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Assessment of protein profile and antibacterial activity in hemolymph of *Bacillus thuringiensis*-immunized muga silkworm

Shibani Kalita[1]*, Sanghamitra Saharia[1], Dimpimoni Kalita[1], Dhirunabh Swargiary[2], Sunayan Bardoloi[3]

[1]Department of Zoology, Gauhati University, Guwahati, Assam, India.
[2]Advanced Level Institutional Biotech Hub, B. Borooah College, Guwahati, Assam, India.
[3]Department of Zoology, Girijananda Chowdhury University, Guwahati, Assam, India.

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ABSTRACT

The major aspect of immune system in insects can be attributed to its humoral immune response that is largely operated by means of some defense proteins or antimicrobial peptides. Muga silkworms have remained mostly unexplored in this matter, and as such in this study, *Bacillus thuringiensis* inoculated fifth-instar muga silkworm larva was analyzed for changes in protein profile, antimicrobial activity, as well toxicity assessment of the bacteria in the body of the silkworm larvae. Toxicity assessment was done by the serial dilution method, which was used to prepare various bacterial doses, and the LC_{50} value was found to be at 5.7×10^6 CFU/mL. A sublethal concentration was used for immunization of the larvae, following which hemolymph from the silkworms were collected at different time intervals, that is, 6 h, 12 h, 18 h, and 24 h. Antimicrobial assessment by well diffusion method showed the hemolymph collected after 24 h to be potent against both *B. thuringiensis* (MTCC 1953) and *Escherichia coli* (MTCC 40). Furthermore, analysis of this hemolymph revealed higher concentration of protein content as well as higher free amino acid concentration compared with a normal group. The appearance of several new bands in SDS-PAGE analysis of the same hemolymph was observed, which was not seen in the protein profile of normal silkworm previously. Proper proteomic analysis and characterization of such proteins produced on immunization might prove to be revolutionary in better understanding the silkworm as well as its defense mechanism providing valuable insights. This might be crucial for the development of natural and potent antibiotics with fewer side effects.

1. INTRODUCTION

Insects are one of the most abundant organisms having remarkable evolutionary eminence. This can be attributed to their immune mechanisms that exhibit potent defenses against the multitude of disease-causing pests and pathogens. The increasing significance of research on insect immunity underscores its pivotal role in contemporary scientific investigations. Numerous insect model organisms have been studied over time in different research projects to unveil the intricacies of the same. Among them, studies on silkworm immunity are quite extensive due to its economic importance and its genetic homogeneity to humans to some extent [1]. As such, an in-depth study of silkworm immune system and a better understanding of the various diseases and their causative organisms are in all probability quite pertinent for future explorations of alternatives against potential pathogens.

Among the various diseases occurring in silkworms, bacterial flacherie is quite common. Just like other breeds of silkworms, the muga silkworm, *Antheraea assama* Helfer, also has quite a high occurrence of flacherie [2]. This causes about 40% loss of muga crops each year. One of the major causative agents of flacherie was reported to be *Bacillus thuringiensis,* having high toxicity levels, causing septicemia, and eventually death of the affected worms [3,4]**.** Though muga silkworms are susceptible to the infection of *B. thuringiensis*, they are not totally vulnerable to them and also to other such potentially pathogenic microbes and are often reported to develop resistance against such pathogens [5]. It is believed to be so because the muga worms are reported to have a well-developed immune system that includes both innate and adaptive elements [6]**.** The innate immune system and its components, especially antimicrobial peptides (AMPs), play a major role since it is the primary line of defense against microbes [7]. The AMPs are low-molecular-weight proteins synthesized by the fat body and then liberated into the hemolymph of the silkworm upon infection or injury [6]. For the synthesis of AMPs, the free amino acids in the hemolymph are utilized on immunization or in response to injury [8]. The AMPs have various modes of action; however, the most prominent mechanism is breakdown of the cell membrane or cell wall of microorganisms [9]**.**

**Corresponding Author:*
Shibani Kalita,
Department of Zoology, Gauhati University, Guwahati, Assam, India.
E-mail: shibanik203@gmail.com

B. thuringiensis, known for its significant role as a causative agent of flacherie, is believed to trigger immune responses in silkworms, leading to the synthesis of immune proteins or AMPs. This production is expected to involve the utilization of free amino acids from the hemolymph repertoire following injury or infection by microbes. Therefore, the primary objective of this study was to assess the total protein content, total free amino acids, and antimicrobial activity in the hemolymph of *B. thuringiensis*-immunized muga silkworm larvae against both *B. thuringiensis* and *Escherichia coli.* While various silkworm breeds have been extensively studied, the muga silkworm, native to the northeastern region of Assam, has not received as much attention in this context. The successful exploration of other breeds has led to the discovery of numerous AMPs. Consequently, investigations into the Muga silkworm may prove crucial and highly significant, offering valuable insights into enhancing the quality of muga silkworm breeds and potentially uncovering novel avenues for antibiotic discovery.

2. MATERIALS AND METHODS

2.1. Insects

Disease-free eggs of *Antheraea assamensis* were procured from CSB sericulture farm, Gandhinagar, Tetelia, Assam, and reared on the farm itself. The fifth-instar healthy larvae (2–3 days post molt) developing from such eggs were considered for collection of hemolymph. Larvae were reared on Som plants (*Machilus bombycina*) in CSB sericulture farm, Gandhinagar, Tetelia, in prevailing climatic conditions with high humidity and temperature ranging from 32 to 35°C.

2.2. Bacterial Species Selection

Two bacterial strains were used in this study. *B. thuringiensis* (MTCC 1953) and *E. coli* (MTCC 40) were procured from the Microbial Type Culture Centre (MTCC), Chandigarh, and culture was carried out as per MTCC instructions using nutrient broth and nutrient agar media followed by reculture of bacteria after every 15–20 days. Immunization of the larvae was carried out by *B. thuringiensis*. Antimicrobial activity was assessed against both *B. thuringiensis* and *E. coli*.

2.3. Determination of Lethal Concentration of *B. thuringiensis*

Lethal concentration (LC_{50}) value was found via the method of [3] with slight modifications. Different concentrations of *B. thuringiensis* (MTCC 1953) were prepared ranging from 10^1 to 10^9 CFU/ml by serial dilution. Ten groups of silkworms, each consisting of 10 individuals per concentration, were included in the experiment, along with a control group. Each group was injected with 20 µl of different bacterial concentrations while the control group was injected with sterilized phosphate-buffered saline (PBS). The number of silkworms dying at various concentrations was recorded for 24 h. Based on the observed data, LC_{50} value was calculated for 24 h using Probit analysis in the Biostat 2009 software package [10].

2.4. Preparation of Control and Treated Groups

The fifth-instar larvae were inoculated with 20 µl of 10^5 CFU/m, that is, the one-tenth dose of the LC_{50} of *B. thuringiensis*, with the help of a sterile 1 ml disposable insulin syringe. The control group was injected with 20 µl of PBS and hemolymph was collected after 24 h. Samples of hemolymph were collected at various time intervals, namely, 6 h, 12 h, 18 h, and 24 h, following the induction of infection. Hemolymph were collected by incision of the last abdominal leg in previously chilled falcon tubes containing crystals of phenylthiourea and phenylmethane sulfonyl fluoride crystals to prevent oxidation as well as degradation of peptides. The hemolymph were mixed with the crystals and centrifuged at 3000×*g* (15 min at 4 °C) to remove any hemocytes, and supernatant with plasma was stored at −20 °C till further use.

2.5. Assessment of Antimicrobial Activity of Hemolymph Extracted at Different Hours

The cell-free hemolymph was assessed for probable antimicrobial activity by the well diffusion method by Haloi [11]. Two sterile Petri plates with nutrient agar medium were swabbed and spread with the test bacterial strains, that is, *B. thuringiensis* and *E. coli*. Five wells were formed in each plate with the help of a sterile 0.5 ml pipette tip, where 20 µl of sample hemolymph taken from four different time intervals (6 h, 12 h, 18 h, and 24 h) and PBS injected hemolymph collected after 24 h (control) were placed on the medium. In each plate, one well was kept as control by adding 20 µl of 1× PBS only. The plates were incubated overnight at 37 °C in an incubator. After 24 h, the diameter of the clear zone of inhibition was measured and documented by photography.

2.6. Comparison of Total Protein Content of Both Normal and Infected Hemolymph Samples of Larvae

The sample showing the highest antimicrobial activity was then analyzed for total protein content following the method of Lowry *et al*. [12] and compared with a control sample. The intensity of the color was measured photometrically using a spectrophotometer at 670 nm.

2.7. Total Free Amino Acid Analysis

Free amino acid content was analyzed using the Ninhydrin method as per the protocol of Moore and Stein [13] using leucine as standard. Analysis was carried out with a spectrophotometer at 570 nm. The values were expressed in milligram of leucine per milliliter of hemolymph.

2.8. Protein Profile Study and Comparison of Hemolymph Samples

The protein profiles of normal and hemolymph sample having maximum antimicrobial activity were analyzed by 12% SDS-PAGE according to Laemmli [14] using electrophoresis apparatus from Bio-Rad and SDS-PAGE chemicals from GeNei. Control and immunized hemolymph samples were loaded into the wells of the gel along with a standard protein molecular marker (PMM), and the gel was run at a constant voltage of 100 V for 120 min. Once electrophoresis was complete, the gel was removed from the apparatus and stained overnight in a staining box with 0.2% Coomassie Brilliant Blue R-250 and destained in methanol and acetic acid in the ratio 2:1 [15]. The protein bands in the gel so obtained were then observed under a gel documentation system (UVITECH, TECHNE).

2.9. Statistical Analysis

Statistical analysis was performed using MS Office 2017 and SPSS 21. For protein content and free amino acid estimation, *t*-test was performed to determine the significance between the two groups using the same software ($p<0.05$). The results are expressed as mean ± standard deviation (X± SD). The data for inhibition zone measurement were subjected to ANOVA for the mean comparison using IBM SPSS

25.0 version. Differences between means were considered significant at $p < 0.05$.

3. RESULTS

3.1. Determination of Lethal Concentration of *B. thuringiensis*

In this study, the mean mortality values of muga silkworm were calculated for *B. thuringiensis* bacterial strains at 24 h. The lethal concentration was found to be 5.7×10^6 CFU/ml [Figure 1]. As such, for the later experiments, one-tenth of the concentration of LC_{50} was used, that is, about 10^5 CFU/ml. Regression plot was the same.

3.2. Assessment of Antimicrobial Activity of Hemolymph Extracted at Different Hours

Hemolymph samples were collected at different times after immunization with bacteria, that is, 6 h, 12 h, 18 h, and 24 h, and their antimicrobial activity was assessed against two test bacteria, *B. thuringiensis* and *Escherichia coli,* respectively. It was found that in both analyses the hemolymph extracted at 24 h showed clear inhibition zones against both bacteria while the hemolymph extracted at 6 h, 12 h, and 18 h failed to show any response [Figure 2]. It was also seen that the diameter of the inhibition zone developed against *E. coli* was 1.67 cm, which is larger than that against *B. thuringiensis* where the inhibition zone was 1.24 cm, suggesting greater antimicrobial activity against *E. coli* than *B. thuringiensis*. However, at $p>0.05$, these values were shown to be significantly different from each other [Figure 3].

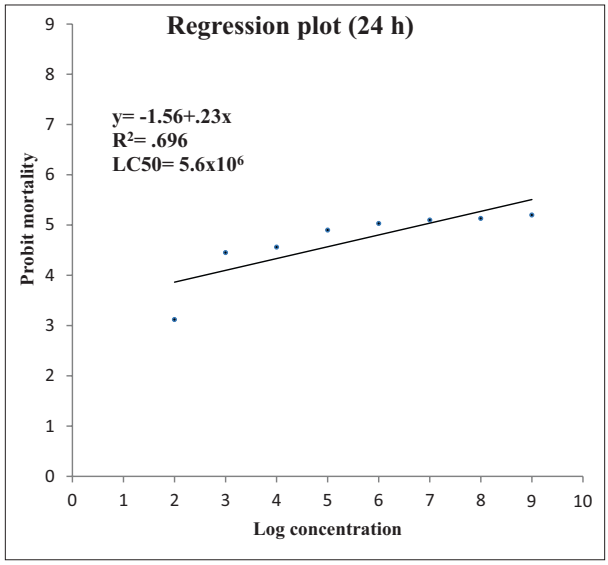

Figure 1: Regression plot of probit mortality and log concentration for *B. thuringiensis* to *A. assamensis* fifth-instar larvae for 24 h.

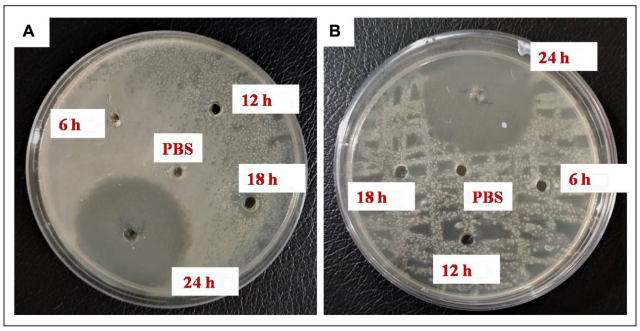

Figure 2: Antimicrobial activity of fifth-instar muga larva hemolymph samples extracted at different time intervals after bacteria inoculation, that is, 6 h, 12 h, 18 h, and 24 h against **(A)** *B. thuringiensis* and **(B)** *E. coli*. PBS is taken as negative control.

3.3. Comparison of Total Protein Content of Both Normal and Infected Hemolymph Samples of Larvae

The results of protein estimation revealed an increase in hemolymph protein concentration in the bacteria-immunized groups of silkworm compared to the normal or control ones. The normal groups of muga silkworm (*Antheraea assamensis*) larvae were found to have a total protein concentration of 31.4 ± 0.36 mg/ml. However, the average total concentration of protein in immunized hemolymph (34.26 ± 0.44 mg/ml after 24 h of immunization) showed a significant increase at $p<0.05$ [Figure 4].

3.4. Total Free Amino Acid Analysis

Variation in the quantity of total free amino acids in the larval hemolymph of the control group (1.53 ± 0.034 mg/ml) and bacteria-inoculated group (2.93 ± 0.056 mg/ml) was also found to be significantly different, suggesting a direct effect of bacterial stress induced in the amino acid concentration values [Figure 5].

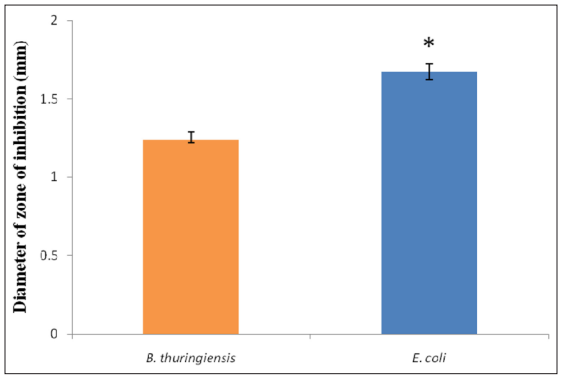

Figure 3: Comparison of antimicrobial activity of induced hemolymph procured at 24 h after immunization against *B. thuringiensis* and *E. coli*. Hemolymph extracted at 6 h, 12 h, and 18 h were not considered as they did not show any antimicrobial activity. * indicates significant differences between groups ($p>0.05$); n = 3.

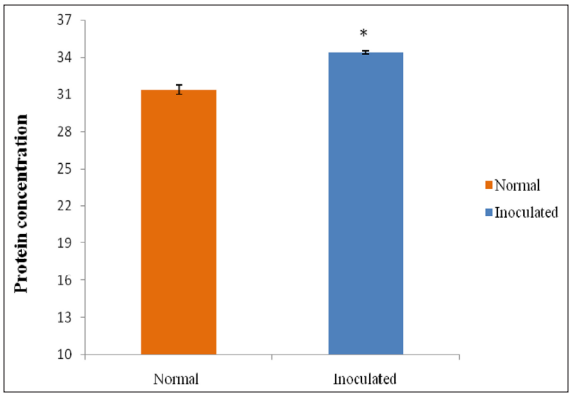

Figure 4: Protein concentration of fifth-instar larval hemolymph of muga silkworm control, that is, normal and bacteria-inoculated (*B. thuringiensis)* groups. Data are expressed in mean ±SEM. n=5. * represents significant differences between groups ($p<0.05$).

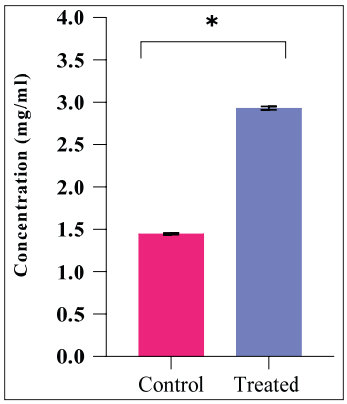

Figure 5: Total free amino acid detection of control silkworm hemolymph and bacteria (*Bacillus thuringiensis*)-injected hemolymph sample of muga (*Antheraea assamensis*) silkworm. Data are expressed in mean ±SEM. n=5. * represents significant differences between groups ($p<0.05$).

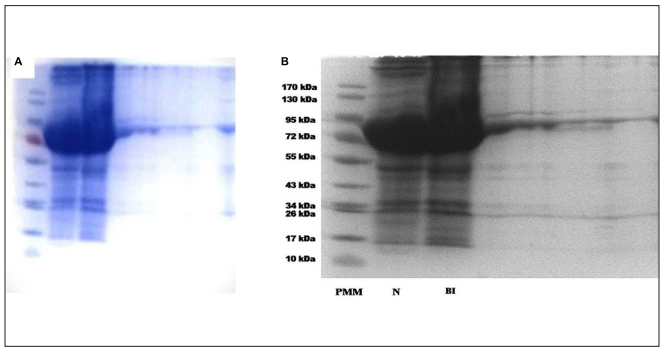

Figure 6: SDS-PAGE protein profile of normal and bacteria-immunized hemolymph. PMM = protein molecular marker, N = normal, BI = bacteria inoculated.

3.5. Electrophoretic Analysis of Protein Profiles for Investigating the Presence of AMP

The comparative protein profile analysis of hemolymph protein of muga silkworm (*A. assamensis*) studied by single-dimension SDS-PAGE for normal and immunized bacteria showed different bands as shown in Figure 6. Some 6–7 bands were seen in the normal sample. Various new bands were seen to arise in the bacteria-inoculated samples between the molecular range 2–66.4 kDa. The bands are more prominent below 23.0 kDa and between 116 kDa and above [Figure 6].

4. DISCUSSION

Proteins associated with defense, in case of lower organisms like insects, serves as the first component and a key contributing factor in humoral immune response on invasion by microbes [16]. Each species is well equipped with their own set of specific AMPs, which are produced in response to different pathogens [17,18]. Our study basically compared and analyzed the protein profiles of noninduced hemolymph and induced hemolymph of muga silkworm larvae for the presence of proteins with potential antimicrobial activity against Gram-positive bacteria *B. thuringiensis* and Gram-negative bacteria *E. coli.*

In almost all the studies conducted on the AMPs of insects or immunity as a whole, it was seen that the immune system of the insect was activated or induced by immunizing with different doses of either bacteria, viruses, fungi, or other microorganisms. An explanation for this might be that the peptides produced as a result of induction are not absent but might be present below the detectable ranges under normal conditions. Only on exposure to a pathogen or on injury are the peptides transcribed rapidly, leading to their detection after a certain time post exposure [6].

Immunization in our study was carried out by *B. thuringiensis* into the abdominal cavity of muga silkworm larvae. It has been reported to cause flacherie in silkworms and is a very potent disease-causing bacteria having multiple toxic effects as it can cause sepsis in the midgut, leading to the penetration of the bacterial strains into the hemocoel that can cause death [3]. The lethal concentration of *B. thuringiensis* in this study was found to be 5.7×10^6 CFU/ml after 24 h, which is similar to that observed by Haloi [3] in muga silkworm with about 4.78×10^6 CFU/ml for 24 h as well. However, it was found to be much less in another study conducted against *B. mori,* with about 3.3×10^4 CFU/ml [19]. This might be due to the involvement of different strains of *B. thuringiensis* and host specificity.

Once defense peptides are synthesized, they are rapidly released into the hemolymph [5]. Hemolymph samples were then collected at different time intervals, that is, 6 h, 12 h, 18 h, and 24 h after inducing infection since AMPs and other immune-related proteins have been reported to be first expressed at about 6 h after infection [20]. In our study, it was found that hemolymph extracted in 6 h, 12 h, and 18 h did not show any activity against both *B. thuringiensis* and *E. coli*. However, hemolymph extracted at 24 h showed very potent antimicrobial activity against both the test bacterial strains. This might be due to the intensity of transcription of AMPs peaking from 18 to 24 h, after which transcription decreases as such major production of AMPs might have occurred in the later hours after immunization. As in the hours just after immunization, the process of production of defense peptides might be slow and reaction against pathogens might not have fully developed as such. This theory was confirmed by Meister *et al.* [20].

In this study, the quantitative protein analysis of muga silkworm hemolymph showed an increase in protein concentration in the *B. thuringiensis* immunized larvae compared to the control group. Similar findings were recorded by Sharma *et al*. [21] in non-mulberry silkworm after inoculation of bacteria. The Eri silkworm *Philosamia ricini* also showed similar kinds of results when injected with *E. coli* and *M. luteus* bacteria [22]. Adamo [23] suggested that the bacteria-injected hemolymph might contain higher concentrations of proteins due to the presence of some inducible antibacterial protein synthesized by silkworm to defend themselves or as immune response against the injected bacteria.

Quantitative changes in free amino acids revealed changes in the hemolymph of control and bacterial-challenged muga silkworm larvae in the present studies. It was found to be higher than that of the normal larvae. Salama et al. [24,25] also reported changes in amino acid studies in *Spodoptera littoralis* hemolymph. Studies on total amino acid in *B. thuringiensis*-treated *Plutella maculipennis* by Narayanan [26] also reported similar observations like that of our study. Likewise, Reddy et al. [27] reported increases in free amino acids in thyroxine-treated larvae of *Antheraea mylitta* (tassar silkworm). It was suggested that amino acids contribute to the general pool for the production of new proteins among various other functions, and as such introduction of bacteria into the body of the insects might lead to the rapid production as well as utilization of

the amino acid to produce defense proteins, which is reflected in our results. This theory is in accordance with Pant and Agrawal [28]. Similarly, Lazar and Mohamed [29] suggested that variations in the total amino acid pool change with corresponding changes in total protein content.

SDS analysis of the protein profiles of normal and bacteria-immunized muga silkworm showed that the bacteria-injected groups had new bands, which were completely absent in the normal groups. Similar results were derived from studies with *Bombyx mori* and *Maduca sexta*, where the hemolymph revealed the occurrence of a variety of new proteins in response to injury or microbe inoculation [30-32]. Ganjendra *et al.* [32] reported that such proteins are produced in the fat body and are released into the hemolymph of *A. mylitta* larvae too after bacterial introduction. It can be assumed that the newly observed proteins represented by the newer bands might be a response to bacterial stress as observed earlier in terms of an increase in free amino acids [33,34]**.** However, further proteomic analysis will be necessary to prove it conclusively.

5. CONCLUSION

This study is a preliminary approach to understand the effects of bacteria immunization on the protein profile of muga silkworm larvae. Inoculation of *B. thuringiensis* into the hemolymph of fifth-instar muga larva causes changes in the total protein content, free amino acids, as well as occurrence of new protein bands in the SDS analysis of the hemolymph post bacteria challenge. Hence, we can conclude that these new proteins might be the compounds associated directly or indirectly with the antimicrobial response of induced muga silkworm larvae but more conclusive results require proper isolation, purification, and characterization of such proteins, which might be performed in later studies. There is also the possibility that the discovery of such new protein capable of antimicrobial activity might pave the path for the development of such proteins as novel antibiotic drugs that may be highly efficient with drug designing.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

13. PUBLISHER'S NOTE

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