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Asessment of antibacterial activity of bacteria immunized Muga silkworm (*Antheraea assamensis* Helfer) and its comparison with market antibiotics

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Abstract

The Muga silkworm, *Antheraea assamensis* Helfer, which plays a huge role in the socio-economic scenario of Assam is susceptible to a wide range of infections due to the outdoor rearing of these silkworms. Infection or injury triggers the immune response of their body, which involves several components including the production of some antimicrobial proteins for defense against the invading microorganism. The current study deals with the immunization of the Muga silkworms with *Bacillus thuringiensis* to observe the response of the activated haemolymph against *E. coli* and *B. thuringiensis*. Analysis was carried out to compare the antibacterial activity of the immunized haemolymph with that of three common market antibiotics, Levofloxacin, Azithromycin and Rifaximin. After immunization, haemolymph was procured at different time intervals i,e 6 hours, 12 hours, 18 hours and 24 hours and each time interval was taken as a different group along with one control group. Analysis for total protein and free amino acid for all groups were carried out which showed increase in protein and free amino acid concentration in all groups as compared to the control haemolymph samples. No antimicrobial action was shown by the samples obtained at 6 hours, 12 hours and 18 hours with only the immunized haemolymph obtained after 24 hours showed positive activity against both *B. thuringiensis* as well as *E.coli* along with potency almost at par with the antibiotics taken for the study. Thus, the study revealed clear roles of bacteria immunized haemolymph to fight against infections and proper characterization of the components of the immunized haemolymph might lead to more specific understanding of such immune mechanisms as well as their role as future antibiotics.

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Introduction

Among all organisms, it is well known that the largest and most successful groups worldwide are the insects (Saito *et al.,* 2004). This can be attributed to their powerful defense system and they have the capacity to elicit both innate and humoral immune responses against different types of pathogens (Lavine and Strand, 2001, 2003). Their innate mechanism mainly includes processes of eliminating the pathogen involving encapsulation and phagocytosis of parasites mediated by the haemocytes (Riberio and Brehelin, 2006). In contrast, the humoral immune response majorly involves the generation and release of antimicrobial proteins (Lavine and Strand, 2002). Generation of such antimicrobial proteins (AMPs) plays a very important role in fighting against the invading microorganism. These proteins or peptides secreted by a wide variety of insects have low molecular weight and are heat stable and typically cationic (Wang and Lai, 2010).

Mechanism of immunity and defense has not been much explored in case of the Muga silkworm, *Antheraea assamensis* Helfer, that belongs to the order Lepidoptera and family Saturniidae. The polyphagous insect, Muga silkworm feeds primarily on Som (*Persea bombycine*) but in case of unavailability of primary host plants, it also feeds on Dighloti (*L. salicifolia*), Soalu (*Litsea monopetala*), and Mejankari (*L. citrata*) (Neog *et al.,* 2005). Due to the outdoor rearing of Muga silkworms, they are highly susceptible to a wide range of infections caused by microorganisms like bacteria, fungi, virus, protozoans etc. Among these, infections resulting due to various bacterial strains often collectively called 'flacherie' is responsible for the highest amount of loss of silkworms (Singh *et al.,* 2013). Various causative organisms have been known to cause flacherie, one among them being Bacillus thuringiensis. (Haloi *et al.,* 2015)

Numerous drugs with potent antibacterial activity have been used to cure or minimize these infections; however, excessive use of these drugs has promoted the development of various treatment resistant bacterial strains (Tacconelli *et al.,* 2018). Furthermore, most of these conventional drugs emerged during 1940s to 1970s, after which new drugs were only formed by chemically modifying these pre-existing molecules (Silver, 2011). Therefore, new bioactive compounds having potent antibacterial activity seem to be the need of the hour that might be an answer to multi drug resistance and hence the search for such compounds continues in various sources.

It was established that these Muga silkworms show natural resistance against some bacterial strains, thereby hinting the presence of some proteins in these worms capable of fighting against the invading bacteria. After infection or injury, just like in case of other insects, these bactericidal proteins are synthesized rapidly in some specific tissue to be secreted into the haemolymph of the Muga silkworms. Proper isolation and purification of such new proteins can lead to better understanding as to ascertain their probable role in usage in-lieu of market antibiotics. There is also an added need to recognize these new molecules with antibacterial activity which might prove to be true alternatives to the different drugs already existing in the market, thereby being a valuable support to the antibiotic therapies used conventionally (Browne *et al.,* 2020). In this study, attempt has been made to study the antibacterial potential of *Bacillus thuringiensis* immunized haemolymph of 5th instar muga silkworm larvae against *B.thuringiensis* and *E.coli* and also compare its activity with three over-the-counter antibiotic drugs.

Materials and methods

Rearing and collection of silkworms

Disease-free eggs of Muga silkworm, *Antheraea assamensis* were collected from the Directorate of Sericulture, Khanapara, Assam. The newly hatched larvae were reared in the Muga farm itself on the Som (*Persea bombycine*) plants. Only the fifth instar larvae (2-3 days post moult) weighing about 2.5 gm (20-25 larvae) were used in this study.

Bacteria for immunization

Bacillus thuringiensis (MTCC 1953), gram-positive bacteria procured earlier from Microbial Type Culture

Centre (MTCC), Chandigarh, were sub cultured in prepared nutrient broth (liquid culture) as per the instructions of MTCC. The nutrient broth solution was prepared by following the methodology of Haloi *et al.,* (2016).

Immunization of the larvae and collection of haemolymph

10^5 CFU/ml (sub lethal dose as found by Haloi *et al.,* 2016) bacterial concentration was prepared by serial dilution method. 20µL suspension of 10^5 CFU/ml (sub lethal dose) was injected into the larvae. For collection of the haemolymph, 5 different groups of larvae were made each containing 10 larvae: 4 groups with treated larvae and 1 group containing the control larvae. From the first group, haemolymph was collected after 6 hours of bacteria immunization by cutting off an abdominal leg into pre-chilled Eppendorf tubes containing pinch of Phenylthiourea crystals and Phenyl methane sulfonyl fluoride. From the second group, haemolymph was collected in the same way after 12 hours of bacteria immunization. From the third and the fourth group, haemolymph was collected after 18 and 24 hours of bacteria immunization respectively. For the fifth group or the control group, no immunization was carried out in larvae and haemolymph was collected just like the previous times. The collected haemolymph samples were centrifuged immediately at 4000 rpm for 10mins for removing the haemocytes. The supernatant containing plasma was stored at -20˚C until use.

Total protein estimation

The estimation of total proteins was carried out by following the methodology of Lowry *et al.,* (1951). The optical density (OD) was noted at 670nm for all the respective test tubes against the standard ones. The same procedure was carried out for all the 4 treated groups of haemolymph (haemolymph collected after 6,12,18,24 hours) and also for the controlled group. The total protein concentration for all the haemolymph samples was shown in terms ofmg (of protein) /ml (of haemolymph).

Total free amino acids estimation

The estimation of total free amino acids was carried out by following the methodology of Moore and Stein (1968). The optical density (OD) was noted at 570nm for all the 4 treated groups of haemolymph (haemolymph collected after 6,12,18,24 hours) and also for the controlled group. The total free amino acids concentration for all the haemolymph samples was shown in terms ofmg (of leucine) /ml (of haemolymph).

Assessment of antibacterial activity

Antibacterial activity of all the haemolymph samples and the chosen antibiotics were tested against the gram-positive bacteria *Bacillus thuringiensis* (MTCC 1953) and the gram-negative bacteria *Escherichia Coli* (MTCC 40) procured earlier from MTCC, Chandigarh and were sub cultured in prepared nutrient broth by incubating them for about 24 hours at 37˚C. Antibacterial activity was detected using solid agar petri dishes. The nutrient broth and nutrient agar preparation as well as the protocol for experiment were carried out using the methodology of Haloi *et al.* (2016) with a few modifications. 3 potent market available antibiotics Azithromycin, Rifaximin and Levofloxacin effective against broad range of gram-positive and gram-negative bacteria were chosen. One tablet of each is crushed and dissolved in 100ml of PBS separately. Sterile petri dishes were prepared with nutrient agar medium. After solidification of the medium, the agar surface was inoculated with 0.1ml of both the test bacterial strains on separate petri dishes and spread with the help of a sterile swab. Wells were formed in the plates with the help of a sterile 0.5ml pipette tip. About 20µL of the haemolymph sample and 20µL of all the three antibiotics were added to separate wells. In each plate 20µL of PBS was added in one well to keep it as control without addition of haemolymph. The plates were incubated overnight in an incubator at 37˚C. The diameters of the clear zones of inhibition were measured and shown by photography.

Results and discussion

Protein estimation by Lowry's method

The total protein concentration present in the normal haemolymph sample was found to be 15 ± 0.33mg/ml. The total concentration of proteins present in the immunized haemolymph samples collected after 6, 12, 18 and 24 hours of bacteria

immunization were found to be 15.6 ± 0.31mg/ml, 16.26 ± 0.53mg/ml, 17.18 ± 0.46mg/ml and 19.18 ± 0.49mg/ml respectively shown in fig. 1.

The protein concentration in the haemolymph samples collected at different time intervals after bacteria immunization (after 6, 12, 18 and 24 hours) was found to be higher as compared to the control haemolymph sample. Notably, the protein concentration gradually increased with the duration of immunization, reaching the highest concentration at 24 hours (19.18 ± 0.49mg/ml) as shown in fig. 1. This finding aligns with studies conducted by Adamo (2004), Florkin and Jeuniaux (1974) and Schafer *et al.* (2023). This increase in protein concentration suggests the secretion of antibacterial proteins as a response to the bacteria injection. The production of these bactericidal proteins in the haemolymph of the larvae requires the generation of new RNA molecules which requires minimum 8 hours as reported by Sharma *et al.* (2005). Similar results were observed in this study as the protein concentration of immunized haemolymph collected after 6 hours of bacteria immunization showed no significant rise as compared to the controlled group.

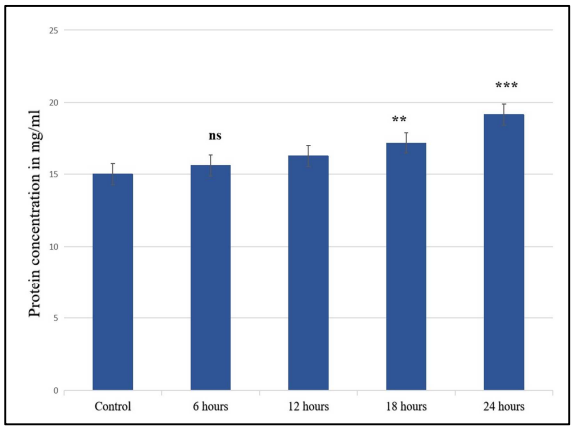

Fig. 1. Comparison of protein concentration present in the haemolymph of different groups of Muga silkworm larvae immunized with bacteria (*Bacillus thuringiensis*) for different time interval with that of the haemolymph collected from the controlled group of larvae.

Data are expressed as Mean ± SEM followed with ns= not significant (p>0.05), *= significant (p<0.05), **= highly significant (p<0.01), ***= very highly significant (p<0.001), df=4,n=5

Total free amino acids estimation

The total free amino acid concentration present in the normal haemolymph sample was found to be 1.72 ± 0.31mg/ml. The total concentration of free amino acids present in the immunized haemolymph samples collected after 6, 12, 18 and 24 hours of bacteria immunization were found to be 1.78 ± 0.35mg/ml, 2.31 ± 0.41mg/ml, 2.97 ± 0.47mg/ml and 3.61 ± 0.52mg/ml respectively shown in fig. 2.

In this study, the free amino acid concentration increased in the immunized haemolymph samples. There was significant increase in amino acid concentration in the haemolymph sample which was collected after 6 hours of bacteria immunization, whereas the other three haemolymph samples which were collected after 12, 18 and 24 hours of bacteria immunization showed significant increase in amino acid concentration as compared to the control haemolymph sample. Similar findings were observed by Gad A. A. (2012) where amino acid concentration were found to increase in the haemolymph samples collected from *Bombyx mori* after being immunized with *Escherichia coli* and *Bacillus thuringiensis* as compared to the control haemolymph samples.

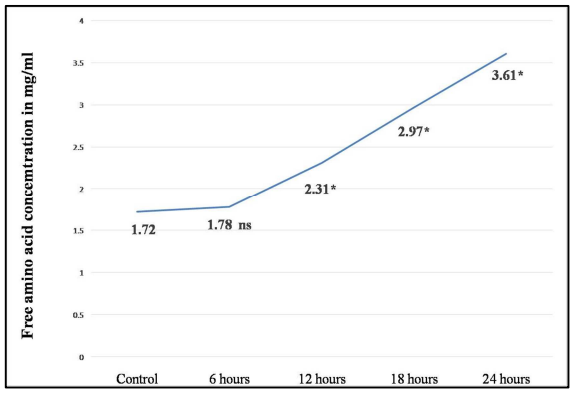

Fig. 2. Comparison of free amino acids concentration present in the haemolymph of different groups of Muga silkworm larvae immunized with bacteria (*Bacillus thuringiensis*) for different time interval with that of the haemolymph collected from the controlled group of larvae.

Data are expressed as Mean ± SEM. ns= not significant (p>0.05), *= significant (p<0.05), **= highly significant (p<0.01), ***= very highly significant (p<0.001), df=4,n=5

The amino acid concentration was found to be more in the *E. coli* induced haemolymph samples than *B. thuringiensis*, suggesting that these induced amino acids can combine to form some proteins that are bactericidal in nature. El-Sadawy *et al.* (2009) reported decrease in the number of some specific amino acids in the haemolymph of flesh-fly larvae, *Parasarcophaga aegyptiaca* and chicken ticks, *Argas persicus* when infected with entomopathogenic nematode, though some other amino acid number increased in the infected haemolymph. The decrease in the number of amino acids can be due to the consideration of different host and different parasite in their study.

Assessment of antibacterial activity

Triplicates of each sample and the antibiotics were taken against each bacterial strain and the diameter of zone of inhibition were measured using vernier calliper. The average diameter of zone of inhibition shown by the antibiotics Levofloxacin, Azithromycin and Rifaximin against *E. coli* were found to be 2.17 ± 0.17cm, 1.77 ± 0.22cm and 1.33 ± 0.18cm respectively.

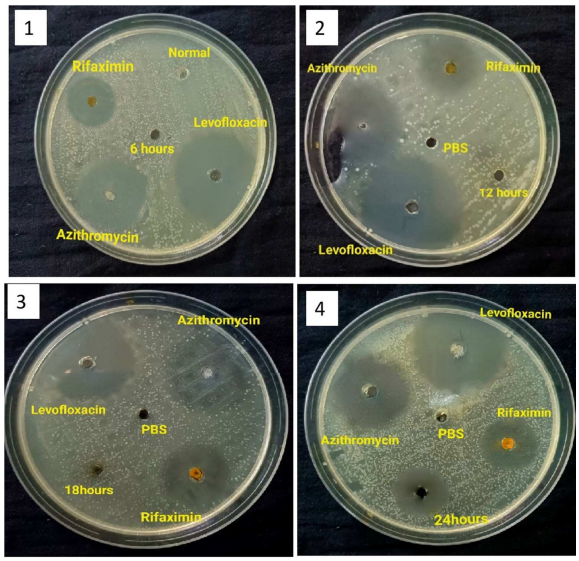

Fig. 3. Antibacterial activity shown by the immunized haemolymph samples collected after different hours of immunization (1) 6 hours (2) 12 hours (3) 18 hours and (4) 24 hours and the chosen market antibiotics (Levofloxacin, Azithromycin and Rifaximin) against *E. coli*. PBS is taken as negative control.

The average diameter of zone of inhibition shown by the normal and the bacteria immunized haemolymph samples collected after 6, 12, 18 and 24 hours of bacteria immunization against *E. coli* were found to be 0.00cm, 0.00cm, 0.00cm, 0.00cm and 1.28 ± 0.16cm respectively as shown in fig. 3.

Likewise, the average diameter of zone of inhibition shown by the antibiotics Levofloxacin, Azithromycin and Rifaximin against *Bacillus thuringiensis* were found to be 2.26 ± 0.21cm, 1.60 ± 0.15cm and 1.20 ± 0.10cm respectively. The average diameter of zone of inhibition shown by the normal and the bacteria immunized haemolymph samples collected after 6, 12, 18 and 24 hours of bacteria immunization against *Bacillus thuringiensis* were found to be 0.00cm, 0.00cm, 0.00cm, 0,00cm and 0.80 ± 0.17cm respectively as shown in fig. 4.

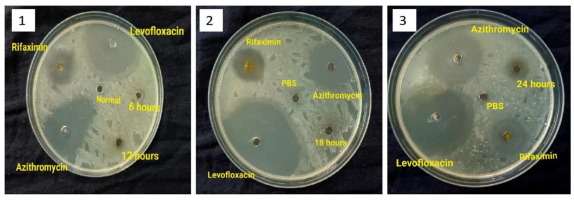

Fig. 4. Antibacterial activity shown by the immunized haemolymph samples collected after different hours of immunization (1) 6 hours and 12 hours (2)18 hours and (3) 24 hours and the chosen market antibiotics (Levofloxacin, Azithromycin and Rifaximin) against *B. thuringiensis*. PBS is taken as negative control.

Among the three chosen antibiotics, Levofloxacin showed the highest antibacterial activity against both *E. coli* and *B. thuringiensis*, followed by Azithromycin. Rifaximin showed the least antibacterial activity against both the bacterial strains (as shown in fig. 5). Interestingly, the haemolymph collected after 24 hours of bacteria immunization demonstrated substantial antibacterial activity by showing clear zones of inhibition whereas no such antibacterial activity was shown by the immunized haemolymph collected after 6, 12 and 18 hours of bacteria immunization. Comparing the results of this study with previous research, Hoffmann (1995) reported antimicrobial protein expression as early as 6 hours after immunization. This difference might be due to variations in the intensity of protein

transcription, with peak activity occurring between 18 hours and 24 hours post-immunization. Immediately after immunization, the production of these bactericidal proteins might be slow, and not enough to react against pathogens. The antibacterial activity shown by the immunized haemolymph samples, when compared with the market available potent antibiotics like Levofloxacin, Azithromycin and Rifaximin showed positive results. These results suggest that these antibacterial proteins and these market available antibiotics show synergistic activities against several pathogens. Similar findings were reported by Portelinha and Angeles (2021) where fish antimicrobial proteins exhibited antimicrobial activity comparable to that of Kanamycin and Ciprofloxacin when tested against *P. aeruginosa*. In this study the antibacterial activity was observed to be more effective against *E. coli* compared to *Bacillus thuringiensis*. This discrepancy in antibacterial activity against different bacterial strains may be attributed to the varying toxicity levels and susceptibilities of these bacteria.

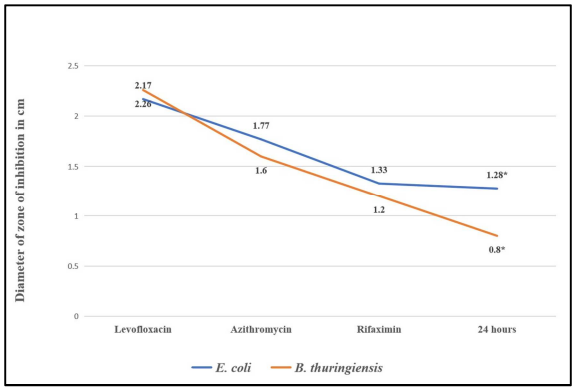

Fig. 5. Comparison of antibacterial activity between the two groups (against *E. coli* and *B. thuringiensis,* for the haemolymph sample collected after 24 hours). n=3, * represents significant differences between their antibacterial activity.

Conclusion

The observations and discussion of this study made it clear that bacterial infection induces new set of proteins in the haemolymph of Muga silkworm larvae to fight against the bacteria and can play a vital role in safeguarding the health of these silkworms and increase their ability to survive against the odds of nature, thereby opening a way to protect the Muga silk industry of Assam. As the antibacterial activity shown by the immunized haemolymph is almost close to that of the chosen antibiotics, these bactericidal proteins can function as a promising group of antimicrobial compounds. Due to increasing threat of multidrug resistance, the discovery and production of new antibiotics is very essential. Proteins derived from *Bacillus thuringiensis* immunized Muga silkworm larvae can prove to be the natural sources for antibiotic development. By reducing reliance on conventional antibiotics, we can mitigate the harmful consequences of overuse and contribute to the development of sustainable and effective antimicrobial therapies.

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Competing interests

The authors declare that there are no conflicts of interest.

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