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Isolation, identification, and characterisation of pathogenic bacteria from the gut tissue of silkworm (bombyx mori, l) and its management using Phyto essential oils

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Abstract
The agriculture industry is an important sector includes the sericulture industry which employs people in its thrust areas like moriculture, rearing, reeling, egg production centers, twisting, weaving, dyeing, and fabrication. Silkworm which is a silk producer is sensitive to different infections caused by pathogens (bacteria) with a loss of 15 -25 %. Among the bacterial diseases, flachreie which was recognised by Pasture as silkworm diarrhoea is a serious disease of Bombyx mori associated with various bacterial pathogens. In the present study, cadavers during summer and rainy seasons from nearby sericulture areas and Kakatiya University, (sericulture lab) were collected, dissected to isolate, identify and characterize based on morphological, biochemical and physicochemical parameters. The shape, size, and colour of the bacterial colony were recorded with Micronauts bacteria identification techniques. For purification and control of flacherie and enhancement of commercial parameters, different phyto essential oils were used to harvest a good cocoon crop in hygienic conditions under optimum environmental conditions.

Keywords: bacteria, bombyx mori, isolation, identification, characterisation

1. Introduction
Silk is a best-known protein which spells luxury, elegance, comfort with unparalleled grandeur continuity is composed of mainly fibroin and sericin obtained from cocoons spun by mulberry silkworms reared in hygienic conditions. It's a fiber which faced many daunting challenges from artificial silk fibres and remained as Queen of textiles globally as a popular and widely appreciated used material [1, 2].
In India 80% of the local area under cultivation is confined to five states, the production under Telangana state has increased drastically in recent years due to the increase in an areage and government efforts.
Immune factors of this lepidopteran insect have drastically lowered due to excessive inbreeding and adverse environmental factors making the silkworm susceptible to various diseases [3, 4].
Based on the experimental studies [5] carried out it was reported that understanding the disease etiology is difficult due to the multiplicity of bacterial types that causes flacherie. Unhygienic conditions prevailing in the rearing house, contaminated mulberry leaves, dead diseased silkworms, and their leftovers are sources for bacterial infection. Bacillus spps, Streptococcus spps, Proteus spps, Staphylo coccus spps, Serria marcescens, Micrococcus spps, Acrobactor cloacae induce flacherie [6, 7].
The flacherie causes the silkworm cadavers to lose elasticity, appetite loss, slow growth due to sluggishness with body shrinkage, oral and anal discharge, thorax swelling, liquification of internal organs, skin rupturing and oozing of brown fluid with foul smell [8].

2. Materials and Methods Source for collecting Silkworm Cadavers
Healthy and disease 4th and 5th silkworm larvae were collected from rearing houses of sericulture unit Kakatiya University and surrounding areas were rearing were carried out (Warangal and Bhupalapally districts) based on the symptoms and then preserved in aseptic containers.

3. Isolation of Bacteria
Bacteria can be isolated from three different sources namely the outer body surface, gut and whole body of silkworm cadavers for the experiments from the silkworm which were weighed separated from gut tissue was dissected and surface sterilized with 70% ethanol and washed thrice with sterile distilled water in a dissecting tray that was disinfected with 95% ethanol and centrifuged at 3000rpm for three minutes, the clear supernatant was re centrifuged after washing with distilled water. The supernatant obtained was pooled with the collected previous samples [9].

4. Preparation of primary culture
With the help of cotton swabs, the inoculums from each of dilutions (10-1, 10-2, 10-3 and 10-4) were spread on Petri dish containing solid agar medium. The best colonies obtained with 10-2 dilutions were used for further experiments.

5. Culturing of Bacteria
From the primary culture, a loop full of the suspension was taken with inoculation needles and streaked on nutrient agar

medium and then incubated at room temperature using a streak plate technique followed by subculturing of the isolated pathogen. The pathogens from isolated single colonies were purified by streaking on the plate. After attaining good growth, the slants were stored in the refrigerator at 40c [10].

6. Morphological and Biochemical Characterization of Bacteria

The characterization of bacteria was carried out after streaking the subcultures on the nutrient agar media at 370c for 48 hours. Cellular, morphological characters such as colony, morphology, and gram staining techniques were carried out for bacterial pathogen identification followed by biochemical tests such as catalase and nitrate reductase tests [11].

7. Antimicrobial screening by disc diffusion

Sterilised whatmans1 papers 6mm diameter discs were prepared and autoclaved for further screening. The sterilized discs were impregnated with 50ml of different concentrations (1:1, 1:5, 1:10and 1:20) of irrespective essential oils were then kept on the agar spread plates with the help of sterilized forceps. In the sealed Petri dishes, the ethanol moistened paper discs were placed which act as vehicle control, followed by incubation at 250c for 24 hours. After the visible growth of bacteria in dishes, the zone of inhibitions (MMS) was measured with the help of caliper. The complete data about the inhibition of bacterial growth were calculated in triplicate for declaring the essential oils as bioactive compounds [12, 13, 14].

Result

Table 1

SI NO	Bacteria	Gram	Shape	Spore	Cata-lase	Nitrate reduc-tase
1	*Bacillus cereus*	+	Rod shape Violet colour	+	+	+
2	*Proteus vulgaris*	_	Rod shape	_	_	_

⇒ * crystal formation
⇒ + positive
⇒ - negative

The colonies developed on the agar plate were characterized based on or, shape and stain. The overall shape of the colony was circular, convex colonies with an irregular edge in the case of B. cerus while in the case of P.v ulgaris it was circular with a regular edge.

8. Selection of Few Essential Oils Based on Antibacterial Assay

The anti-microbial activity of seven essential oils was studied against isolated strains of silkworm bacteria (B.cereus and P.vulgaris). The potency of the essential oils was assayed by the presence or absence of inhibition zones and its diameters. Results showed that the oils were active against both the strains with different inhibition zones ranging from 1- 8mm.

Lavender, Cinnamon, Clove and Rose oils showed activity against the gram-positive bacteria, while clove and cinnamon oils at low concentrations (1:20 and 1:10) showed high antibacterial activity than at higher levels(1:5 and 1:1). High inhibition zones (8mm) at 1:10 were obtained with lavender and cinnamon oils against B.cereus (gram-positive), while low values were observed with orange, olive, clove and lemongrass oil at 1: 20 ratio. Cinnamon oil at 1:10 concentration showed the highest inhibition zone of 6mm against P.vulgaris(gram-negative). Clove oil showed an inhibition zone of 6mm against both strains at 1:5 concentrations. Among the oils tested, lavender and cinnamon oils showed high antibacterial activity against silkworm bacterial strains.

Table 2: The effect of different essential oils on the growth of the bacteria Bacillus cereus and Proteus Vulgaris isolated from the flacherie infected silkworm.

S.no	Name of essential oil	Essential oils concen-trations	*Bacillus cereus*	*Prot-eus vulga-ris*
1	Clove oil	1:1	6	6
		1:5	3	3
		1:10	3	2
		1:20	3	2
2	Olive oil	1:1	3	3
		1:5	3	3
		1:10	5	3
		1:20	4	4
3	Orange oil	1:1	2	2
		1:5	2	2
		1:10	3	2
		1:20	2	3
4	Rose oil	1:1	5	3
		1:5	2	5
		1:10	4	5
		1:20	2	5
5	Lemon grass oil	1:1	2	5
		1:5	2	4
		1:10	3	3
		1:20	3	2
6	Lavender oil	1:1	3	6
		1:5	4	7
		1:10	5	8
		1:20	2	4
7	Cinnamon oil	1:1	5	5
		1:5	6	6
		1:10	8	4
		1:20	5	2

Values are the mean of three individual determinations

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