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Research Article

Isolation and identification of bacterial pathogens causing infections in *Bombyx mori*

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Abstract

The present study was planned to assess the influence of *Morus alba*, *Morus nigra* and mixed mulberry leaves diet on the health and disease susceptibility to *Bombyx mori*. 5g of dried leaves samples of *Morus alba*, *Morus nigra* and mixed mulberry leaves were used to done proximate analysis. The silkworms were reared for 32 days under semi-controlled conditions, with an optimum temperature of 25-28°C and humidity maintained at 70-80% throughout the experimental period. Diseased silkworms showing clinical signs such as dark brown bodies, slow growth, secretions from the body, slow movement, and shrinkages of the body were collected from the experimental site and were used for bacterial enumeration and isolation. The bacteria were isolated from the gut, whole body, and outer body surface. The isolation of bacteria from diseased silkworms revealed 157 distinct colonies from different sources. The highest number of colonies was observed in samples taken from the gut (62 colonies), followed by the whole body (53 colonies) and the outer body surface (42 colonies). The Pearson's correlation analysis (Pearson's $r = 0.87$, $p < 0.001$) revealed a strong association between the severity of disease symptoms and bacterial load, especially in the gut-derived samples. Preliminary identification based on colony morphology, growth characteristics, and simple biochemical tests suggested the presence of potential bacterial pathogens such as *Staphylococcus spp.*, and *Escherichia coli*. It is suggested that adopting mixed mulberry leaves diets and enhancing sanitary measures can improve overall health and productivity in sericulture.

Keywords: *Staphylococcus* spp., *Escherichia coli*, *Bacillus spp.*, Infection, Bacteria

Introduction

Sericulture is an agricultural industry, and it is the science of rearing silkworms and food plants. The two sectors of sericulture are industry and agriculture. Growing food plants for silkworms and raising them for cocoons are both part of the farming sector. The industry sector includes knitting, twisting, dyeing, printing, finishing, and reeling (Sharma & Kapoor, 2020). There are four types of natural silk. The category of non-mulberry silks includes three varieties namely Eri, Tasar, and Muga silk (Massot et al., 2021). The cocoons of the mulberry silkworm *Bombyx mori*, which is raised in captivity, yield the best silk (Raheem et al., 2019). About a thousand different strains of *Bombyx mori* have been reported so far, but bivoltine and multivoltine races are especially useful for raising. The traditional breeding activities between members of different groups add new varieties every year (Giora et al., 2022). Three main components are necessary for successful silkworm rearing: mulberry leaf, climatic conditions, and silkworm strain (Tulu et al., 2022). The dietary needs, body mass, and cocoon characteristics of different hybrid silkworms may differ (Andadari et al., 2023). From hatching to cocoon formation, the weight of a silkworm grows ten thousand times. A single larval worm can consume up to 20 grams of mulberry leaves, with 90% of that being consumed in the final two instars when the worm is producing cocoons (Marin et al., 2023).

Sericulture makes a substantial contribution to the GDP and national economies of many nations, including India, China, and Thailand. In Pakistan, sericulture continued to be a thriving and profitable business project until the 1990s. Sericulture is important because of its enormous capacity to employ labor, particularly young people and women (Akram, 2016). All five of Pakistan’s provinces engage in sericulture. However, the primary silk production activity takes place in the vicinity of the irrigated forest plantations in Punjab province, including Changa Manga, Kamalia, Chichawatni, and Multan (Salman et al., 2022). It is commonly recognized that the quantity and quality of mulberry leaves influence the larvae's growth rate, developmental stage, body weight, and survival rate. By adding additional nutrients, mulberry leaves can be made more nutrient-rich, which will boost larval growth and enhance cocoon properties (Salman et al., 2022). Sericulture not only creates job opportunities and boosts farmers' income but also stimulates regional economic growth and enhances the environment **(**Bhuyan et al., 2022).

Numerous diseases can affect silkworm growth, development, and cocoon production. Silkworms are very sensitive to moisture levels and other environmental factors, making them more vulnerable to diseases (Gupta & Dubey, 2021). In the Indian subcontinent, there are four prevalent silkworm diseases: pebrine (protozoan), muscardine (fungal), flacherie (bacterial), and grasserie (viral) (Bhattacharyya et al., 2016). Among these, bacterial flacherie is more dangerous as compared to others. Depending on the type of bacteria involved, bacterial diseases are categorized as bacterial gastroenteric diseases, bacterial toxicosis, and bacterial septicemia (Mehdizadeh et al., 2021). *Bacillus spp.*, *Serratia marcescens*, *Bacillus thuringiensis*, *Streptococcus spp.*, *Staphylococcus spp.*, *Aerobacter cloacae*, *Micrococcus spp.*,, *Proteus spp.*, *Pseudomonas spp.*, and *Achromobacter delmarvae* are among those that cause flacherie (Gurgulova et al., 2017). Flacherie is a significant disease causing major economic losses in sericulture. The flacherie disease is caused by bacterial pathogens, and reduced yield and quality of cocoons. Despite knowing the fact that comprehensive research on the identification of specific bacterial pathogens responsible for flacherie is lacking. Identification of bacterial pathogens is are critical step toward developing effective management and prevention strategies to ensure the sustainability and productivity of the sericulture.

Material and methods

Study area

The present study was conducted at the Sericulture Unit, Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Ravi campus. The experiment was conducted from February to March 2024. The sericulture unit has an area of 12×08 ft. The rearing room, all the instruments, including the rearing trays, stands, and incubator, were sanitized with formaldehyde (2%) at the start of the experiment.

Rearing of Silkworm

The rearing of silkworms (Figs 1 and 2) was done under semi-controlled environmental conditions, and optimum temperature (25-28°C) and relative humidity (70-80%) were maintained throughout the growing period.

Proximate analysis of mulberry leaves

The mulberry species including black mulberry (*Morus nigra*), white mulberry (*Morus alba)* and mixed mulberry leaves were utilized as a source of feed for rearing of silkworms. The chemical composition of mulberry leaves, including dry matter (%), crude fat (%), crude fiber (%), crude protein (%), and gross energy (MJ/kg), was determined according to standard protocols (Sree & Vijayalakshmi, 2018).

Figure 1. Rearing of silkworm in semi-controlled environmental conditions.

Figure 2. Silkworm (*Bombyx mori*) larvae after the 3rd instar during the experiment.

Collection of samples

Diseased silkworm specimens were collected during the cleaning of rearing trays. Infected silkworms were placed in petri plates separately for further processing and analysis. Physical symptoms of infected specimens were a dark brown body, slow growth, secretion from their body, slow movement, and shrinkage of the body. Isolation of bacteria was done from three sources as follows;

Outer body surface

The infected specimens were washed with 70% ethanol and 3 times with distilled water. The specimen was triturated in sterile water using a glass rod. The 0.1 mL of suspension was used to inoculate on nutrient agar using the spread plate method and was incubated at 37°C for 24 hours. The standard plate count method was used to observe colony growth, and the total number of colonies was counted. Distinct colonies were picked for further analysis.

Gut of a silkworm

The infected specimens were placed on dissecting trays and were cut along the back to get the digestive tract. The digestive tract was placed in 200µL sterile solution. The 0.1mL of suspension was used to inoculate on nutrient agar using the spread plate method and was incubated at 37°C for 24 hours. The standard plate count method was used to observe colony growth, and a total number of colonies was counted. Distinct colonies were picked for further analysis.

The whole body of the silkworm

The diseased specimens were crushed by using a mortar and pestle, and the solution was filtered. The filtrate was centrifuged at 4000-5000 rpm for 10 minutes. The supernatant was discarded, and the pellet was used for bacterial culture after resuspending in distilled water.

Culturing of bacteria

After sample collection, the bacteria were cultured on the nutrient agar under aseptic conditions. The inoculated nutrient agar plates were placed in an incubator at 37°C for 24 hours. A sterile loop was used to pick a single colony and streak it onto the surface of each selected medium. MacConkey Agar was used for *E. coli*, and Mannitol Salt Agar was used for *Staphylococcus* spp. Culturing was done under aseptic conditions and then incubated at 37°C overnight (Fig. 3).

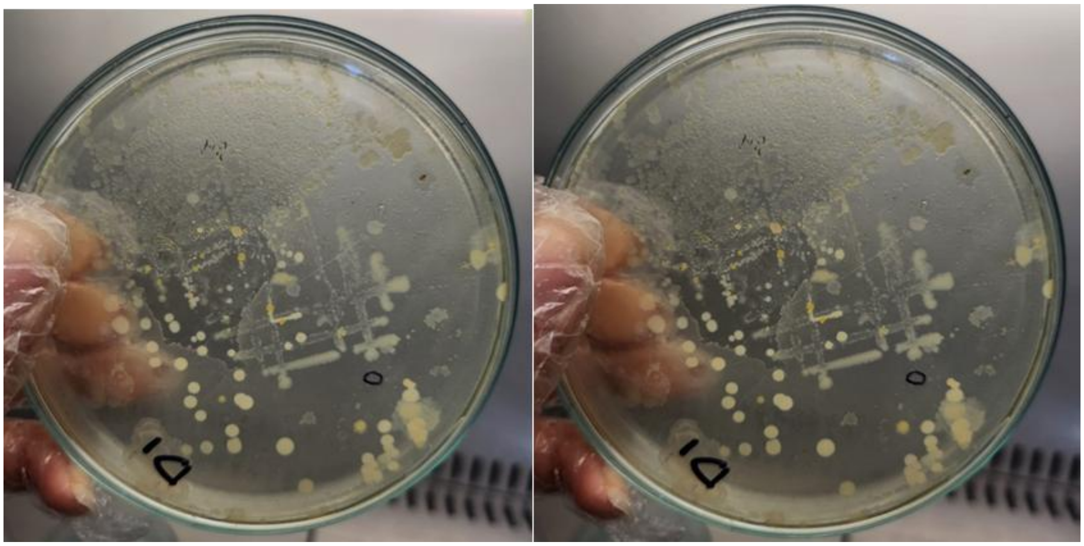

Figure 3. Culturing of bacteria on the nutrient agar under aseptic conditions at 37°C.

Statistical analysis

Results were presented as mean ± standard deviation (SD), and Pearson's correlation coefficient was calculated to explore the relationship between severity of disease symptoms and bacterial load using SPSS software (Version 26.0). The One-Way ANOVA was used to compare the mean colony counts across the different sources of isolation.

Results

Proximate analysis of mulberry leaves

The proximate analysis results revealed distinct variations in dry matter, crude protein, crude fat, crude fiber, and gross energy content. Table 1 summarizes the proximate analysis results of *Morus alba*, *Morus nigra* and mixed mulberry leaves. The results showed that black mulberry had the highest crude protein content, whereas white mulberry leaves were richest in crude fat. Mixed mulberry leaves exhibited intermediate values for most nutritional parameters. These variations were significant as they influence the growth rate and cocoon yield of silkworms.

Table 1. Nutritional composition of different mulberry species used during the present study.

Species	Dry Matter (%)	Crude Protein (%)	Crude Fat (%)	Crude Fiber (%)	Gross Energy (MJ/kg)
	Mean ± S.D				
Morus nigra	92.78±0.605	19.43±0.544	7.15±0.208	12.13±0.224	18.11±0.198
Morus alba	90.88±0.349	18.34±0.556	4.28±0.324	9.88±0.195	16.95±0.144

Mixed mulberry leaves	91.42±0.828	19.01±0.130	6.01±0.123	11.04±0.150	17.66±0.075

Bacterial isolation and growth

A total of 157 distinct bacterial colonies were isolated across all specimens and sources. The highest number of colonies was observed in samples taken from the gut (62 colonies), followed by the whole body (53 colonies) and the outer body surface (42 colonies).

Quantitative analysis

The standard plate count method revealed significant variability in colony numbers among different specimens and isolation sources using one-way ANOVA (Table 2).

Table 2. The mean colony count from multiple plates.

Source of isolation	Mean colony count per plate ± S.D	p-value
Gut	62±8	< 0.05
Outer Body Surface	42±6	< 0.05
Whole Body	53±7	< 0.05

Morphological and cultural characteristics

Distinct morphological differences were observed among the isolated colonies on both MacConkey Agar and Mannitol Salt Agar (Figure 4). Colonies isolated from the gut exhibited a red coloration on MacConkey Agar, indicative of lactose fermentation, whereas colonies from the outer body and whole body samples were predominantly pale, suggesting non-lactose fermenting bacteria. On Mannitol Salt Agar, colonies from the whole body samples showed yellow pigmentation, suggesting mannitol fermentation, which was less common in samples from other sources (Table 3).

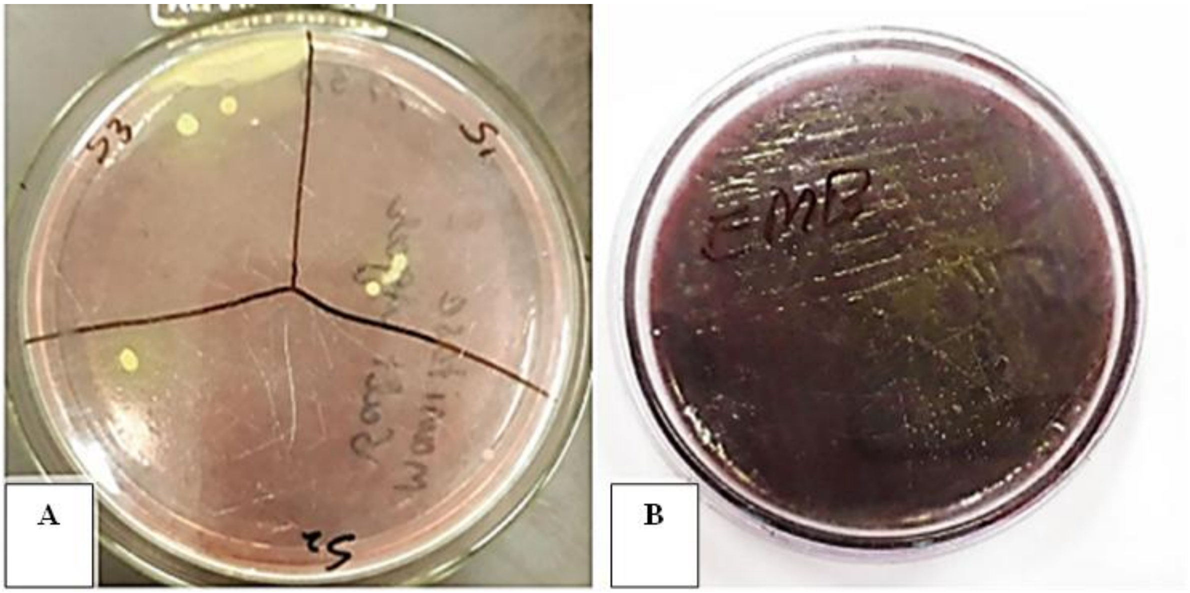

Figure 4. Morphological and biochemical identification of bacterial species on their respective media. A: *Staphylococcus aureus* and B: *Escherichia coli*.

Table 3. Culturing of bacteria on selective media.

Bacterial Species	**Selective Media**	**Growth Characteristics**	**Confirmatory Test**
Escherichia coli	MacConkey Agar	Pink colonies, lactose-fermenting	Positive Growth
Staphylococcus aureus	Mannitol Salt Agar	Yellow colonies, mannitol-fermenting	Positive Growth

Correlation between bacterial load and disease severity

Preliminary identification based on colony morphology, growth characteristics, and simple biochemical tests (lactose and mannitol fermentation) suggested the presence of bacterial species, including potential pathogens such as *Staphylococcus* spp., and *Escherichia coli*. Pearson's correlation coefficient (r) was calculated to determine the relationship between bacterial load and disease severity in *Bombyx mori*. A strong positive correlation was observed, particularly in the gut-derived samples, with a correlation value of $r = 0.87$ and a p-value < 0.001 (Table 4). Specimens with higher bacterial counts exhibited more severe symptoms, including darkened body color, pronounced shrinkage, and lethargy, potentially indicating a higher virulence of gut-colonizing bacteria. Samples from the outer body surface and whole body showed weaker correlations, with $r = 0.65$ ($p < 0.05$) and $r = 0.72$ ($p < 0.01$), respectively. These results indicate a moderate positive correlation between bacterial load and disease severity, suggesting that while there is an association, it may not be as pronounced as that observed in the gut samples.

Table 4. Pearson's correlation analysis of bacterial load and disease severity.

Source of isolation	Bacterial load (Mean ± S.D)	Disease severity (Pearson's r)
Gut	62 ± 8	$r = 0.87, p < 0.001$
Outer Body Surface	42 ± 6	$r = 0.65, p < 0.05$
Whole Body	53 ± 7	$r = 0.72, p < 0.01$

During the study, disease resistance associated with different mulberry leaf diets was closely monitored. The incidence of diseased silkworm specimens varied markedly among the groups fed with *Morus alba*, *Morus nigra*, and a mix of both leaves. The *Bombyx mori* fed with *Morus nigra* leaves shown a reduced frequency of disease symptoms. Approximately 8% of the total silkworms' larvae fed with *Morus nigra* shown the signs of infection including darkened body color and reduced activity. Similar symptoms were shown by 18% of *Bombyx mori* group fed *Morus alba*. The mixed mulberry leaves group displayed intermediate results, with around 15% of silkworms showing disease symptoms. The results suggest that *Morus nigra* leaves increase the resistance against bacterial pathogens in *Bombyx mori* due to their higher protein content and certain phytochemicals that can enhance silkworms' immune system.

Discussion

The present study was planned to assess the influence of *Morus alba*, *Morus nigra* and mixed mulberry leaves diet on the health and disease susceptibility to *Bombyx mori* The proximate analysis showed the significant differences in the nutritional composition of *Morus alba*, *Morus nigra* and mixed mulberry leaves. The analysis shown that *Morus nigra* had the highest crude protein contents while *Morus alba* have the highest crude fat. These differences are significant as silkworm growth and cocoon production are closely linked to the nutritional quality of their diet. Previous studies have shown that higher protein content in mulberry leaves enhances larval growth and silk yield (Andadari et al., 2023). Additionally, the balance of nutrients, including fats, plays a vital role in the overall health and productivity of *Bombyx mori*.

Protein is a vital component for the growth and development of silkworms. Higher protein content in mulberry leaves has been associated with increased larval weight and better cocoon characteristics. The elevated crude protein in *Morus nigra* suggests that feeding *Bombyx mori* with *Morus nigra* leaves could enhance growth rates and cocoon quality. Similarly, the higher crude fat content in *Morus alba* can provide additional energy reserves, potentially benefiting larval survival and resilience to diseases. The findings of our study are similar to Iqbal et al. (2021), who reported that *Morus nigra* leaves are rich in protein as compared to *Morus alba* while Yu et al. (2018), reported that the higher protein contents in mulberry leaves are related to better quality of cocoon production. However, the higher crude fat contents in *Morus alba* leaves can also provide additional energy, but it does not seem to positively affect the silk quality. Recent studies have shown that the types and quality of mulberry leaves in sericulture play a vital role in the silkworm's health and cocoon production. Sajgotra et al. (2018) reported the effect of different mulberry species on the growth and economic traits of the bivoltine silkworm species. The results showed that the nutritional composition of mulberry leaves significantly affects overall silkworm larvae development and cocoon production. Gobena & Bhaskar (2015) reported that adding a few plant extracts to the silkworm diet can positively affect growth and cocoon quality. Similarly, Thrilekha et al. (2024) documented that using protein-rich mulberry leaves maximizes cocoon production and silk quality.

The proximate analysis of *Morus alba* (white mulberry), *Morus nigra* (black mulberry) and a mixed mulberry leaves during present study have shown that these species have unique nutritional composition. These results align with prior studies that underscore the importance of specific nutrient compositions in influencing silkworm growth and cocoon quality (Murugesh, 2021; Hossain et al., 2022). According to Murugesh et al. (2021), protein availability in silkworm diets directly correlates with higher silk yield, suggesting that the use of *Morus nigra* could enhance cocoon production in sericulture practices. Dharanipriya (2019), reported that protein-enriched diets positively influence larval biomass and silk yield. Additionally, the moderate nutritional profile observed in mixed mulberry leaves suggests a balanced dietary approach for fluctuating climatic conditions, similar to insights by Tao et al. (2011) on the adaptability of silkworms to mixed diets for sustained growth rates.

E. coli and *S. aureus* are opportunistic pathogens that can cause infections under favorable conditions. In silkworms, these bacteria can invade the gut, multiply, and disseminate throughout the body, leading to symptoms such as lethargy, loss of appetite, and eventual death. The identification of these bacteria in infected silkworms aligns with previous reports linking them to flacherie outbreaks (Haloi et al., 2016).

The present study preliminarily identified *Escherichia coli* and *Staphylococcus aureus* as predominant bacteria in infected silkworms. These pathogens are known to cause flacherie disease, leading to significant losses in sericulture. The presence of these bacteria in the gut and whole-body samples aligns with previous findings reported by Sakthivel et al. (2012), highlighting the gut as a primary site for bacterial infections in silkworms. These bacteria were present in varying loads across different silkworm tissues. The highest number of colonies was observed in samples taken from the gut (62 colonies), followed by the whole body (53 colonies) and the outer body surface (42 colonies). The Pearson's correlation analysis (Pearson's $r = 0.87$, $p < 0.001$) revealed a strong association between the severity of disease symptoms and bacterial load, especially in the gut-derived samples. The presence of these pathogens is significant, as both *E. coli* and *S. aureus* are known to cause flacherie, a disease that leads to significant losses in sericulture. The bacterial counts from different silkworm body regions in our study indicated a higher bacterial load in gut and whole-body samples, resonating with Haloi et al. (2016), who noted that the silkworm gut hosts a diverse microbial population essential for digestion and pathogen resistance. In particular, the isolation of *E. coli* and *Staphylococcus aureus* in your study is consistent with observations by Subrahmanyam et al. (2023), who identified these pathogens as contributors to disease symptoms in the muga silkworm. Notably, symptoms such as sluggish movement and discoloration were observed in infected silkworms, as discussed by MsangoSoko et al. (2020), who also emphasized that such infections compromise growth and silk production.

Similarly, Sakthivel et al. (2012), isolated and identified bacteria responsible for flacherie in silkworms, reporting the presence of *Bacillus subtilis, Streptococcus pneumoniae, Staphylococcus aureus, E. coli, Pseudomonas fluorescens,* and *Bacillus cereus*. The results of the study revealed that the bacterial pathogens causing flacherie need comprehensive disease management strategies. Chen et al. (2023), reported that the gut of *Bombyx mori* harbors complex microbiota due to exposure to different leaf types and the environmental conditions. Moreover, the bacterial diversity and their relative abundance in the gut suggested that the silkworms maintain a unique microbiome, which in turn plays a role in digestion, immunity, and pathogen resistance. Rahul et al. (2019), reported the characterization and identification of the bacteria that cause flacherie in *Bombyx mori*. The larvae displaying typical flacherie symptoms and their midgut contents were examined. After being serially diluted, the same was plated onto nutrient agar plates. The 16S rRNA gene was sequenced to confirm the identity of five bacterial cultures that were isolated in axenic form. The results of BLAST analysis showed that they had the greatest sequence similarity to *Staphylococcus argenteus*. Li et al. (2015), identified and described *Bacillus cereus* in *Bombyx mori.* The project aimed to separate and identify a pathogen from diseased silkworms. The 16S rRNA gene sequence and the strain's bacteriological characteristics were used to identify *Bacillus cereus.*

Bacterial pathogens viz., *Escherichia coli* and *Staphylococcus aureus* can cause infection in silkworm through contaminated mulberry leaves. These findings highlight the importance of maintaining good sanitary conditions in sericulture. Singh et al. (2011) reported similar findings and documented that enhanced sanitary conditions, along with routine microbial monitoring, are effective in reducing overall bacterial infections in silkworms. Furthermore, MsangoSoko et al. (2020), documented that environmental factors and diet significantly influence the gut microbiota of *Bombyx mori*. Thus, the mulberry leaves have a secondary effect on the microbial community in the gut of the silkworm.

Conclusion

The present study successfully explored the nutritional composition of different mulberry species (*Morus alba*, *Morus nigra* and mixed mulberry leaves). The proximate analysis revealed significant variations in the crude protein, crude fat, crude fiber, and gross energy content with *Morus nigra* showing the highest crude protein content, suggesting its potential as a more beneficial diet for silkworms. The bacterial isolation showed distinct morphological characteristics, with the gut-derived samples presenting the highest bacterial count, Furthermore, a strong positive correlation ($r = 0.87$, $p < 0.001$) between bacterial load and disease severity was observed in the gut samples, indicating that higher bacterial counts are linked to more severe symptoms. It is suggested that adopting mixed mulberry leaves diets and enhancing sanitary measures can improve overall health and productivity in sericulture.

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