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Research Article

Attempt on the Control of Bacterial Disease: Flacherrie in the Larval Instars of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) Through the Utilization Garamycin

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Abstract

The present attempt was planned with the aim of enhancement of resistance in the body of silkworm, *Bombyx mori* (L) against the infections of bacterial pathogens through the use of Garamycin antibiotics. The bacterial disease: flacherrie is the most significant parameter associated with the loss of silk yield. The loss of appetite; discharge watery feces and vomiting are the common symptoms of infection of bacteria to the larval instars of silkworm, *Bombyx mori* (L). For the bacterial pathogens, the diseased black thorax septicemia infected larvae of silkworm, *Bombyx mori* (L) were crushed through the use of using mortar and pestle; the solution was filtered (the filtration method of Levin., *et al.* 1974); the filtrate was centrifuged (at 4000 - 5000 rpm) for ten minutes; the precipitate (in the form of the pellet) was used for bacterial inoculum. The bacterial sample (inoculum) was streaked in Luria Agar under aseptic conditions and processed for incubation (at 37ºC overnight). After 24 hours, the growth of bacteria was noticed, and it was further processed for sub culture. A bacterial sample was taken through the use of loop; centrifuged for 15 minutes at 4000 rpm and the precipitate (in the form of the pellet) was dissolved in distilled water. Soon after the second moult, larval instars were divided into four groups (Untreated control group; Water treated group; Bacterial inoculum *(Genus: Streptococcus bombycis*) treated (infected) group and the group treated with *Garamycin antibiotics* (40 microgam/ml distal water), each with hundred individuals. The larvae of bacterial inoculum treated (infected) group and the group of larvae for antibiotics treatment were infected (treated) with the aqueous solution of bacterial inoculum. This treatment was carried out through smearing the solution bacterial solution onto the surface of leaves of mulberry, *Morus alba* (L) (M.5 Variety) leaf surface. The treated leaves were allowed for draining. The treated leaves were fed four times to the third instar larvae on the first day (100 grams of leaves for the group of hundred larvae for each time). For the second day and third day, the larvae were fed with normal untreated mulberry leaves. The water treated group of larvae was fed with mulberry leaves smeared with distilled water. The larvae of untreated control and antibiotics treated group were fed with normal untreated leaves for the days: first, second and third. The antibiotics treatment was followed on the fourth day of the third instar. Hundred grams of mulberry leaves were immersed in four hundred milliliter aqueous solution of Garamycin (40 microgam/ml distal water) for half an hour. The leaves were drained completely. The Garamycin treated leaves were used for the feeding on the fourth day (four feedings at the rate of 100 grams of leaves for the group of hundred larvae for each time). Thereafter, the larvae were fed with untreated mulberry leaves to all the groups of larvae of third, fourth and fifth instars. The haemolymph from the larvae (ten larvae from each group) was collected on the fifth day of the fifth instar and processed for electrophoresis. The hundred percent effective rate of rearing (ERR) were reported for the Garamycin treated group. Single female cocoon weight: 1.564 (±0.429) units with the shell ratio: 24.744 units and single male cocoon weight: 1.193 (±0.055) with the shell ratio: 22.967 units were reported for the Garamycin treated group. The variation was detected in the pattern of banding of the protein with significant polymorphism (88.3 percent) with two bands of monomorphic nature; twelve bands of polymorphic nature and three bands of "unique" nature.

Keywords: Antibiotics; Garamycin; Bacterial Flacherrie; *Bombyx mori*

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Introduction

Silk is considered as the most superior fiber for human protection. Therefore, the silk deserves economic significance. The larval instars of the silkworm, *Bombyx mori* (L) feed on the leaves of mulberry, *Morus alba* (L). The mature fifth instar larvae spin the silky cocoon around their body and enter into the pupal stage. The quality of silk depends on many factors like: the race of silkworm; the race of host plant (mulberry, *Morus alba*); health of individual instars in the life cycle; environmental conditions etc. There are several reports on attempts on the improvement of quality of health of larval instars of the silkworm, *Bombyx mori* (L) for the qualitative yield of the silk. Most of the attempts on this line are concerned with the management of control of the diseases of silkworm and it's host plant mulberry. According to Taha [1], the bacterial flacherrie is the most common disease of larval instars of the mulberry silkworm. The bacterial infection in the larval instars of the silkworm, *Bombyx mori* (L) deserves multiplicity [2]. The pathological knowledge on microbial diseases in silkworm is vast area and it should be fully understood.

According to Tanda and Kaya [3], the loss in appetite; discharge of watery fecal matter (diarrhea) (discharge of watery feces) and vomiting are the significantly common symptoms of infection of bacteria to the larval instars of the silkworm, *Bombyx mori* (L). Then, the larval body wall starts for softening and die emitting a foul odor. The only available practice at present is to discard large stocks of infected to avoid the spread of disease. There are no specific preventive methods known to the Indian sericulturist farmers. According to Acharya., *et al*. [4], sanitizations of the rearing room and rearing beds are going to help to avoid the spread of disease. Adoption of methods of the "Prophylactic and Curative" category should be aimed for the management of the control of the microbial diseases in the mulberry silkworm, *Bombyx mori* (L) is the prime concern for sericulturist farmers. The disease control methods should be aimed at taking into account the eco-friendly nature and cost-effectiveness. According to Subramanian., *et al.* [5], the sericulture should be with the use of the antibiotics as a component of bed disinfectants. The antibiotics are used as therapeutic applications against bacterial diseases. In the sericulture industries, the silk productivity and the silk quality mostly depend on the health of larval instars of the silkworm; quality of mulberry leaves used for feeding; the growth of the silkworm larvae and the favorable conditions of the environment. The physiological process within the body of larval instars of the silkworm, *Bombyx mori* (L) is contributing to growth and development. Improvement in the methods of rearing; quality of the mulberry leaves (nutrition) and upkeep of the health of larval instars of the silkworm is going to orchestrate the progression of sericulture practice towards the improvement of silk quality. The silkworm larvae are highly susceptible to the infection through significant group of microbial pathogens. Attempt on the use of antibiotics for the control of bacterial diseases in silkworm, *Bombyx mori* (L) is not new. The use of antibiotics (examples: penicillin, streptomycin, tetracycline and chloramphenicol) for the control of bacterial diseases in silkworm, *Bombyx mori* (L) was already reported. According to Venkatesh and Srivastava [6], the use of antibiotics (examples: penicillin, streptomycin, tetracycline and chloramphenicol) for the control of bacterial diseases in silkworm, *Bombyx mori* (L) was found successful. Recently, Aarti Sanjay Dhumal., *et al*. [7] of Dr. APIS of Baramati reported the significant improvement in the haemolymph proteins through the use of norfloxacin antibiotics for treating the leaves of mulberry, *Morus alba* (L) and feeding to the fifth instar larvae of silkworm, *Bombyx mori* (L). The content of the total protein in the silk glands; the fat body tissues and the haemolymph was reported for the improvement (61.519 to 114.667; 79.928 to 90.055 and 30.983 to 31.010 percents, respectively) in the attempt of the feeding the fifth instars of the silkworm, *Bombyx mori* (L) (Double Hybrid Race) with the mulberry leaves treated with aqueous solution of Norfloxacin antibiotics [7].

The use of antibiotics in silkworm rearing is allowed for four reasons. These reasons (purposes of use of antibiotics for the rearing of larval instars of the silkworm) include: treating the diseased larvae with antibiotics stops the heavy loss through the bacterial diseases in silkworms; antibiotic treatment prevents the larvae from diseases; antibiotic treatment control diseases in silkworms; antibiotic treatment help for health maintenance and promotion of growth of silkworms. For the innate immunity in silkworms, the haemolymph deserves a key role. According to Hou., *et al*. [8], the response of the body of larva (through the innate immunity) in the form of triggered through the entry of microbial pathogens. Tanaka and Yamakawa [9] opined the effective innate immunity system in the body of insects against foreign microbial pathogens. The humoral reactions and cellular reactions are the two significant types in the body of insects for the innate immunity response. Humoral reactions involve soluble proteins in the hemolymph such as The production of soluble proteins like an enzyme (ex. Phenoloxidase); anti-microbial protein (AMP); the lysozymes and the lectins in the

are the examples for humoral reactions in insects like, silkworm, *Bombyx mori* (L). The processes like phagocytosis, encapsulation and nodule formation are contributing as the cellular reactions in insects like, a silkworm, *Bombyx mori* (L). According to Jannatun Nesa., *et al*. [10], in the body of larval instars of the silkworm, *Bombyx mori* (L), there are six different groups of anti-microbial proteins (AMPs) and they include: the cecropin; the attacin; the lebocin; the moricin; the gloverin and the defensin. One lysozyme is reported in the silkworm, *Bombyx mori* (L). The three lysozyme-like proteins are reported in the silkworm, *Bombyx mori* (L). One of the lysozymes like protein is reported for involvement in elimination of invading microbial pathogens. There are no reports on use of Garamycin antibiotics for the management of bacterial diseases in of silkworm, *Bombyx mori* (L). Therefore, the present attempt was planned with the aim of enhancement of resistance in the body of silkworm, *Bombyx mori* (L) against the infections of bacterial pathogens through the use of Garamycin antibiotics.

Material and Methods

The attempt on the utilization of antibiotic compound, "Garamycin" for the control of bacterial disease: flacherrie in the larval instars of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) was carried out through the steps like: Silkworm larval stages [Race: (CSR6 x CSR26) x CSR2 x CSR27)] rearing; Bacterial isolation; Luria Agar (LA) medium preparation and bacterial culture; Infecting the larval instars of silkworm with bacteria and antibiotic treatment; Preparation of haemolymph Sample for the Protein; the qualitative analysis of the haemolymph proteins through the electrophoresis; Analysis of commercial parameters (characters of cocoons and silk filament) and Analysis of the data through the method of statistics.

Rearing of larval stages of the silkworm, *Bombyx mori* (L) [Race: Bivoltine Double Hybrid-(CSR6 x CSR26) x CSR2 x CSR27)]: Through the standard method prescribed by Krishnaswami and Krishnaswami., *et al*. [11,12] for the rearing of silkworm larvae appearing in the document authorized by V. B. Khyade [13] and Khyade abd Khyade [14]; Vitthalrao B. Khyade [15]; Khyade and Slama [16] and through the use of leaves of mulberry, *Morus alba* (L) (M.5 variety), the rearing of silkworm instars was carried out. The DFLs (disease-free layings) of double hybrid bivoltine race (CSR6 x CSR26) x CSR2 x CSR27) of the silkworm, *Bombyx mori* (L) were procured through the "Dr. APIS" Laboratory and processed for black boxing, rearing of early instars, rearing of late age instars, regular feeding with leaves of mulberry, provision of mountages for spinning the cocoon and cocoon harvesting through the standard methods.

Isolation of Bacteria Causing the Disease: Flacherrie in the Larval instars of the silkworm, *Bombyx mori* (L): Bacteria associated with the silkworm larvae of the fourth and fifth instars of silkworm were isolated according to two main techniques:- Instar have abnormal symptoms such as change in color into brown and black, cessation of feeding, loss of body luster, flaccidity, sluggishness and exiting of fluid from the anal lip and rectal protrusion. These larvae were removed and stored in sterilized tightly closed vials at 4°C in refrigerator until they had been needed for complete the isolation and identification techniques for associated bacteria. So, in the present investigation an attempt was made to isolate, characterize, describe and identify unknown bacteria associated with fourth and fifth larval instars of silkworm, using biochemical, morphological and physiological tests. In order to reveal any bacteria associated with the subjected larvae, each of the refrigerated individuals was examined through 24-72 h from the time of storage under aseptic conditions where the larvae were sterilized by dipping in 2% sodium hypochloride (10% commercial solution) for 3-5 min, then passed through five separate washing with sterile distilled water [17,18]. The solution on nutrient agar medium using the spread plate method and incubated at 30°C. Sterilized larvae were dried between two sterilized filter papers, cultured on nutrient agar medium in Petri-dishes of 9 cm. diameter, then incubated at 37°C for 24 h. Incubated dishes were daily observed inspected bacterial colonies which purified and stored on slants of EPPXOPRBTE media at 4°C and cultured periodically and used for the subsequent experiments. Healthy silkworm larvae were subjected to the same procedures of isolation for the expected dormancy of bacteria.

The method explained by Aneja [19] was utilized for the isolation of the bacteria causing the flacherrie disease in the larval instars of the silkworm, *Bombyx mori* (L). According to Tanda and Kaya [3], the loss in appetite; discharge of watery fecal matter (diarrhea) (discharge of watery feces) and vomiting are the significantly common symptoms of infection of bacteria to the larval instars of the silkworm, *Bombyx mori* (L). Then, the larval body wall starts for softening and die emitting a foul odor. The flacherrie diseased larval stages of the silkworm, *Bombyx mori* (L) exhibit the black thorax. This black thorax condition of the larval instars of the silkworm, *Bombyx mori* (L) is recognized as "Septicemia" in sericulture practice. The larval instars of silkworm, *Bombyx mori* (L)

exhibiting "Septicemia" were collected from the fifth instar bed of Malegaon Sheti Farm of Agricultural Development Trust, Baramati (India). The diseased larval instars of the silkworm, *Bombyx mori* (L) were crushed through the use of the little amount of distilled water; mortar and pestle. The aqueous solution was processed for filtration through the method of Levin., *et al.* [20]. In this method, the sample was passed through a Balston type AA cartridge filter (6.4 by 2.5 cm, 0.2-, micrometer pore size; Balston, Lexington, Mass.) under vacuum. The filter was then placed directly into a suitable culture medium for incubation. The filtrate was then processed for centrifugation (at 4000 - 5000 rpm) for ten minutes. According to Aneja [19], the supernatant should be discarded and the precipitate in the form of pellet should be used for further processing. Accordingly, the supernatant was discarded and the precipitate (in the form of the pellet) was used for bacterial culture after resuspending in distilled water.

Luria Agar (LA) Medium Preparation and the Preparation of Bacterial Culture: The sample of bacterial was streaked in LA under aseptic conditions in a laminar airflow chamber with the help of streaking loop. It was then incubated at 37ºC overnight. After twenty-four hours, the growth of bacteria was noticed. The system was then processed for further sub culture. The bacterial sample was taken away with the help of a loop. It was allowed for centrifugation (at 4000 rpm) for 15 minutes. The supernatant was discarded. The precipitate was in the form of pellet. It was sedimented at the bottom of the centrifuge tube. This pellet was dissolved in distilled water. The presence of bacteria was confirmed through the method explained by Suparna., *et al*. [21]. The basic stains: crystal violet and methylene blue were utilized for the confirmation of presence of the bacteria.

Infecting the Larval Instars of the silkworm, *Bombyx mori* (L) [Race: Bivoltine Double Hybrid -(CSR6 x CSR26) x CSR2 x CSR27)] with the Bacteria and Antibiotics Treatment: Through the standard method prescribed by Krishnaswamy and Krishnaswami., *et al*. [11,12] for the rearing of silkworm larvae appearing in the document authorized by V. B. Khyade [13] and Khyade and Khyade [14] and through the use of leaves of mulberry, *Morus alba* (L) (M.5 variety), the rearing of the silkworm, *Bombyx mori* (L) [Race: Bivoltine Double Hybrid -(CSR6 x CSR26) x CSR2 x CSR27)] instars was carried out. Soon after the second moult, the third instared larvae were divided into four groups: The untreated control group; the water treated group; the bacterial culture treated (infected) group and the group treated with *Garamycin antibiotics*, each with hundred individuals.

The untreated control group were fed with normal untreated leaves for the days: first, second, third and fourth (four feedings per day at the rate of hundred grams leaves for the group of hundred larvae for each feeding) (Table- 1: Schedule of feeding the larvae of silkworm, *Bombyx mori* (L) (Race: Double Hybrid).

The larvae of the water untreated control group were fed with water treated leaves for the first day (four feedings at the rate of hundred grams leaves for the group of hundred larvae for each feeding). For the second, third and fourth days, this group (water treated group of the larvae) was fed with normal untreated leaves (four feedings per day at the rate of hundred grams leaves for the group of hundred larvae for each feeding) (Table- 1: Schedule of feeding the larvae of silkworm, *Bombyx mori* (L) (Race: Double Hybrid).

The larvae of the bacterial culture *(Genus: Streptococcus bombycis*) treated (infected) group were fed with the bacterial culture treated leaves of mulberry for the first day (four feedings at the rate of hundred grams leaves for the group of hundred larvae for each feeding). For the second, third and fourth days, this group (bacterial culture treated group of the larvae) was fed with normal untreated leaves (four feedings per day at the rate of hundred grams leaves for the group of hundred larvae for each feeding). The treatment was carried out through smearing the solution of bacterial culture onto the surface of leaves of mulberry, *Morus alba* (L) (M.5 Variety) leaf surface. The treated leaves were allowed for draining and then used for feeding to the respective group of the larvae (Table- 1: Schedule of feeding the larvae of silkworm, *Bombyx mori* (L) (Race: Double Hybrid).

The group of larvae of antibiotic treatment was fed with the bacterial culture treated leaves of mulberry for the first day (four feedings at the rate of hundred grams leaves for the group of hundred larvae for each feeding). For the second and third days, this group (antibiotics treatment group of the larvae) was fed with normal untreated leaves (four feedings per day at the rate of hundred grams leaves for the group of hundred larvae for each feeding).

The antibiotics treatment was followed on the fourth day of the third instar. A hundred grams of mulberry leaves were immersed in four hundred milliliter aqueous solution of Garamycin (40 mi-

crogams/ml distal water) for half an hour. The leaves were drained completely. The Garamycin treated leaves were used for the feeding on the fourth day of the third in stared larvae of the group of antibiotic treatment (four feedings at the rate of hundred grams of leaves for the group of hundred larvae for each time).

Group	Nature of Leaves Fed Day	U.T	W.T	B. C. T.	A. T.
I	1	+			
I	2	+			
I	3	+			
I	4	+			
II	1		+		
II	2	+			
II	3	+			
II	4	+			
III	1			+	
III	2	+			
III	3	+			
III	4	+			
IV	1			+	
IV	2	+			
IV	3	+			
IV	4				+

Table 1: Schedule of feeding the larvae of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid).

Thereafter, the larvae of all the groups (in the third instar, fourth instar and fifth instar) were fed with normal untreated mulberry leaves of mulberry.

Preparation of Haemolymph Sample for the Protein: Preparation of the haemolymph sample for the protein analysis was carried out according to method described by V.N.Yogananda Murthy [22]. Preparation of the haemolymph sample for the protein analysis was carried on the fifth day of the fifth instar. Ten larvae from each group were selected randomly. The hemolymph was collected from larvae (on the fifth day of the fifth instar). The abdominal legs were cut through the use of sterilized scissor. The haemolymph from each group was collected separately in an eppendrof tube. An equal volume of buffer solution (Tris buffer with pH 6.8) was taken. The buffer solution was mixed in the haemolymph sample. Thus, the dilution of the haemolymph was carried out. From each group of the larvae in the attempt, fifteen microliters diluted (with Tris buffer solution pH 6.8) haemolymph sample was taken in another eppendrof tube. The haemolymph sample in the eppendrof tube was mixed thoroughly. This mixing was carried out through the use of the vertex. The sample solution was heated in boiling water for 2-3 min to ensure complete interaction between proteins and SDS, and a pinch of Bromophenol blue is used as a tracking dye.

The Qualitative Analysis of the Haemolymph Proteins through the Electrophoresis: The qualitative pattern of proteins in the haemolymph of the fifth in stared larvae of silkworm, *Bombyx mori* (L) (Race: Double Hybrid) were analyzed by sodium dodecylsulfate (SDS) polyacrylamide slab gels through the use of method explained by Laemmli [23]. The method of Laemmli [23] is dealing with a discontinuous system of sodium dodecylsulfate (SDS) system. This method is the most widely used in electrophoretic system in recent times. The treated peptides are concentrated in a stacking gel before gets entered into the separating gel. Therefore, the method of Laemmli gel deserves excellent quality of resolution.

The SDS-PAGE using 4% stacking gel and 10% separating gel was performed under denaturing condition. The electrophoresis of the assay sample was carried out in the four percent stacking gel and ten percent separating gel. The gel was allowed to run at 150V for about ten to twenty minutes until the tracking dye moved till the end of the gel. After the completion of the electrophoretic run, the gel was removed and it was stained with the Coomassie Brilliant Blue for 45 min^{-1}hr. Standard molecular weight markers were used for estimating the molecular weight.

Analysis of commercial parameters (characters of cocoons): The method explained by V. B. Khyade [13] was followed for the analysis of commercial parameters (characters of cocoons). The cocoons were harvested (separated from the mountage) on the sixth day after the provision of mountage for spinning. Fifty cocoons from each group were selected randomly. The weight of each individual cocoon was recorded. Each individual cocoon was deflossed. The weight of individual deflossed cocoon was recorded. Each cocoon in particular group was cut vertically using the blade. The weight of individual silk shell from individual cocoon was recorded. For knowing the weight of the pupa within the individual cocoon, the reading of the weight of the shell of respective individual cocoon was subtracted from weight of respective individual deflossed cocoon. Weight of entire deflossed cocoon; the weight of the silk

shell of individual cocoon and the weight of pupa from individual cocoon were noted. The shell ratio of the cocoon is commercial or economic parameter. The shell ratio is the percentage of content of silk within the individual entire cocoon. The silk shell percentage (correctly called as shell ratio) was calculated through the use of readings of weight of the whole deflossed cocoon and the weight of silk shell in cocoon. The reading of silk shell weight was divided by reading of the weight of whole cocoon without floss. The quotient thus obtained was processed for multiplication with hundred. Shell ratio or shell percentage is the outcome of this attempt. In sericulture, this silk shell percentage is called as the shell ratio. Sericulture farmers get the price for the cocoon yield on the basis of "Shell Ratio".

Statistical Analysis of the data: The present attempts in experimentation were repeated for three times for consistency in the results. The data, in the form of mean, standard deviation and percent change was collected. This data was subjected for statistical analysis. The statistical parameters considered in the attempt include: mean, standard deviation, percent variation and student "t" – test. The data, in the form of mean, standard deviation and percent change was collected. This data was subjected for statistical analysis. The statistical parameters considered in the attempt include: mean, standard deviation, percent variation and student "t" - test [24].

Results and Discussion

The results on the attempt on the utilization of antibiotic compound, Garamycin for the control of bacterial disease: flacherrie in the larval instars of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] are summarized in table 2-4 and presented in figure 1 and 2.

The 98.786 percent of effective rate of rearing (ERR) was reported for the untreated control group and water-treated control group. The 81.431 percent of effective rate of rearing (ERR) was reported for the group of larvae fed with mulberry leaves treated with solution of bacterial culture. The highest (100 hundred percent) effective rate of rearing (ERR) was recorded for the group of larvae of antibiotics treatment (Table 2).

The larval life (duration in hours) of the untreated control group; water-treated control group; group infected with bacterial culture and the group of larvae infected with bacterial culture followed by antibiotics treatment were recorded: 792 (± 9.265); 792 (± 11.395); 916 (± 13.394) and 792 (± 14.161) respectively (Table 2).

The weight (gm) of entire deflossed female cocoon; weight (gm) of female silk shell (gm) and the weight (gm) of female pupa (gm) of the untreated control group were measured 1.413 (±0.211); 0.259 (±0.052) and 1.154 respectively (Figure 1A). The ratio of female silk shell to the entire cocoon in the group of "untreated control" was recorded 18.329 (Table 2) (Figure 1B).

The weight (gm) of entire deflossed female cocoon; weight (gm) of female silk shell (gm) and the weight (gm) of female pupa (gm) of the water treated control group were measured 1.413 (±0.263); 0.259 (±0.078) and 1.154 respectively (Figure 1A). The ratio of female silk shell to the entire cocoon in the group of "water treated control" was recorded 18.329 (Table 2) (Figure 1B).

The weight (gm) of entire deflossed female cocoon; weight (gm) of female silk shell (gm) and the weight (gm) of female pupa (gm) of the bacterial cultured treated group were measured 1.167 (±0.269); 0.198 (±0.057) and 0.969 respectively (Figure 1A). The ratio of female silk shell to the entire cocoon in the group of "bacterial cultured treated" was recorded 16.966 (Table 2) (Figure 1B).

The weight (gm) of entire deflossed female cocoon; weight (gm) of female silk shell (gm) and the weight (gm) of female pupa (gm) of the group of the bacterial cultured treated followed by the treatment with antibiotics (Garamycin) group were measured 1.564 (±0.429); 0.387 (±0.059) and 1.177 respectively (Figure 1A). The ratio of female silk shell to the entire cocoon in the group of "bacterial cultured treated followed by the treatment with antibiotics (Garamycin)" was recorded 24.744 (Table 2) (Figure 1B).

The weight (gm) of entire deflossed male cocoon; weight (gm) of male silk shell (gm) and the weight (gm) of male pupa (gm) of the untreated control group were measured 1.158 (±0.053); 0.238 (±0.019) and 0.920 respectively (Figure 1A). The ratio of male silk shell to the entire cocoon in the group of "untreated control" was recorded 20.552 (Table 2) (Figure 1B).

The weight (gm) of entire deflossed male cocoon; weight (gm) of male silk shell (gm) and the weight (gm) of male pupa (gm) of the water-treated control group were measured 1.158 (±0.069); 0.238 (±0.098) and 0.920 respectively (Figure 1A). The ratio of male silk shell to the entire cocoon in the group of "water treated control" was recorded 20.552 (Table 2) (Figure 1B).

Groups Parameters	Untreated Control	Water Treated Control	Bacterial Culture Treated Group (Inoculated with Bacterial Culture)	Bacterial Culture Treated (Inoculated with Bacterial Culture) followed by Antibiotic Treatment group
Larval Life Duration (Hours)	792 b (±9.265) 00.000	792 (±11.395) 00.000	916 b (±13.394) 00.000	792 b (±14.161) 00.000
Effective Rate of Rearing (Percentage)	98.786 ab	98.786 ab	81.431 b	100 a
Female cocoon weight (Grams)	1.413 (±0.211) 00.000	1.413 (±0.263) 00.000	1.167 (±0.269) 82.590	1.564 (±0.429) 10.686
Female cocoon shell weight (Grams)	0.259 (±0.052) 00.000	0.259 (±0.078) 00.000	0.198 (±0.055) 76.447	0.387 (±0.059) 149.42
Female Shell Ratio	18.329	18.329	16.966	24.744
Male cocoon weight (Grams)	1.158 (±0.053) 00.000	1.158 (±0.069) 00.000	0.786 (±0.057) 67.875	1.713 (±0.055) 147.92
Male cocoon shell weight (Grams)	0.208 (±0.019) 00.000	0.208 (±0.098) 00.000	0.133 (±0.018) 63.942	0.381 (±0.023) 183.17
Male Shell Ratio	17.962	17.962	16.921	22.241

Table 2: The economical parameters in the silkworm, *Bombyx mori* (L) (Race: Bivoltine Double Hybrid) fed with the leaves of mulberry, *Morus alba* (L) (M.5 Variety) treated with the aqueous solution of Garamycin antibiotics.

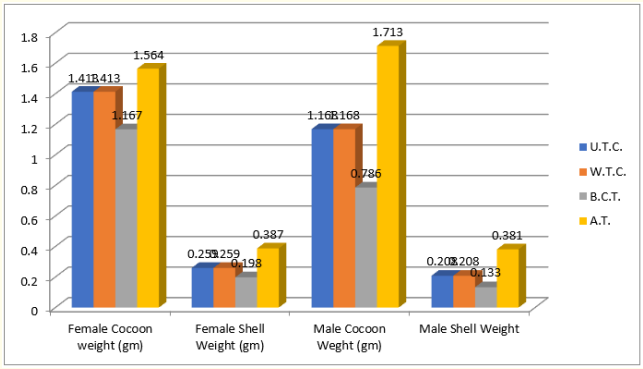

Figure 1A: The economical parameters in the silkworm, *Bombyx mori* (L) (Race: Bivoltine Double Hybrid) fed with the leaves of mulberry, *Morus alba* (L) (M.5 Variety) treated with the aqueous solution of Garamycin antibiotics. (U.T.C.: Untreated control; W.T.C.: Water treated control; B.C.T.: Bacterial culture treated; A.T.: Antibiotic treated).

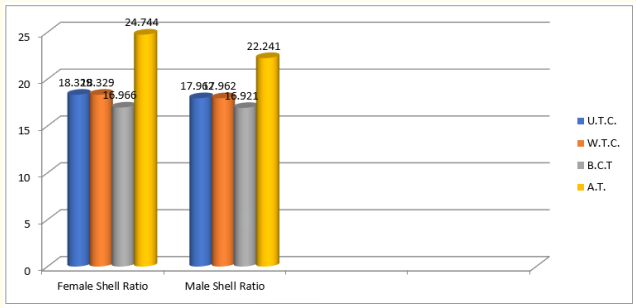

Figure 1B: The economical parameters in the silkworm, *Bombyx mori* (L) (Race: Bivoltine Double Hybrid) fed with the leaves of mulberry, *Morus alba* (L) (M.5 Variety) treated with the aqueous solution of Garamycin antibiotics. (U.T.C.: Untreated control; W.T.C.: Water treated control; B.C.T.: Bacterial culture treated; A.T.: Antibiotic treated).

The weight (gm) of entire deflossed male cocoon; weight (gm) of male silk shell (gm) and the weight (gm) of male pupa (gm) of the bacterial cultured treated group were measured 1.074 (±0.055); 0.221 (±0.018) and 0.853 respectively (Figure 1A). The ratio of male silk shell to the entire cocoon in the group of "bacterial cultured treated" was recorded 20.577 (Table 2) (Figure 1B).

The weight (gm) of entire deflossed male cocoon; weight (gm) of male silk shell (gm) and the weight (gm) of male pupa (gm) of the group of the bacterial cultured treated followed by the treatment with antibiotics (Garamycin) group were measured 1.193 (±0.059); 0.274 (±0.021) and 0.919 respectively (Figure 1A). The ratio of male silk shell to the entire cocoon in the group of "bacterial cultured treated followed by the treatment with antibiotics (Garamycin)" was recorded 22.967 (Table 2) (Figure 1B).

The sole aim in sericulture through the rearing the larval instars of silkworm, *Bombyx mori* (L) is production of superior silky cocoons (qualitatively and quantitatively). According to Aarati Dhumal., *et al*. (2019), there is infection to the larval instars of the silkworm through some microbial pathogens. The microbial infection to the larval instars of the silkworm is caused by various biological, chemical, physical, nutritional and environmental factors. Aarati Dhumal., *et al*. (2019) [17] listed the favorable factors for the infection to the larval instars of the silkworm through several microbial pathogens, which include: the wrong methods of silkworm rearing; the low nutritional quality of leaves of mulberry, *Morus alba* (L) and the ill health of silkworm. These favorable factors are helping the microbial pathogens for rapid multiplication and contribute to the significant loss of yield of cocoon crop. The Indian practices in sericulture reported the annual crop loss through the microbial pathogens in larval instars of the silkworm, *Bombyx mori* (L). In the body of poikilotherm animals, internal temperature exhibit significant variations. In the body of poikilotherm animals the situation is exactly opposite to that of homeotherm animals (an animal that maintains thermal homeostasis). Silkworm, *Bombyx mori* (L) belongs to the "Poikilotherm" group. The larval instars of the silkworm, *Bombyx mori* (L) use to respond very quickly for the changes in the environment. The temperature and the relative humidity are the environmental factors that use to affect the quality of life of the larval instars of the silkworm, *Bombyx mori* (L). The environmental condition of higher or lower temperature and humidity, ventilation and quality of food material exert adverse influence on the physiological functions of the silkworm, *Bombyx mori* (L). The larval instars of the silkworm, *Bombyx mori* (L) become highly susceptible to diseases.

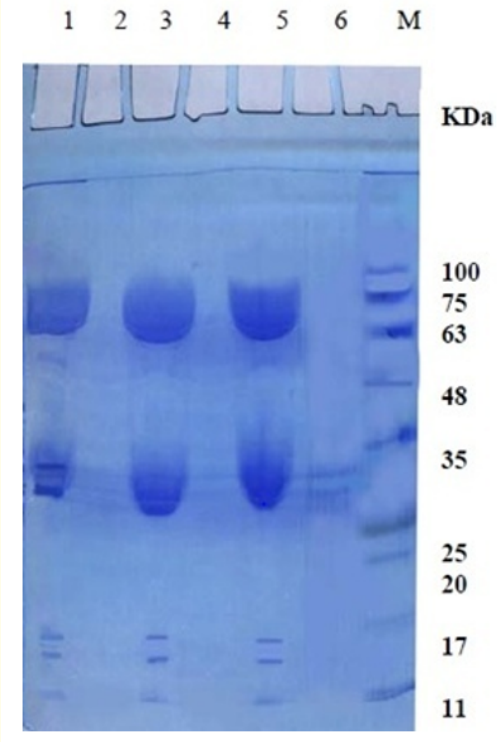

Figure 2: The electrophoretic pattern of the haemolymph proteins [M: molecular weights of marker protein (KDa); Lane-1: The protein of haemolymph sample from untreated healthy larvae of control group; Lane-2: The protein of haemolymph sample from flacherrie diseased larvae of untreated control group; Lane-3: The protein of haemolymph sample from healthy larvae of infected (Bacterial culture treated) group; Lane-4: The protein of haemolymph sample from flacherrie diseased larvae of infected (Bacterial culture treated) group; Lane-5: The protein of haemolymph sample from healthy larvae of antibiotics treated group. Lane-6: The protein of haemolymph sample from flacherrie diseased larvae of antibiotics treated group].

The electrophoretic pattern of the haemolymph samples of the larvae in the attempt is presented in figure – 2. It demonstrates the SDS-protein profile of the haemolymphal samples from the larvae in the attempt. The lane-1 is dealing with the pattern of the haemolymph proteins of sample from healthy larvae of untreated group (Figure 2). The lane-2 is dealing with the pattern of the protein of haemolymph sample from flacherrie diseased larvae of the untreated control group (Figure 2). The lane-3 is dealing with the pattern of the protein of haemolymph sample from from healthy larvae of infected (Bacterial culture treated) group (Figure 2). The

lane-4 is dealing with the pattern of the protein of haemolymph sample from flacherrie diseased larvae of infected (Bacterial culture treated) group (Figure 2). The lane-5 is dealing with the pattern of the protein of haemolymph sample from healthy larvae of antibiotics treated group (Figure 2). The lane-6 is dealing with the pattern of the protein of haemolymph sample from flacherrie diseased larvae of antibiotics treated group (Figure 2).

The table 3 and the table 4 are dealing with the computer analysis of the bands of proteins. The results reported presence (+) and or absence (-) of the protein bands. The electrophoretic pattern of the protein of haemolymph sample from untreated healthy larvae of control group; the protein of haemolymph sample from flacherrie diseased larvae of untreated control group; the protein of haemolymph sample from healthy larvae of infected (Bacterial culture treated) group; the protein of haemolymph sample from flacherrie diseased larvae of infected (Bacterial culture treated) group; the protein of haemolymph sample from healthy larvae of antibiotics treated group and the protein of haemolymph sample from flacherrie diseased larvae of antibiotics treated group exhibited a total of fourteen bands of the proteins.

The electrophoretic pattern of haemolymph samples of the "Healthy Larvae" of the untreated control group, the "Healthy Larvae" of infected (bacterial culture treated) group and "Healthy Larvae" of antibiotics treated group exhibited total "Fourteen" protein bands with molecular weight as listed here: 68.30; 65.09; 54.51; 52.07; 49.08; 45.04; 42.32; 41.44; 31.82; 29.77; 27.77; 14.25; 12.85 and 10.56 KDa, respectively (Lane-1, 3 and 5; Figure 2).

The electrophoretic pattern of haemolymph samples of the "Flacherrie Diseased Larvae" of the untreated control group exhibited total "Eight" protein bands with molecular weight as listed here: 68.30; 65.09; 54.51; 52.07; 45.04; 31.82; 29.77; and 28.97 KDa, respectively (Lane-2; Figure 2).

The electrophoretic pattern of the haemolymph samples of from flacherrie diseased larvae of infected (Bacterial culture treated) group exhibited total "Seven" protein bands with molecular weight as listed here: 68.30; 65.09; 54.51; 52.07; 45.04; 31.82 and 29.77 KDa, respectively (Lane-4; Figure 2).

The electrophoretic pattern of the haemolymph samples of from flacherrie diseased larvae of antibiotics treatment group exhibited total "Five" protein bands with molecular weight as listed here: 37.02; 36.13; 31.82; 29.75 and 27.77 KDa, respectively (Lane-6; Figure 2).

Totally, seventeen bands of the protein were reported in the screened (tested) samples of haemolymph of fifth instar larvae of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] with polymorphism of 88.24 percent (Table 4).

Two monomorphic bands of the protein were reported in the screened (tested) samples of haemolymph of the fifth instar larvae of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] were reported to be recognized (Table 4).

The twelve bands of the protein reported in the screened (tested) samples of haemolymph of fifth instar larvae of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] were considered as polymorphic (Table 4).

The three bands of the protein reported in the screened (tested) samples of haemolymph of fifth instar larvae of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] were considered as "Unique" (Table 4).

The polymorphism in the profile of the proteins in the haemolymph of the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] detected in present attempt could be attributed to some of the stress conditions of environment [25]. It may also be due to the events of mutation. According to Rottenberg., *et al*. [26], mutation is to alter the performance of the proteins through their encoding genes. The percentage of polymorphism resulted in the present attempt could support the issue opined by Rottenberg., *et al*. [26].

The two unique bands (with the molecular weight: 37.02 and 36.13 KDa) of the haemolymph proteins appeared in the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] in the present attempt belongs to the larvae diseased with bacterial flacherrie (for both control and treated groups).

The one unique band (with the molecular weight: 28.97 KDa) of the haemolymph proteins appeared in the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] in the present attempt belongs to the larvae diseased with bacterial flacherrie (in control group).

MW Band	Lane:1	Lane:2	Lane:3	Lane:4	Lane:5	Lane:6	Marker
Band:1	68.30	68.30	68.30	68.30	68.30	37.02	87.76
Band:2	65.09	65.09	65.09	65.09	65.09	36.13	77.65
Band:3	54.51	54.51	54.51	54.51	54.51	31.82	65.44
Band:4	52.07	52.07	52.07	52.07	52.07	29.75	50.03
Band:5	49.08	45.04	49.08	45.04	49.08	27.77	37.32
Band:6	45.04	31.82	45.04	31.82	45.04	-	24.41
Band:7	42.32	29.77	42.32	29.77	42.32	-	21.27
Band:8	41.44	28.97	41.44	-	41.44	-	15.72
Band:9	31.82	-	31.82	-	31.82	-	10.89
Band:10	29.75	-	29.75	-	29.75	-	-
Band:11	27.77	-	27.77	-	27.77	-	-
Band:12	14.25	-	14.25	-	14.25	-	-
Band:13	12.85	-	12.85	-	12.85	-	-
Band:14	10.56	-	10.56	-	10.56	-	-

Table 3: The molecular weight of the different protein bands.

MW	Lane:1	Lane:2	Lane:3	Lane:4	Lane:5	Lane:6	Polymorphism
68.30	+	+	+	+	+	-	Polymorphic
65.09	+	+	+	+	+	-	Polymorphic
54.51	+	+	+	+	+	-	Polymorphic
52.07	+	+	+	+	+	-	Polymorphic
49.08	+	-	+	-	+	-	Polymorphic
45.04	+	+	+	+	+	-	Polymorphic
42.32	+	-	+	-	+	-	Polymorphic
41.44	+	-	+	-	+	-	Polymorphic
37.02	-	-	-	-	-	+	Unique
36.13	-	-	-	-	-	+	Unique
31.82	+	+	+	+	+	+	Monomorphic
29.75	+	+	+	+	+	+	Monomorphic
28.97	-	+	-	-	-	-	Unique
27.77	+	-	+	-	+	+	Polymorphic
14.25	+	-	+	-	+	-	Polymorphic
12.85	+	-	+	-	+	-	Polymorphic
10.56	+	-	+	-	+	-	Polymorphic

Table 4: The presence (+), absence (-) of bands and type of bands in all tested haemolymph samples.

The appearance of unique bands of the haemolymph proteins appeared in the silkworm, *Bombyx mori* (L) (Race: Double Hybrid) [Race: Double Hybrid - (CSR6 x CSR26) x CSR2 x CSR27)] in the diseased larvae in the present attempt may be related to the proteins of the immune system. Feeding the silkworm with mulberry leaves treated with bacterial culture (the bacterial infection) may be used

for surveying the genome of the host (silkworm larvae) as significant reactions (or response). The most significant reactions (or the response) by the larval instars of the silkworm, *Bombyx mori* (L) for feeding them with mulberry leaves treated with bacterial culture (the bacterial infection) is the "Innate Immune Response" to the microbial pathogen at the level of transcription. The provision of "Another Detailed Comprehension of the Interaction between the Pathogen and the Host" is supposed to be one more expected reaction (or the response) by the larval instars of the silkworm, *Bombyx mori* (L) for feeding them with mulberry leaves treated with bacterial culture (the bacterial infection). A lot of "Basal Metabolic Pathways" were modulated significantly. According to Huang., *et al*. [27], the genes with reference to the poisoning are also regulated. Further, the genes with reference to the poisoning might be with a key role to control (naturally) the bacterial septicemia disease in the silkworm, *Bombyx mori* (L).

The antimicrobial proteins (AMPs) and the lysozymes are produced in the body of the silkworm, *Bombyx mori* (L) as a reaction to the infection of microbial pathogen. The antimicrobial proteins (AMPs) and the lysozymes are rapidly produced firstly in the fat body (FB). They are subsequently secreted into the haemolymph. The purpose of release of the antimicrobial proteins (AMPs) and the lysozymes is elimination of invading microbial pathogens. Tanaka and Yamakawa [9] listed six groups of the antimicrobial proteins (AMPs) in silkworm, *Bombyx mori* (L) and they include: the cecropin; the attacin; the lebocin; the moricin; gloverin and the defensin. These antimicrobial proteins (AMPs) in silkworm, *Bombyx mori* (L) are supposed to be up-regulated at 24 hours post the infection. In the present attempt of study, there is a common band in all tested haemolymph samples with molecular weight of 29.75 ≈ 30 KDa. Fujiwara and Yamashita (1992) named this protein type as the "Haemolymph Protein" or the "Bomyx mori Larval Serum Protein (BmLSP). This, "Haemolymph Protein" or the "Bomyx mori Larval Serum Protein (BmLSP) is with two hundred sixty two amino acid residues. According to Izumi., *et al*. [28], the "Haemolymph Protein" or the "Bomyx mori Larval Serum Protein (BmLSP) is a group of structurally related proteins, entitled, "30 K Proteins". This is because of their approximate molecular weights of 30 KDa. The "30 K Proteins" were found to be stored in the haemolymph of larval instars of silkworms in a stage dependent fashion. The "30 K Proteins" distinguished by their minimal detectable feature. The "30 K Proteins" appear in the haemolymph before the third day of the fifth instars of silkworm, *Bombyx mori* (L). The "30 K Proteins" becomes the major proteins in the haemolymph at the early stage of the pupa within the silky shell. This may be due to their progressive increase in expression after the third day of fifth instared larvae of silkworm, *Bombyx mori* (L). The efforts Kim., *et al*. [29] get resulted for the identification of the "30 K Proteins" as a component of an anti-apoptotic system. Kim., *et al*. [29] reported inhibition of poptosis in the instars of silkworm, *Bombyx mori* (L) by the novel "30 K Proteins". It inhibited the virus or chemical-induced The apoptosis in human cells and the insect cells are reported for inhibition through the action of "30 K Proteins". Kim., *et al*. [29] recommend effective (and efficient too) utilization of the "30 K Proteins" for minimizing the death of cells and to increase the productivity through extending the time of production in host cells in the animal cell culture. The efforts of Naletova., *et al*. [30] are concerned with the identification of the protein with the molecular weight of 69 KDa as the carboxylesterase. The carboxylesterase is the enzyme with the antigenic activity. In the present attempt the protein with molecular weight 68.30 KDa is observed in all tested haemolymph samples (except the flacherrie diseased haemolymph sample from antibiotics treatment group). According to Nakahara., *et al*. [31], the protein with molecular weight of 49.08 KDa may be the paralytic peptide binding protein with 421 amino acid residues. According to Tanaka and Yamakawa [9], this 49.08 KDa protein deserve the significant role in the immunity system in silkworms. According to Kaito., *et al*. [32], the antibiotic compounds are used clinically for the human health and also they have therapeutic effects against silkworms injected with *Staphylococcus aureus* and *Pseudomonas aeruginosa* (L). The attempts of Hossain., *et al.* (2006) were concerned with the estimation of the bacterial exotoxins with the capability of killing the silkworms. The fifty percent lethal dosage (LD50) of the staphylococcal alpha-toxin is 12 μg/g. The fifty percent lethal dosage (LD50) of the staphylococcal beta-toxin is 9 μg/g. The fifty percent lethal dosage (LD50) of the *Pseudomonas* exotoxin A is 0.14 μg/g. The fifty percent lethal dosage (LD50) of the diphtheria toxin is 1.1 μg/g. Most of the fifty percent lethal dosage (LD50) values obtained in silkworm, *Bombyx mori* (L) were similar to that reported fifty percent lethal dosage (LD50) values in mice. This is suggesting that, silkworms can be utilized as a model animal to study the general effects of the bacterial exotoxins on multicellular organisms, including human beings. If we agree the opinion of Yamakawa and Tanaka [9], we are free for a broad range of expectations from the studies on immunity system of silkworm for the contribution for the field of medicine, in the field of production of antibiotic compounds based on the

bacterial proteins. The field of agriculture is also waiting for the establishment of the transgenic crops (at least in sericulture) with antimicrobial gene proteins through such attempts.

Conclusion

The hundred percent effective rate of rearing (ERR) were reported for the Garamycin treated group. Single female cocoon weight: 1.564 (± 0.429) units with the shell ratio: 24.744 units and single male cocoon weight: 1.193 (± 0.055) with the shell ratio: 22.967 units were reported for the Garamycin treated group. The variation was detected in the pattern of banding of the protein with significant polymorphism (88.3 percent) with two bands of monomorphic nature; twelve bands of polymorphic nature and three bands of "unique" nature. Feeding the silkworm with mulberry leaves treated with bacterial culture (the bacterial infection) may be used for surveying the genome of the host (silkworm larvae) as significant reactions (or response). The most significant reactions (or the response) by the larval instars of the silkworm, *Bombyx mori* (L) for feeding them with mulberry leaves treated with bacterial culture (the bacterial infection) is the "Innate Immune Response" to the microbial pathogen at the level of transcription. The provision of "Another Detailed Comprehension of the Interaction between the Pathogen and the Host" is supposed to be one more expected reaction (or the response) by the larval instars of the silkworm, *Bombyx mori* (L) for feeding them with mulberry leaves treated with bacterial culture (the bacterial infection). A lot of "Basal Metabolic Pathways" were modulated significantly.

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