Assessment of Gut Microflora in Silkworm Fed on Mulberry Leaf treated with *Spirulina* mediated TiO_2Nps.

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Abstract
Investigations were undertaken to Assessment of gut microflora in silkworm fed on mulberry leaf treated with *Spirulina* mediated TiO_2NPs at Department of Sericulture, Forest College and Research Institute, Mettupalayam. The nanoparticles were synthesized with TiO_2 using aqueous extract of *Spirulina platensis* as reducing and capping agent. The *S. Platensis* mediated TiO_2NPs at the concentration of 50 ppm treated silkworms gut bacterial isolates were identified through morphological and biochemical characteristics. The identified microbes are *Pseudomonas sp, Leuconostoc sp, Bacillus sp and Staphylococcus sp.*

Introduction
Silkworm obtain required nutrients entirely from mulberry leaves because mulberry silkworm is monophagous in nature. Generally, vitamins and other essential nutrients present in the mulberry leaves fulfils the minimum needs of silkworms but the amount of nutrients present in the mulberry leaves diverged on the basis of environmental conditions, usage of fertilizers, mulberry varieties and other cultivation practices. *B. mori* takes essential sugars, amino acids, proteins and vitamins for its normal growth and development.

Application of nanotechnology in sericulture for improving the silk yield made a great avenue in the last decade. Nanotechnology deals with the most advanced applications in multidisciplinary fields including targeted drug delivery, molecular diagnosis and electronic imaging (Sankar *et al*., 2015). Recently nanotechnology created a greater impact in agriculture and allied sciences including sericulture. Many researchers were made various attempts in increasing silk production, midgut flora assessment and enhancement of reproduction ability in silkworms through nanotechnology (Kumar *et al.,* 2013; Shyed and Ahmad, 2013).

Recently many researchers have made attempts to increase raw silk production in various ways like silkworm hybridization, usage of artificial diet and application of phytojuvenoids. Breeding of silkworm races has been a key strategy to improve silk production, little improvement in silk production has been achieved to date. As a result, the development of sericulture economy has not progressed well, pointing to the need of new ways for improvement of silk production (Ni *et al.,* 2015).

The TiO_2NPs exhibit excellent antibacterial, anti-inflammatory, anti-fungal, anti-microbial and several biological activities. TiO_2NPs had been widely used as feed additives with significant biological activity. Recent studies had shown that low concentrations of TiO_2NPs can promote silkworm growth and improve cocoon quality (Li *et al.,* 2015).

In mulberry silkworm, there are several types of bacteria present in midgut portion which aids in nutritional and symbiotic benefits. The application of TiO_2 nano formulation enhanced digestive utilization of feeds and detoxification of metabolites. They also promote the production of vitamins, increase host resistance and compete with pathogenic bacteria by producing organic and antibiotic substances. Also, gut bacteria improved commercial characteristics, disease resistance and protein synthesis (Rani *et al.,* 2011).

Materials and methods

Glasswares and Chemicals

The glasswares of Borosil grade were used in all experiments. Glasswares were cleaned by soaking in chromic acid solution (100 g potassium dichromate dissolved in 1 litre of water with 500 ml of concentrated sulphuric acid) for two hours and finally washed with distilled water. The glasswares were rinsed once again with distilled water and sterilized in hot air oven before use. All the chemicals used were of Analytical Reagent (AR) grade.

Media

For this experiment, Nutrient agar media was used. The specified quantity of nutrients was dissolved in water, pH was adjusted to 7. Added agar mixed with distilled water at the rate of 1.5%. The mouth of conical flask was closed with cotton plug, The stiff paper was covered with cotton plug. Finally sterilized in autoclave at 15 lbs for 15 mins at 121°C.

Sterilization techniques

All glasswares were sterilized in a hot air oven at 180^0C for 3 hours. All growth medium and broth were sterilized in an autoclave at 15 lb pressure for 20 minutes. Isolation, purification, inoculation and other microbiological works were carried out in laminar air flow chamber.

Isolation of gut

For isolation of gut microflora, the 5^{th} instar larvae were isolated from 50 ppm *S. Platensis* mediated TiO_2NPs (10^{-6}), kept in starvation upto 6 hours prior to dissection under sterile condition and anesthetized with 70% chloroform. The oral and anal ends of larvae dipped in 70% alcohol for 10 seconds. The alimentary canal was dissected out and transferred to water blank.

Gut preservation

After isolation, the guts were homogenized immediately in 0.1 M Potassium phosphate buffer (pH 7.0). The homogenate was transferred to vials and kept at 5^0C for several hours to overnight before washing three times with glutaraldehyde-free phosphate buffer.

Culturing of gut microflora

The total gut microflora (Bacteria) was isolated after growing them in a Nutrient Agar (NA) medium. The streak plate technique, most popular and easier method for isolating single colonies from large number of different gut microflora was followed for further subculturing of isolated gut microbes.

Streak plate assay

The media was poured in sterile petriplates and the plates were rotated in clockwise and anticlockwise directions for uniform spread of the medium. The plated media were divided into four quadrants and cultures of gut microflora were streaked on all the four quadrants. The plates were incubated at an inverted position at 28-32^0C and the growth of microbes was recorded.

The colonies were repeatedly purified by streaking on the plate. The isolated single colonies of gut microbes were picked up and streaked on respective NA slants. After attaining good growth, slants were stored in a refrigerator at 4^0C for further studies.

Identification of Bacteria

The morphological characteristics of colony were studied and staining technique was carried out for characterizing the gut microbes of silkworm.

Morphological characteristics

Colony morphology

The colony surface characteristics such as concentric, radiated, contoured, smooth and wrinkled and individual colony edge characteristics like circular, pinpoint, irregular and filamentous were used for the identification of various isolated bacterial strains. The cellular morphology was used for distinguishing the coccus and bacillus forms of bacteria under microscope.

Staining techniques

Simple staining

A simple staining was done to determine cell shape, size and arrangement of bacterial cells. In this method, the cell smear was stained by the application of a single staining reagent.

Gram staining

The differential staining method was followed to differentiate microbes into two basic groups *viz.,* gram positive microbes and gram negative microbes. Gram positive organisms retained the primary dye complex (Crystal violet-iodine) while gram negative cells lost the primary dye complex at the time of rinsing.

The smear was saturated with crystal violet for 60 sec and gently rinsed with water. The smear was then stained with iodine for 60 sec and rinsed with water. The smeared slide was discolourized with 95% ethanol for 3-5 sec, rinsed with water, counter stained with Safranin for 1 min and rinsed with water. The slide was blotted dry carefully and observed under the microscope. By this staining technique, gram positive bacteria stained purple and gram negative bacteria stained red or pink.

Characterization of bacterial culture

The culture characteristics were observed using Nutrient agar media, whereas biochemical test such as Coagulase test, Catalase test, Sugar fermentation and IMViC tests were conducted to characterize the bacterial cultures (Robert Pollock *et al*., 2002).

Biochemical tests

Coagulase test

The isolates were aseptically placed on the coagulase plasma and mixed well. The obvious clumping of the bacteria was indicated as the positive reaction.

Catalase test

One to two drops of hydrogen peroxide was added to the colonies on the plates and observed for the characteristic bubbling which indicated the presence of catalase that catalyzed the breakdown of hydrogen peroxide (H_2O_2) with the release of free oxygen.

Fermentation of sugars

The change of colour of test medium to yellow for Glucose, Lactose, Sucrose and Mannitol indicated positive reaction whereas the colour change to dark red indicated negative reaction.

IMViC tests

The IMViC series of biochemical tests including indole production [I], Methyl red test [M], Voges-Proskauer test [Vi] and Citrate test [C] were conducted (Robert Pollock *et al*., 2002).

Indole production

Two tubes containing peptone broth were labeled, of which one tube was inoculated with the bacterial culture and incubated for 24-48 hrs, other tube was kept as a control. An amount 0.5 ml of Kovac's reagent (Para-dimethyl aminobenzaldehyde in alcohol) was slowly added to the peptone broth culture. Appearance of dark red colour indicated the positive reaction.

Methyl red test

Five drops of methyl red was added to the MR–VP tubes and mixed gently. Appearance of red colour indicated the presence of high concentration of acid (positive), whereas the yellow colour indicated a negative reaction.

Voges-Proskauer test

One ml of 5% Naphthol and 0.5 ml of 40% potassium glyroxide was added to MR-VP tube and left standing for several minutes. Development of red colour indicated the positive reaction.

Citrate utilization

The citrate broth with bacterial culture was observed for cloudiness or turbidity which indicated a positive reaction and if broth remained clears without growth and then it was recorded as a negative reaction.

Oxidase test

The 24 hr old bacterial cultures were spot inoculated on oxidase disc and change in colour of the disc from white to purple or blue was observed (Robert Pollock *et al*., 2002).

Starch hydrolysis

Nutrient agar containing 0.2 per cent soluble starch was used. The test cultures were spotted on the petri plates. Starch hydrolysis was tested after 48 hours of incubation by flooding the agar surface with lugol's iodine solution. A colourless

zone around the bacterial growth in contrast to the blue background of the medium indicated positive reaction (Laskin and Lechevailer, 1977).

Gelatin hydrolysis
The test medium containing beef extract 3 g, peptone 5 g, gelatin 120 g, distilled water 1litre was dispensed in a test tube, autoclave at 120^0C for 12-15 min and cooled until inoculation. After inoculation, the tubes were inoculated at 20-22^0C for 3 days and kept at 4^0C for 30 min and observations were recorded (Schaad, 1992).

Results
Isolation and characterization of bacteria
The gut microflora isolated was identified based on colony morphology, cell shape and staining reactions. The characteristic details are furnished in Table.1.

The experiments on the isolation of gut microflora of double hybrid silkworm breed *Bombyx mori* (CSR 6 × CSR 26) × (CSR 2 × CSR 27) encountered consistently four dominant species of bacteria. Pure cultures of four gut bacterial isolates were designated as 'Silkworm Bacterial Isolates - 1, 2, 3 & 4 (BI- 1, BI-2, BI-3 and BI-4). The bacterial cultures were identified through morphological and biochemical characteristics as detailed below (Fig.1).

Colony morphology
From the observed colonies, the strain of BI-1 grew as circular, fluorescent green, raised and margin was entire. BI-2 appears to be spherical, but often lenticular coccoid cells in pairs and chains. Sometimes short rods with rounded ends in long chains. BI -3 was shown as circular, bright and convex colonies with irregular edges. BI – 4 was visible as Large, circular, convex and golden yellow colonies showed a painted appearance.

Cell morphology
The cells of organisms were viewed under the compound microscope. BI-1 produces short rods; BI-2 shows coccus forms and were visible as clusters. BI -3 appears to be rod-shaped and BI – 4 had coccus forms.

Staining reactions -Gram staining
Gram staining reactions showed that the strains of BI-1 and BI- 3 were found to be Gram-positive. The strains BI- 2 and BI- 4 were found to be Gram-negative.

Biochemical characterization of selected bacterial isolates
Coagulase test
The strains of BI-1, BI- 3 and BI-4 showed a positive reaction with coagulase by forming a clot, whereas BI-2 seems to be negative.

Catalase test
The BI-1 and BI-4 exhibited a reaction to the catalase test with the release of free oxygen gas bubbles, whereas BI-2 and BI- 3 showed no bubbling effects.

Aesculin hydrolysis
The strains of BI-1, BI- 3 and BI-4 showed a negative reaction, whereas BI-2 seems to be positive reaction.

Sugar fermentation
The three bacterial strains showed positive reactions for glucose fermentation reaction except for BI- 1 which showed negative. For lactose fermentation, SBI- 3 showed negative but not other strains. BI-2 and BI- 4 showed a negative reaction for sucrose fermentation. BI- 4 showed a positive reaction for mannitol. BI- 2 showed a positive reaction for arabinose.

IMViC test
The Indole production test showed a negative reaction only for the strain BI- 4 and all the others seem to be positive. For Methyl red and Citrate tests, all strains were shown as positive reactions and all strains showed negative reactions for the Voges Proskauer test. The citrate test showed BI- 4 was negative reaction, BI- 1, BI-2 and BI- 3 were positive. Based on these characters, BI-1, 2, 3 and 4 were identified as *Pseudomonas sp, Leuconostoc sp, Bacillus sp and Staphylococcus sp*.

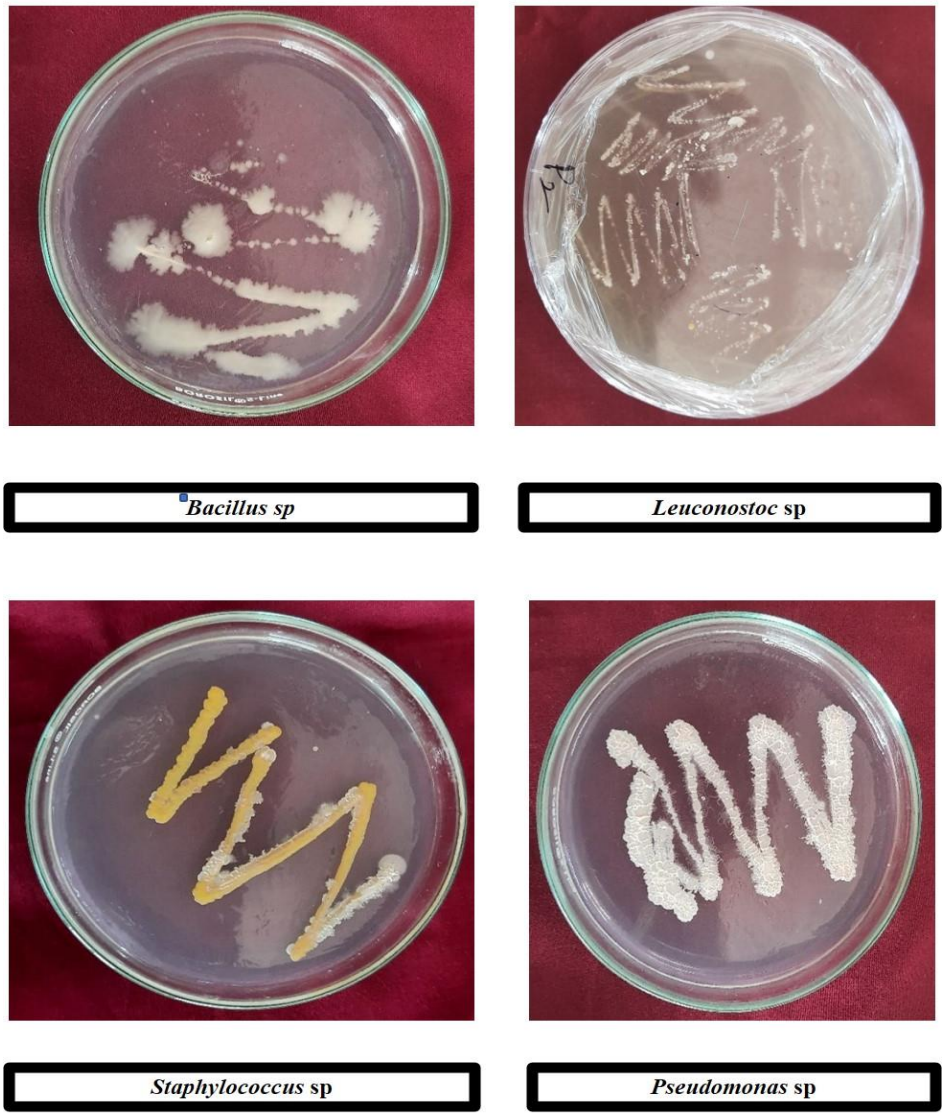

Fig 1. Identification of bacterial isolates

Table. 1 Isolation and Characterization of bacteria on treated silkworm

Bacterial isolates	***BI-1**	**BI –2**	**BI –3**	**BI –4**
Morphological/ Microscopic Characters				
Colony	Circular, fluorescent green, raised and margin was entire	Spherical, but often lenticular coccoid cells in pairs and chains. Sometimes short rods with rounded ends in long chains	Circular, bright and convex colonies with irregular edges	Large, circular, convex and golden yellow colonies showed painted appearance
Cell shape	Short rods	Coccus form	Rod shape	Coccus form
Motility	+	-	+	-
Spore formation	-	-	+	-
Gram staining	-	+	+	+
Biochemical test				
Catalase	+	-	-	+
Coagulase	+	-	+	+
Aesculin hydrolysis	-	+	-	-
Carbohydrate Fermentation reaction				
Glucose	-	+	+	+
Lactose	+	+	-	+
Sucrose	+	-	+	-
Mannitol	-	-	-	+

Arabinose	-	+	-	-
IMViC test				
Indole production	+	+	+	-
Methyl red	+	+	+	+
Voges Proskauser	-	-	-	-
Citrate	+	+	+	-
	Pseudomonas sp.	*Leuconostoc sp.*	*Bacillus sp.*	*Staphylococcus sp.*

***BI-** Bacterial isolates
+ Positive reaction, - Negative reaction

Discussions

Investigations were carried out to isolate dominant aerobic gut bacteria from double hybrid silkworm. The studies have shown that four kinds of bacteria were consistently encountered, with distinct colony characters and they were designated as silkworm bacteria isolate BI-1, 2, 3 and 4. The bacterial isolates were identified by their morphological and biochemical characteristics as *Pseudomonas sp, Leuconostoc sp, Bacillus sp and Staphylococcus sp*. The present study falls in line with Liu *et al*. (2016) who reported *Staphylococcus* had sturdy resistance and enhanced lipase synthesis, which developed growth of silkworm was noticed. The present result also made an agreement with Kaminski *et al.* (2018) who registered *Pseudomonas* is effective in degrading insecticides and also improve growth and development of silkworm.

The four gut bacterial isolates of the present investigation *viz*., *Pseudomonas sp., Leuconostoc sp., Bacillus sp*. and *Staphylococcus sp.* have been encountered as dominant gut bacteria of silkworm breeds in earlier studies too. Thus, the present studies also reassure the fact that *Pseudomonas sp., Leuconostoc sp., Bacillus sp. and Staphylococcus sp*. among the dominant gut microflora of silkworm breeds.

Conclusion

The *S. Platensis* mediated TiO2NPs at the concentration of 50 ppm treated silkworms gut bacterial isolates were identified through morphological and biochemical characteristics. The identified microbes are *Pseudomonas sp, Leuconostoc sp, Bacillus sp and Staphylococcus sp.*

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