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Detection of Probiotic Bacteria *Ligilactobacillus salivarius* in the Midgut of Eri Silkworm (*Samia ricini*, donovan) Collected from Borduar, Assam

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ABSTRACT

Background: Eri silkworm rearing, crucial to Assam's economy, faces severe productivity threats from disease and crop loss. These challenges impact farmers' livelihoods, highlighting the urgent need for innovative solutions to boost disease resistance and crop yield, ensuring the industry's sustainability and profitability.

Methods: The study investigated the detection of Lactobacillus species in the midgut of the Eri silkworm (*Samia ricini*, Donovan) collected from Borduar, Assam. The research involved identifying *Ligilactobacillus salivarius* in the midgut of 5th instar larvae of the Borduar ecorace of Eri silkworm. The larvae, reared separately on castor and kesseru host plants, were utilized for isolating *Lactobacillus* on MRS agar. Molecular identification of these isolates was achieved through 16S rRNA sequence analysis.

Result: *Ligilactobacillus salivarius*, a species under the genus *Lactobacillus*, was identified for the first time in the midgut of Eri silkworm larvae. This bacterium is widely known as a potential probiotic in higher animals, particularly poultry, indicating its possible benefits for silkworm health. The discovery of *Ligilactobacillus salivarius* in silkworms paves the way for further research aimed at developing novel probiotics tailored specifically for the sericulture industry, potentially improving silkworm health and boosting productivity. Implementing these probiotics presents a promising solution to the challenges facing sericulture in Assam, offering enhanced disease resistance and crop yield.

Key words: Borduar ecorace, Eri silkworm, *Lactobacillus, Ligilactobacillus salivarius,* Midgut, Probiotic.

INTRODUCTION

Eri-culture has been a longstanding tradition in the North-East region of India, with Assam being a prominent state where this practice thrives, providing substantial employment opportunities (Kakoti, 2012; Kom *et al.,* 2022). Among the non-mulberry silks, the Eri silkworm, *Samia ricini* (Donovan), is particularly valued for its adaptability to indoor rearing and its multi-voltine and polyphagous nature. The primary host plant for Eri silkworm is castor (*Ricinus communis*), though various secondary and tertiary host plants are also utilized (Hazarika *et al*., 2003). Out of the 26 identified ecoraces of Eri silkworm, the Borduar ecorace is noted for its superior productivity and high commercial value (Velayudhan *et al*., 2014).

The growth and silk production of silkworms are heavily influenced by the quality and quantity of their food, with different food plants offering varying nutritional values (Borah *et al*., 2020). The gut of lepidopteran insects plays a crucial role in digesting leaf components to ensure proper nutrition. The gut microbial community of insects has been extensively studied, highlighting the beneficial roles of gut microbiota in food digestion and the release of nutritionally important compounds (Paniagua *et al*., 2018). In lepidopteran insects, the midgut bacterial community not only produces enzymes that assist digestion but also enhances the host's overall health by improving innate immunity, aiding in detoxification and providing growth-promoting metabolites (Anand *et al.,* 2010; Storelli *et al*., 2011; Ceja *et al*., 2015). In higher organisms, the microbial community is crucial for metabolism and lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium* species, are commonly used as probiotics for their health benefits (Montville *et al.,* 2005; Habib *et al.,* 2020; Lalitha *et al*., 2022). Several studies have reported that the application of probiotic bacteria

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improves the growth and productivity of silkworms (Bai *et al*., 2012; Mala *et al.,* 2018). Despite the high significance of gut microbiota in other organisms, there are limited studies on the beneficial gut microbiota of *Samia ricini*. Lactic acid bacteria are significant inhabitants of the intestinal tract of humans and other vertebrates. Members of this family, such as *Enterococcus*, *Lactobacillus and Bifidobacterium*, are prevalent in food and fermentation processes (Chadli *et al.,* 2024). Species of the *Lactobacillus* genus help maintain the intestinal microbial ecosystem and the gastrointestinal health of the host (Naidu *et al*., 1999). Due to their beneficial properties, *Lactobacillus* members are widely used as probiotics.

This study was undertaken to investigate the presence of probiotic bacteria, specifically *Lactobacillu*s species, in the midgut of the Eri silkworm, *Samia ricini* (Donovan), collected from Borduar and reared on two different host plants, castor and kesseru. The research aimed to identify and characterize the *Lactobacillus* species present, focusing on their potential probiotic benefits. The Eri silkworms were reared separately on castor and kesseru host plants and their midguts were used to isolate *Lactobacillus* species using MRS agar. Molecular identification of the isolates was conducted through 16S rRNA sequence analysis.

The study revealed the presence of *Ligilactobacillus salivarius* in the midgut of 5th instar larvae of the Borduar ecorace of Eri silkworm. This species, under the genus *Lactobacillus*, was identified for the first time in the midgut of Eri silkworms. *Ligilactobacillus salivarius* is widely recognized as a potential probiotic in higher animals, particularly poultry, suggesting its possible benefits for silkworm health. This discovery opens new avenues for further research to develop novel probiotics tailored specifically for the sericulture industry, addressing the current lack of commercial probiotics designed for silkworms. Implementing these probiotics could significantly enhance silkworm health and productivity, offering a promising solution to the challenges faced by sericulture in Assam.

The findings underscore the importance of the gut microbiome in the health and productivity of Eri silkworms. By exploring the beneficial roles of gut microbiota, particularly *Lactobacillus* species, this research contributes to a deeper understanding of how probiotics can be leveraged to improve sericulture practices. The potential development of targeted probiotics for Eri silkworms could lead to enhanced disease resistance, improved nutrition and increased silk production, ultimately benefiting the sericulture industry in Assam and beyond.

MATERIALS AND METHODS

The experiment was conducted during the period of November 2020 to February 2022 at Department of Animal Biotechnology, College of Veterinary Science, Khanapara, Guwahati.

Sample collection and isolation of lactobacillus

20 larvae of Eri silkworm at 5th instar stage were collected from Borduar, Assam; which were reared on castor and kesseru food plant separately. Larvae were collected aseptically, 6 larvae from each batch subjected to overnight starvation so that leaf materials are eliminated from the midgut. Prior to dissection the selected larvae were sterilized with 70% ethyl alcohol for 60 sec followed by rinsing with sterile distilled water and the midgut was removed in sterile condition. The midgut homogenate was prepared using 0.85% (w/v) NaCl solution. The sterile homogenate was centrifuged (eppendorf 5430) at 8000 rpm for 10 minutes and the supernatant was inoculated in MRS broth (Catalog no: GM369, Himedia). After 24 hr of incubation, cultures were streaked on MRS plates and incubated aerobically for 24 hr at 37°C.

Morphological and biochemical tests

Morphological observation was carried out using gram staining. Carbohydrate fermentation pattern and basic biochemical tests are carried out for *lactobacillus*.

DNA extraction and 16srRNA gene amplification

Purified colonies of bacteria ware selected for identification based on 16srRNA gene probe. Isolates were grown on MRS broth for 24 hr at 37°C. The culture after 24 hr of growth were centrifuged at 12000 rpm and pellet was used for DNA extraction using phenol-chloroform method. The 16srRNA gene was amplified using genomic DNA with standard polymerase chain reaction (PCR). Forward primer 341f, 5′- CCTACGGGNGGCWGCAG-3′ and reverse primer 805r, 5′-GACTACHVGGGTATCTAATCC-3′ were used for 16srRNA partial gene amplification. The PCR reaction was carried out on Pro-flex PCR system (applied biosystems by Life technologies). The amplification cycles were carried out as follows- one cycle of initial denaturation at 95°C for 5 min followed by 35 cycles denaturation at 95°C for 30 sec, annealing at 52°C for 2 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were examined in gel electrophoresis using 1.5% agarose gel and bands were visualized under UV in the Gel Documentation system (BIO-RAD, Universal hood a!. Purified PCR amplicons were outsourced for sanger's sequencing The comparison of 16SrRNA gene sequences was performed with the genetic database from NCBI (National Center for Biotechnology Information) GenBank. The multiple sequence alignment was performed using BioEdit 7.0 software tool and MEGA 11. Following neighbor-joining method the phylogenetic tree was constructed with 1000 replications bootstrap analysis. All 16S rRNA gene sequences generated in this study have been deposited in the NCBI GenBank database under accession number OP512540, OP518292, OP503443 and OP518289.

RESULTS AND DISCUSSION

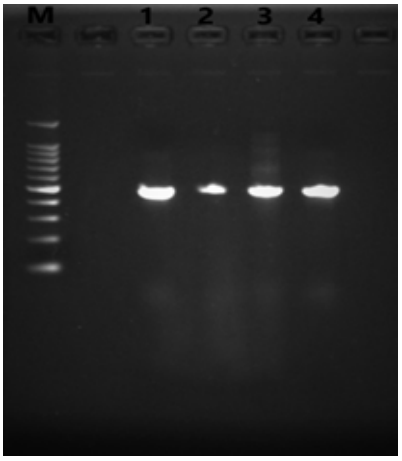
5th instar larvae of Eri silkworm collected from Borduar, Assam reared on castor and kesseru plant were subjected

to isolation of *lactobacillus* from the midgut. The colonies isolated from Borduar ecorace grown on MRS agar plates are examined for grams staining and biochemical tests as per Bergy's Manual of Determinative Bacteriology. The results of biochemical tests are shown in Table 1; isolate 1 and isolate 2 are from castor feeding Eri silkworm larvae whereas isolate 3 and isolate 4 are from kesseru feeding Eri silkworm.

Molecular identification of Bacterial isolates: Species specific identification of the 4 isolates was derived from 16srRNA sequence analysis. The V3-V4 region was amplified by the primer pair 341f (5'-CCTACGGGNGGCWGCAG-3') and 805r (5'-GACTACHVGGGTATCTAATCC-3'). The PCR result using the above primer revealed that *lactobacillus sp*. is present in the midgut of Eri silkworm. The PCR product size is ~465bp was observed using 1.5% agarose gel, amplification of 16srRNA of bacterial isolates is shown in Fig 1; Lane 1: isolate 1, Lane 2: isolate 2, Lane 3: isolate 3, Lane 4: isolate 4 and Lane M: 100 bp marker.

The Basic Local Alignment Search Tool (BLAST) algorithm was used to retrieve for homologous sequence in NCBI Gen Bank, accession no AB733112 is used as out-group. Phylogenetic relationship of all the isolates under accession number OP512540, OP518292, OP503443 and OP518289 showed that they belong to the same bacterial species which is *Ligilactobacillus salivarius. Ligilactobacillus salivarius* is well known probiotic in higher animal also present in midgut of Eri silkworm and it has been reported for the first time through this study.

The present study aimed to detect the presence of *lactobacillus* species in the midgut of Eri silkworm. PCR results and 16srRNA sequencing revealed the presence of Fig 2 *Ligilactobacillus salivarius* in the midgut of 5th instar larvae of Eri silkworm which was collected from Borduar, reared on castor and kesseru food plant and it might act as probiotic bacteria in the midgut of Eri silkworm. The gut microbes of insects play a crucial role in development, metabolism, protection, disease resistance and reproduction also the diversity of microbes differs significantly according to the diet, environment and genetics of the host (Dillon *et al*., 2004, Lee *et al*., 2013). Digestion of leaf components occurs in the gut of lepidopteron insects and beneficial gut microbes as they have symbiotic relationship, helps by releasing metabolically active enzymes which assist digestion of substrate as well as releases nutritionally rich compounds. The midgut bacterial community of lepidopteran has been studied extensively for production of enzyme that assists digestion of leaf components (Feng *et al*., 2011, Nandy *et al*., 2021). Few studies have already revealed the presence of diverse microbial community and composition in the gut of Eri silkworm (MsangoSoko *et al*., 2020) but the relationship between microbiota and its role for advancement of Eri silkworm production is limited. As reported earlier total seven microbial groups were detected



Lane 1: Isolate 1, Lane 2: Isolate 2, Lane 3: Isolate 3, Lane 4: Isolate 4 and Lane M: 100bp marker.

Fig 1: Amplification of 16srRNA of bacterial isolates.

Table 1: The results of biochemical tests; isolate 1 and 2 are from castor feeding Eri silkworm larvae whereas isolate 3 and isolate 4 are from kesseru feeding Eri silkworm.

Characteristics features	Isolate 1 (castor)	Isolate 2 (castor)	Isolate 3 (kesseru)	Isolate 4 (kesseru)
Grams staining	+	+	+	+
Morphology	Rod	Rod	Rod	Rod
Catalase test	-	-	-	-
Citrate utilization test	-	-	-	-
Methyl red test	-	-	-	-
Voges prausker test	-	-	-	-
Indole test	-	-	-	-
Carbohydrate fermentation test:				
Glucose	+	+	+	+
Lactose	+	+	+	+
Sucrose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
dextrose	+	+	+	+

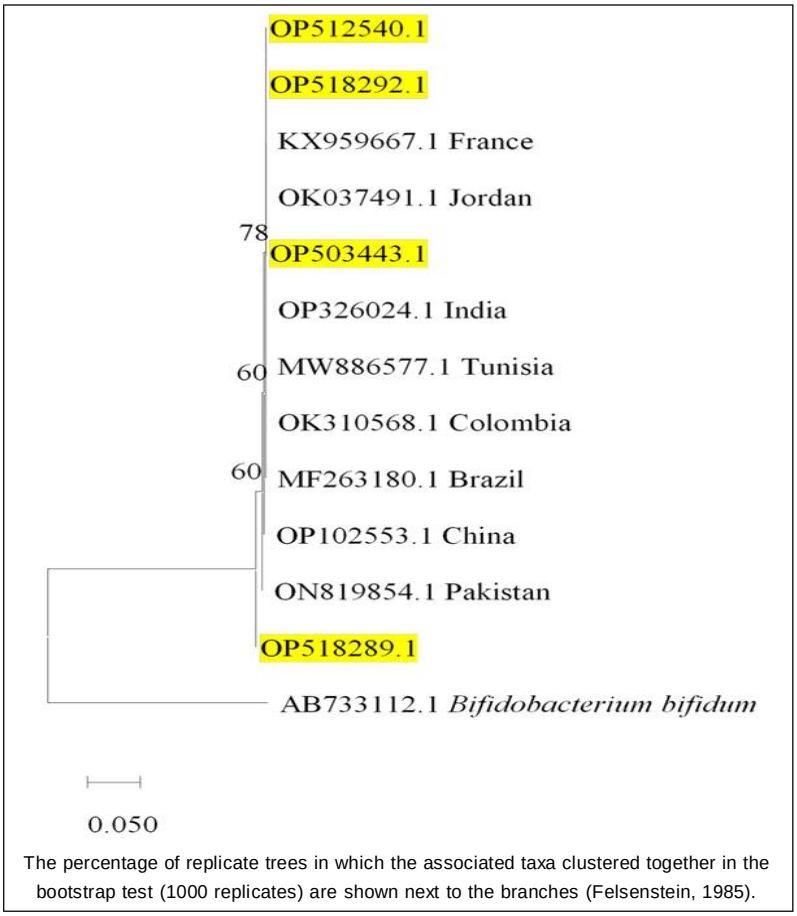


Fig 2: Phylogenetic relationship inferred by Neighbor- joining method using MEGA 11.

in the foregut, midgut and hindgut of Eri silkworm: Gram-ve bacteria, gram + ve bacteria, anaerobes, actinomycetes, methanotrophs, micro eucaryotes and fungi. Culturable aerobic gut bacterial isolates comprises Firmicutes (54%) and proteobacteria (46%) as well as anaerobic bacteria comprises Proteobacteria (92%) and firmicutes (8%) in the gut of Eri silkworm. Lactic acid bacteria (LAB) have traditionally been used for fermenting Dairy food and probiotics. Some beneficial species of *Lactobacillus* and *bifidobacteria* are used as potential probiotics for the improvement of human and animal health. Production of antimicrobial metabolites is also a significant characteristic of LAB (Quinto *et al*., 2014). Lactic acid bacteria as probiotics has also been applied for better growth and production of silkworm and previous studies mostly investigated in mulberry silkworm (*Bombyx mori*) (Anand *et al.,* 2010, Bai *et al*., 2012 and Mala *et al*., 2018) but there are very limited works reported which focused on Eri silkworm (Non-mulberry silkworm). The host might be benefited by the probiotic microbial flora that inhabit in the gut as they might increase efficiency of digestion and nutrition assimilation. It is already reported that lactic acid bacteria, Enterococci present in the mid gut of Eri silkworm and potential use of *Enterococcus hirae* as probiotic, as it enhances larval weight and survival in Eri silkworm (Unban *et al*., 2022). Presence of *Lactobacillus* has been reported in the midgut of mulberry silkworm and its relative abundance depends on the species and physiological activity of silkworm (Yeruva *et al*., 2020). *Ligilactobacillus salivarius,* Lactic acid bacteria of the genus Lactobacillus has gained attention as promising probiotics, it exhibit health benefits as having antimicrobial activity and also modulate gut microbiota (Messaoudi *et al*., 2012). L. *salivarius* mostly isolated from the intestine and faeces of birds and mammals and also present in the gut of honey bee (Audisio *et al.,* 2018). Presence of *Ligilactobacillus salivarius* in the midgut of Eri silkworm, ecorace Borduar has been reported for the first time and it might help in eco-friendly management of Eri silkworm disease. Current finding could also be used for further development of novel probiotic for better production of Eri silkworm and sericulture research.

CONCLUSION

This study marks the first successful isolation of the lactic acid bacteria *Ligilactobacillus salivarius* from the midgut of the Eri silkworm (*Samia ricini*). Known for its probiotic

properties in higher animals, *Ligilactobacillus salivarius* is widely used as a probiotic supplement to enhance gut health and overall well-being. The discovery of this bacteria in the Eri silkworm's midgut represents a significant breakthrough, suggesting potential probiotic benefits for silkworms. This finding paves the way for future research to explore the impact of *Ligilactobacillus salivarius* on silkworm health and productivity, potentially leading to innovations that could boost the sericulture industry. Validating the probiotic potential of this bacteria in silkworms could result in enhanced disease resistance, improved nutrition and increased silk yield, offering a valuable tool for the upliftment of sericulture practices.

Significance statements

Mortality due to disease in Eri silkworms significantly hampers productivity, leading to frequent crop failures. This investigation focuses on the microbial diversity within the midgut of the Eri silkworm, aiming to address this critical issue. Notably, the well-known probiotic *Ligilactobacillus salivarius* has been identified in the midgut, marking a pioneering discovery. Given its established probiotic benefits in higher animals, this bacteria holds promise for enhancing disease resistance and boosting production in Eri silkworms. This finding underscores the potential of leveraging gut microbiota to develop probiotic solutions, which could revolutionize sericulture by reducing mortality rates and improving overall silk yield.

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Declarations

We, the submitting authors declare that the research work is original and has not been published previously to any other journal. Each author has made substantial contribution to the conception or design of the work with analysis or interpretation of data.

Contribution of authors

This study was a collaborative effort among all authors, who collectively contributed to its design and execution. Hena Parbin conducted the laboratory work, while Hena Parbin, Simanta Koushik and Anjela Ahmed drafted the initial manuscript. Girin Hazarika and Probodh Borah performed data analysis. Bhuban Chandra Chutia provided oversight, managed the study's execution and supervised the entire project. All authors reviewed and approved the final manuscript, ensuring comprehensive input and consensus throughout the research process.

Conflict of interest

I declare on behalf of all authors that there are no conflicts of interest.

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