85 substitution model. Sequences were submitted to the NCBI nucleotide database for GenBank accession numbers.

Further, to confirm the pathogenic effects of the isolated bacterial species, a pathogenicity test was performed in vitro on healthy fifth instar larvae. Bacterial culture (40 ml) was added to 5 sterile 50 ml Falcon tubes, centrifuged for 7 minutes at 7000 rpm, and the pellets were resuspended in 20 ml PBS buffer. Pathogenicity was tested by injecting 10 µl bacterial suspension into silkworms (n = 30) by U-40 insulin injection (0.25 mm (31G) × 6 mm needle) (BD Ultra-FineTM Needle Insulin Syringes) and dipping them in 100 µl suspension (n = 30) into petri plates (90 × 17 mm), with continuous supply of *R. communis* leaves, and observations were recorded.

RESULTS

The study found a total of eight colonies, of which three colonies (S1, S2, and S3) were selected for identification. These three colonies were dominant in the nutrient media and exhibited intriguing characteristics, such as a colour change corresponding to the incubation time, rapid growth within 24 hours, and their presence in all the petri plates. All the bacterial colonies showed luxuriant growth at 37 °C after 48 hours. Although many bacterial species prefer low temperatures to grow, the inherent phenotypic variability, adaptability, and genetic similarities

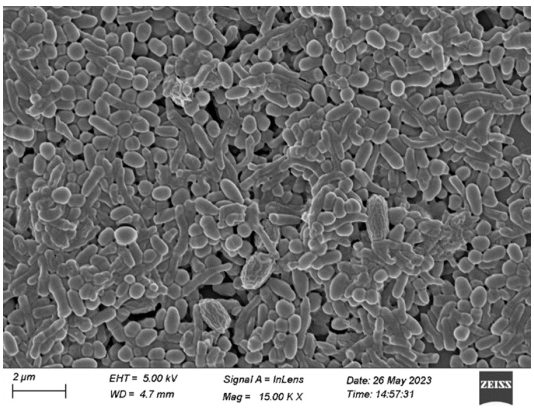

Figure 4. FESEM of S1 isolate (15.00 KX)

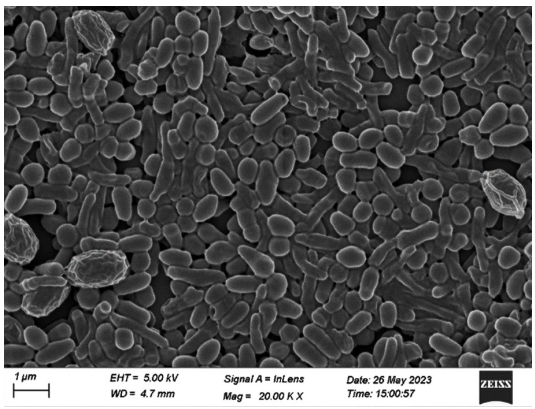

Figure 5. FESEM of S1 isolate (20. 00 KX)

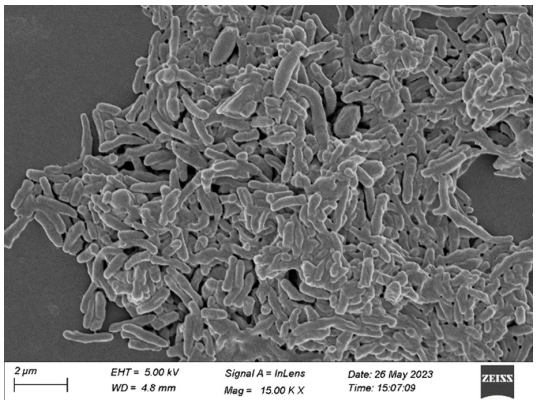

Figure 6. FESEM of S2 isolate (15.00 KX)

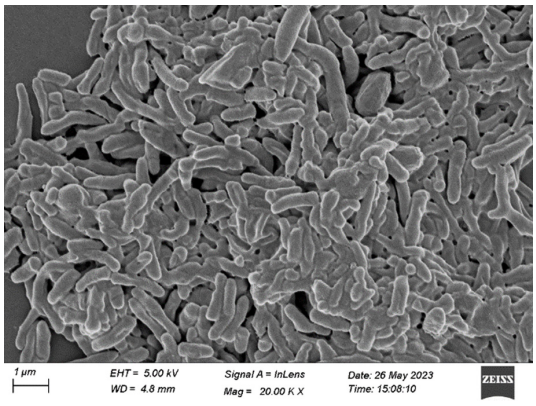

Figure 7. FESEM of S2 isolate (20.00 KX)

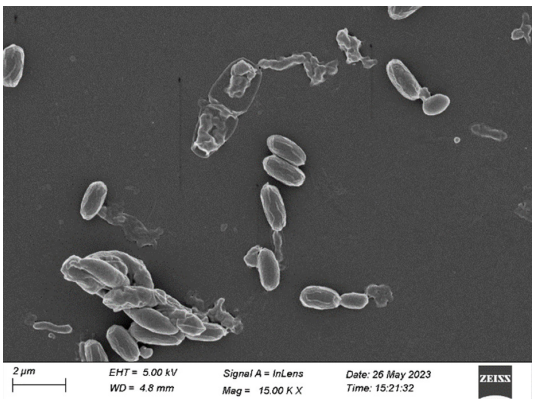

Figure 8. FESEM of S3 isolate (15.00 KX)

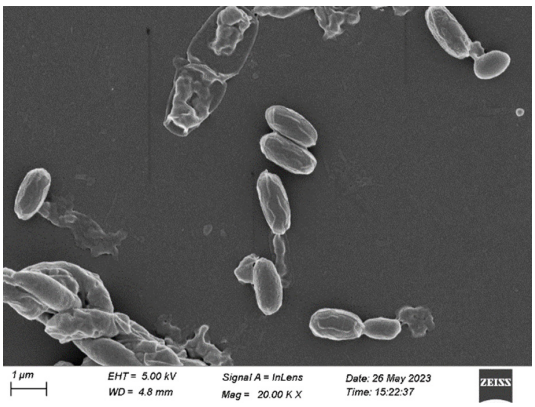

Figure 9. FESEM of S3 isolate (20.00 KX)

enable a species to withstand rising temperatures and attain good growth. Additionally, the capacity of a species to grow at 37 °C is often considered a key trait in differentiating potential pathogens from harmless environmental strains within a particular genus.[25,26]

Colony morphology of the isolates

After 48 hours of incubation at 37 °C, the dominant colonies (S1, S2, S3) on nutrient agar appeared circular with entire margins and formed small (2-3 mm) yellowish colonies in the culture media. The S3 colony had a slight convex elevation, while S1 and S2 had total convex elevation. The S1 and S2 colonies were rod-shaped, pinkish-red Gram-negative *bacilli*, and the circular S3 colonies were stained purple and were arranged in various patterns, such as short chains, tetrads, grapelike packets, and irregular clusters, representing the Gram-positive coccus type of bacteria (Figures 1-3). Scanning electron microscopy of S1 and S2 revealed the presence of rod-shaped structures having lipopolysaccharide layers on their surfaces that facilitate cell-to-cell adhesion, confirming the *bacillus* type of bacteria and S3 as coccus (Figures 4-9) (Table 1).

Biochemical tests

All the selected bacterial isolates (S1, S2, and S3) showed a negative result for the indole and methyl red tests, and a positive result for the citrate utilization test. Colony S1 and S2 showed negative results for Voges Proskauer's test, while S3 showed positive results for the same (Table 2).

In the carbohydrate utilization test, the selected isolates demonstrated an inability to ferment lactose, sorbitol, mannitol, and rhamnose, resulting in negative results after 18-24 hours of incubation. However, the isolates were able to ferment glucose. Apart from this, S1 and S2 isolates were able to ferment sucrose and were unable to ferment adonitol and arabinose, while S3 was unable to ferment sucrose and able to ferment adonitol and arabinose (Table 2).

Molecular identification method

The 16S rRNA gene sequencing identified the bacterial isolates, S1, S2, and S3 as *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila*, and *Kocuria rhizophila*. The identified sequences of both the isolates were submitted to the NCBI nucleotide database which were accepted and available under GenBank accession numbers OQ220315, OQ220316, and OQ220317 against *Stenotrophomonas maltophilia, Stenotrophomonas rhizophila,* and *Kocuria rhizophila,* respectively.

The results after the in vitro pathogenicity test confirmed the bacterial contamination in healthy larvae, thereby providing evidence for *S. maltophilia, S. rhizophila*, and *K. rhizophila* as flacherie-causing agents in *S. ricini*. After exposure to the bacterial suspension, silkworms showed reduced activity, appetite, clasping power, and movement within 72-100 hours. Notably, larvae injected with bacterial suspensions began to vomit and excrete semi-solid, chain-like faeces with a foul odour within 72 hours.

The mortality rate varied among the different bacterial treatments, with silkworms treated with *K. rhizophila* succumbing to infection within 5-6 days, while those treated with *S. maltophilia* and *S. rhizophila* died within 6-7 days. As the infection progressed, the silkworms' bodies underwent notable changes, including a foul odour, a brownish-black discoloration, and softening of the body. Post-mortem examination of the cadavers revealed liquefaction of internal organs, a hallmark of flacherie disease. Furthermore, the hemolymph collected from the treated larvae turned brownish-black, mirroring the symptoms observed in flacherie-infected silkworms collected from the field. These findings suggest that the bacterial isolates used in the study are capable of inducing flacherie-like symptoms in silkworms, consistent with the disease's characteristic pathology.

DISCUSSION

The study identified bacterial colonies isolated from flacherie-infected eri silkworms, and all the colonies showed unique characteristics and gave analogous evidence of *Stenotrophomonas maltophilia, Stenotrophomonas rhizophila,* and *Kocuria rhizophila* based on colony characteristics, biochemical tests, and multiple sequence alignment. *S. maltophilia* and *S. rhizophila* hold substantial applications in agricultural biotechnology.[27-30] These two species are always associated and persist as endosymbionts or endophytes[31-33] with various plant species such as wheat plants, potatoes, tomatoes, cotton, and sweet peppers.[28,30,34,35] Both play major roles such as promoting plant growth, helping in biocontrol, acting as a stress-protecting agent, protecting from draught and salinity, producing osmoprotective substances, degrading lignocellulose and xenobiotics, etc.[30,36-39] Despite their agricultural benefits, *S. maltophilia* has been recognized as a nosocomial pathogen since the 1980s.[40] Its close genetic relationship with *S. rhizophila* raises concerns about the potential pathogenic risks associated with agricultural use.[30] However, *S. rhizophila* has not demonstrated pathogenicity in humans, being incapable of growth at human body temperatures.[37] Nonetheless, its ability to form biofilms raises apprehensions about lateral gene transfer, potentially converting it into a pathogenic entity.[41]

Presently, *S. maltophilia* is deemed an emerging opportunistic pathogen due to its multidrug-resistance, high morbidity, and mortality rates among immunocompromised individuals due to its association with multidrug-resistance.[18,42] The World Health Organization (WHO) has labelled it as an "underestimated important multidrug-resistant organism".[43,44] With an ecological presence in soil, water, hospital equipment, and soda lakes,[40,45,46] *S. maltophilia* can colonize healthcare settings, causing outbreaks through contaminated water systems and appliances.[18] In humans, *S. maltophilia* is associated with bacteremia, respiratory infections, and hematological malignancies.[47-49] Its pathogenicity extends to plants, where it causes crippling white stripe disease in *Oryza sativa*,[50,51] fruit soft rot in *Lilium davidii*,[52] seedling soft rot in *Zea mays*,[53] root rot in *Panax notoginseng*,[54] and also responsible for the Persian oak (*Quercus brantii*) decline in Zagros forests, Iran.[55] Such infections highlight its broad host range and ecological adaptability. Conversely, *S. rhizophila* is primarily recognized for its agricultural benefits, including plant growth promotion and phytopathogen inhibition.[30,56-58] It colonizes plant roots as a non-pathogenic endophyte,[33] presenting opportunities for sustainable agriculture. However, the mechanisms of its action remain largely unknown.

Similarly, the genus *Kocuria*, often overlooked as a common laboratory and specimen contaminant. *K. rhizophila*, another species within the genus, was previously reported as non-pathogenic to humans.[59] Comprehensive studies on its interactions with other organisms are scarce. In *Salmo trutta* and *Oncorhynchus mykiss*, this species was responsible for developing exophthalmic symptoms, skin petechiae, increased skin melanization, focal lesions, intestinal inflammation, tail muscle hemorrhages, and liver congestion.[14,60] *K. rhizophila* is a common commensal found in mammalian skin, mucous membranes, oropharynx, and in chicken meat treated with oxalic acid.[14,61,62] It is also present in contaminated dust, fresh air, soil, marine sediments, freshwater, and various foods.[14,62] Research into its occurrence as a contaminant

in other organisms has been undervalued, as its accurate identification and risk assessment remain elusive.[59] This species was frequently misidentified as coagulase-negative staphylococci (CoNS) because both share similar biochemical characteristics, such as a positive Gram test, a positive catalase reaction, and coagulase-negative properties.[59,62] Furthermore, many clinical microbiology laboratories struggle to accurately identify the species due to diagnostic challenges and the lack of access to advanced molecular techniques.[59]

The interplay between the course of contamination and physiological changes due to pathogenic invasion in *S. ricini* is a field where no detailed studies have been conducted. The possibility that this type of contamination may increase the likelihood of colonization by specific pathogenic agents on the host's body warrants examination due to its hazardous side effects. Bacterial flacherie, a common disease in the ericulture sites of India, especially in Assam, lacks comprehensive information regarding causative agents and pathogenic mechanisms. A study performed on ericulture in Papumpare district of Arunachal Pradesh, India only provides preliminary information on the bacterial types, coccus and bacillus, isolated from the hemolymph of contaminated *S. ricini* larvae.[63] This aligns with the present findings, which elucidated the presence of two bacillus types (*S. maltophilia* and *S. rhizophila*) and one coccus type (*K. rhizophila*) of bacteria. No detailed reports of contamination by these three species have ever been recorded in *S. ricini*. Additionally, no data representing the sequence or strain of *S. maltophilia*, *S. rhizophila*, and *K. rhizophila* isolated from infected *S. ricini* is available in GenBank. This study, however, also addresses the gap in the molecular database for *S. maltophilia*, *S. rhizophila*, and *K. rhizophila* strains obtained from infected *S. ricini* larvae, thereby supplementing the knowledge concerning the pathogenic properties of these bacteria for *S. ricini*. The extensive host range of these species, from soil to skin, leaf to meat, and air to water, favours easy transmission to rearing beds while handling utensils, cleaning, leaf supply, and verbal communication. Moreover, being poikilothermic and immunologically sensitive, any changes in abiotic conditions such as temperature, relative humidity, dampness, and heavy rainfall create favorable conditions for the survival of various pathogenic microorganisms in the eri-rearing areas. Notably, sample collection for this study revealed that most eri silkworm rearers lacked awareness of modern tools and scientific rearing methods. Consequently, they often conducted ericulture in unhygienic and chaotic conditions. Furthermore, the seasonal scarcity of suitable host plants and limited plantation sites forced rearers to rely on decayed, waterlogged, and senescent foliage, and various host plants within a single generation. These factors significantly increase the risk of microbial infestation, creating an environment conducive to the proliferation of bacteria that can exert pathogenic effects on eri silkworm larvae. Interestingly, in the in vitro pathogenicity test, all three bacteria were found to exert pathogenic effects on the fifth instar larvae, and the symptoms were found consistent with the characteristic features of flacherie observed in the field study. Thus, the present study revealed *S. maltophilia, S. rhizophila,* and *K. rhizophila* as key contaminants of bacterial flacherie in *S. ricini*, filling the gap for detailed experimental work on causative agents of bacterial flacherie in *S. ricini* and highlighting the need for stringent rearing practices and regular microbial monitoring.

CONCLUSION

The present study unveils the role of *S. maltophilia, S. rhizophila,* and *K. rhizophila* in eri silkworm infestation and exposes the microbial menace threatening the sericulture industry. By tracing the pathogenic trajectory of these contaminants, the current investigation reveals a startling correlation between their presence and the degradation of *S. ricini* grainage parameters. This research fills a critical knowledge gap, providing a nuanced understanding of the complex interplay between microbial contaminants and their hosts. As the study established *S. maltophilia, S. rhizophila,* and *K. rhizophila* as formidable pathogens of *S. ricini*, findings of the present study could lay the groundwork for targeted interventions to safeguard the health and productivity of eri silkworms, ultimately fortifying the resilience of the sericulture sector.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

DB conceptualized the study and applied the methodology. DB and RRK visualized and investigated the study. RRK performed sample collection and experiments. DB performed data curation. RRK wrote the manuscript. DB reviewed the manuscript. Both authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in this manuscript.

ETHICS STATEMENT

Not applicable.

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