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Staphylococcus aureus-induced sepsis in the lobster cockroach *Nauphoeta cinerea*

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ABSTRACT

Invertebrates have been instrumental in understanding the mechanisms involved in infectious diseases, considering the idea to replace, reduce and refine the use of mammals as well as to understand the basic principles of immune response in insect. We evaluated the consequences of *Staphylococcus aureus*-induced sepsis in the last instar nymphs of *Nauphoeta cinerea* injected with different concentrations of bacteria preserved in two culture media. Infected groups had a decrease in hemolymph metabolites (glucose, amino acids, total proteins, and cholesterol), in contrast to the proteins in the fat body. Higher concentrations of *S. aureus* caused permanent morphological alterations in adults, decrease in food consumption, increase in isolation, and increase in CFU until death of the cockroaches. Survival and protection of nymphs against a repeated and stronger challenge with the same bacteria varied according to the medium they were conserved. *N. cinerea* proves to be a suitable and promising model for studies related to bacterial infections.

1. Introduction

The utilization of invertebrates, mainly insects, as models to study the immune system and pathophysiology of human diseases broadly increased in the last decades. The ethical concerns about the use of vertebrate models that involves pain and extreme suffering (as in the case of sepsis) are certainly one important motivator to adhere to the recent idea to replace, reduce and refine (3RS) the use of mammals in exploratory scientific studies [1]. Consequently, the development of new techniques designed to study the immune system in non-vertebrate organisms is needed not only to avoid the use of vertebrates but also to work with simple models that can be very informative to understand basic principles [1,2].

In this direction, the nematode *Caenorhabditis elegans* [3,4], the fruit fly *Drosophila melanogaster* [5,6], the silkworm larvae *Bombyx mori* [7–10], the wax moth *Galleria mellonella* [11,12], the beetle *Tenebrio molitor* [13], the honeybee *Apis mellifera* [14], and the tobacco hornworm *Manduca sexta* [15] have been used to study the immunological responses after the infection with human pathogens. Of particular significance, the insect models have been instrumental in understanding the complex molecular mechanisms involved in response to infectious diseases, and in studying bacterial virulence factors [2,9,10,14,16].

Immune response in insects is thought to be innate, broad, non-specific, and similar against different types of challenges [17]. Insect cellular response involves phagocytosis, nodulation, and encapsulation, whereas humoral responses can involve the production of antimicrobial peptides, melanization, and clotting [18,19]. However, the study of adaptive immunity of insects started many decades ago. Accordingly, works of literature have indicated the adaptive immune response in the cockroach *Periplaneta americana* after the injection of venom from honeybee [20,21]. In other studies, *P. americana* did not recognize the cuticle of other insects as foreign, whereas *Schistocerca gregaria* encapsulated the cuticle transplants from the other insects (xenografts) [22]. However, both species did not recognize the allografts as foreign tissues, which refers to the implantation of the cuticle from the same species [22].

Recently, studies demonstrated acquisition of immunization through a passive transference of molecules from the parental generation to the offspring [17,23], and an active transference by the contact with the relatives [24]. According to Masri and Cremer [24], the individual level of immunization can be explained in two possible ways, (1) the host can sustain an upregulation of the genes involved in

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immune response or slow down the rate of degradation of the proteins after the first exposure to a pathogen, (2) the immune effectors [especific hemocytes, peptidoglycan recognition proteins (PPRs), integrins, down syndrome cell adhesion molecule (Dscam)] of the host can decline to baseline levels shortly after an exposure to a pathogen, but the host acquired memory. In any case, the mechanisms are not well elucidated [25,26].

Because of the importance to adhere to the 3Rs prerogatives and to find substitutes for the mice and rats models, we focused on the use of lobster cockroach *Nauphoeta cinerea*. The *N. cinerea* is easy to handle and has been instrumental in toxicological and pharmacological studies [27–33]. Some advantages of *N. cinerea* include the size, life cycle, inability to fly, and ease of maintenance. We examined the appropriateness of *N. cinerea* as a model of infection using the human pathogenic bacteria *Staphylococcus aureus*. The effects of different concentrations of bacteria solutions preserved in two culture media on host survival, physiological consequences, alterations in the homeostasis of metabolism and immunization of nymphs against a second challenge with the same bacteria were evaluated.

2. Material and methods

2.1. Cockroach husbandry

The last instar nymphs of *N. cinerea* with 2.0 to 2.5 cm of length (from Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas (CCNE), Universidade Federal de Santa Maria, Brasil) were separated in groups of 15–30 individuals. They were kept in plastic boxes (19 × 19 x 7 cm) containing a yellow plastic part from an egg-holder (Supplementary Fig. 1), water and food ad libitum (the dog diet composition is provided in the supplementary Table 1). Nymphs were maintained under natural lighting conditions, temperature and humidity, written at a later part of this study.

Nymphs were injected with 20 µL of saline or *S. aureus* suspensions (with a 1 ml syringe; Shandong Weigao Group Medical Polymer CO, China) at the base of the leg, in the second thoracic segment, close to the junction of the ventral and dorsal cuticles (Supplementary Fig. 2). The cockroaches were anesthetized on ice for about 6–10 minutes before injecting with saline or bacterial solution.

2.2. Bacterial preparation

Staphylococcus aureus subsp. aureus (ATCC® 25923™; USA) was utilized in this study. The strain was routinely preserved on Nutrient Agar (NA; peptic digest of animal tissue 5 g/L, beef extract 1.5 g/L, sodium chloride 5 g/L, yeast extract 1.5 g/L, agar 15 g/L; Himedia Laboratories, India) or Brain Heart Infusion (BHI) broth (calf brain infusion 200 g/L, beef heart infusion 250 g/L, proteose peptone 10 g/L, dextrose 2 g/L, sodium chloride 5 g/L, disodium phosphate 2.5 g/L; Himedia Laboratories, India), and stored at −2 °C. After overnight growth on NA plate at 37°C, the colonies were used to prepare bacterial suspensions diluted in 1% sterile saline. The concentrations of the bacterial suspensions were determined by measuring optical density at 600 nm (OD600). The bacterial suspensions were diluted with 1% saline to give adequate bacteria concentrations (colony forming units/mL-CFU/mL).

2.3. Survival assay

Nymphs were inoculated with 20 µL of saline (sham) or the indicated bacterial solutions. A not injected group was included as an

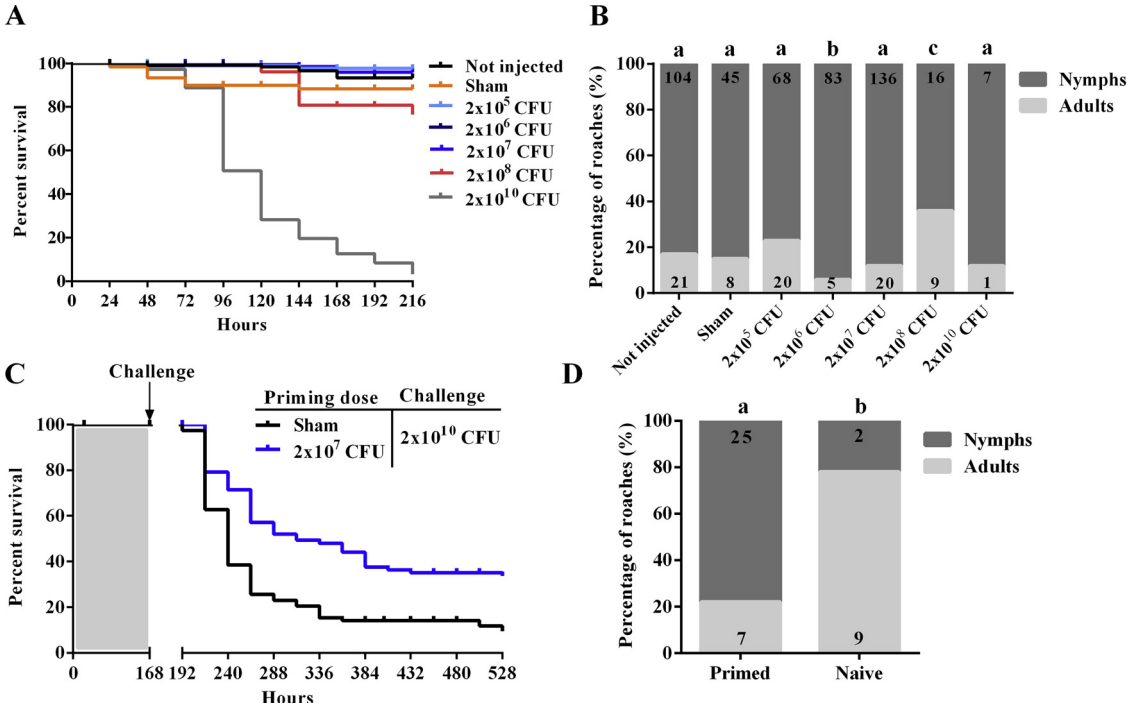

Fig. 1. Sensitivity of *N. cinerea* injected with *S. aureus* stored on NA. (A) Survival of nymphs infected with 2×10^5 CFU/cockroach (N = 90 nymphs), 2×10^6 CFU/cockroach (N = 90 nymphs), 2×10^7 CFU/cockroach (N = 163 nymphs), 2×10^8 CFU/cockroach (N = 30 nymphs), 2×10^{10} CFU/cockroach (N = 73 nymphs), sham (N = 60 nymphs) or not injected (N = 133 nymphs). The Log-rank (Mantel-Cox) test indicates significant differences in the survival of groups infected with higher concentrated doses ($p < 0.05$). (B) Percentage of nymphs and adults after 216 h (9 days) post-infection. The absolute number of cockroaches alive is inside the bars. A significant difference in the percentages of adults by Fisher's exact test (letters). (C) Survival of nymphs primed with 2×10^7 CFU/cockroach of *S. aureus* solution (N = 80 nymphs) to induce sepsis, or sham (saline) (N = 80 nymphs). Primed nymphs with bacteria were protected when challenged with a higher concentration of the same bacteria (2×10^{10} CFU/cockroach) after one week. The Log-rank (Mantel-Cox) test indicates that concentrations tested varied significantly in the survival of groups ($p < 0.05$). (D) Percentage of nymphs and adults after 528 h (22 days) post-infection. The absolute number of survived cockroaches is inside the bars. Statistical differences ($p < 0.05$) were determined by Fisher's exact test (letters).

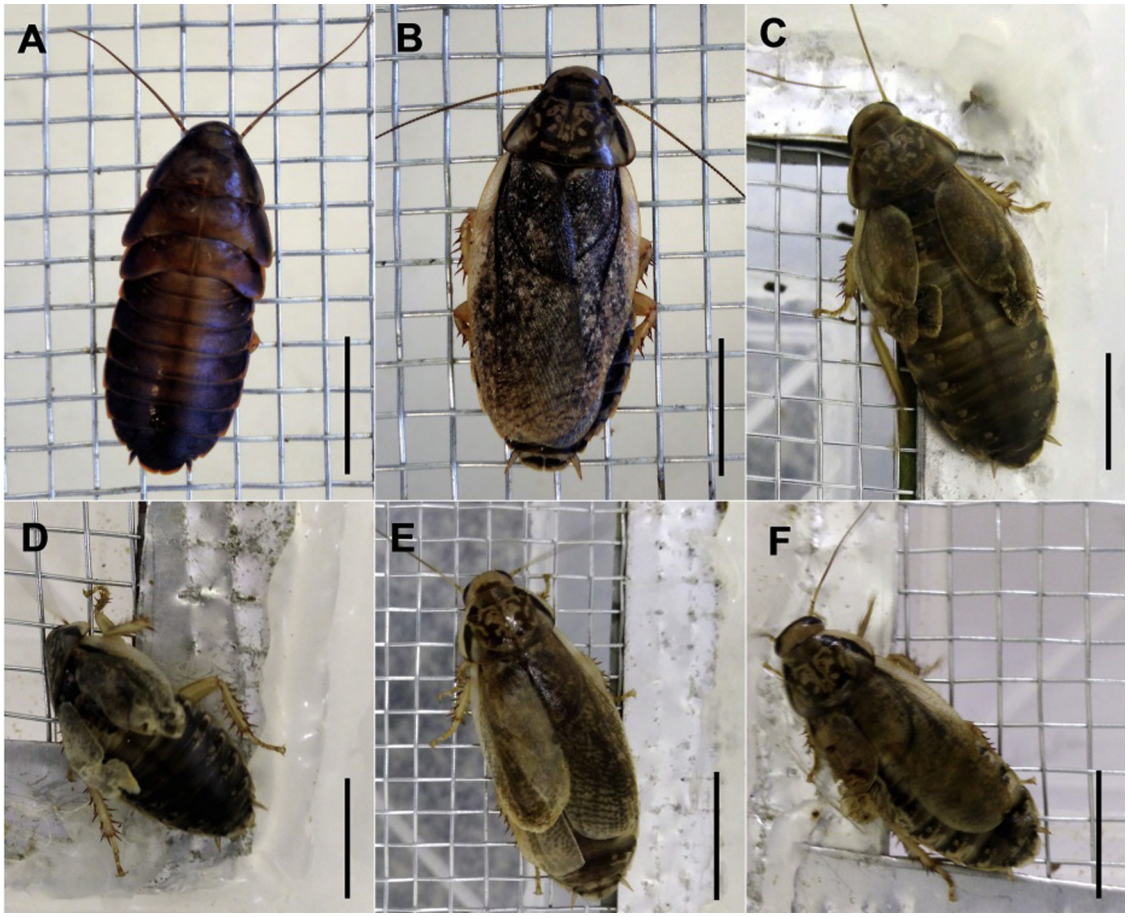

Fig. 2. Alteration in the phenotype of newly formed adults after molting of nymphs infected with higher concentrations of *S. aureus* (2×10^8 CFU/cockroach and 2×10^{10} CFU/cockroach) preserved in NA. (A) Normal nymph. (B) Normal adult. (C–F) Morphological alterations in adults from infected groups. Black lines represent 1 cm.

additional control group. A minimum of 30 nymphs was injected for each condition, and the experiments were performed at least two times.

2.3.1. Survival of nymphs injected with bacteria preserved on Nutrient agar

S. aureus, grown in NA plate at 37 °C for 24 h, were collected and diluted with sterile saline to give five different suspensions. The first solution (which contained the highest concentration of bacteria (10^{12} CFU/mL or 2×10^{10} CFU/cockroach) was diluted 20 times to obtain an OD_{600} 0.8, and the second solution (10^{10} CFU/mL or 2×10^8 CFU/cockroach) was diluted ten times to obtain an OD_{600} 0.8. The dilutions of the first and the second solutions were done to standardize the optical density to obtain 0.8, but the nymphs were injected with the concentrated solutions before being diluted. The third solution was diluted to obtain an OD_{600} 0.8 (10^9 CFU/mL or 2×10^7 CFU/cockroach), the fourth solution was diluted to obtain an OD_{600} 0.2 (10^8 CFU/mL or 2×10^6 CFU/cockroach), and the fifth solution was diluted to obtain an OD_{600} 0.1 (10^7 CFU/mL or 2×10^5 CFU/cockroach).

Test and control nymphs were maintained at a minimum temperature of 19.3℃ ± 1.5 and a maximum temperature of 24.8 ℃ ± 1.4 while minimum and maximum humidity were maintained at 42.8% ± 9.7 and 59.9% ± 12.9, respectively. The survival and molts (number of adults formed) were recorded daily for 216 h post-injection. Three independent tests were performed between July and August 2017.

2.3.2. Survival of nymphs injected with bacteria preserved on BHI

For the second set of survival tests, the same protocol for bacteria preserved and maintained in NA was utilized. The test and control nymphs were maintained at a minimum temperature of 21.4 ℃ ± 1.2 and a maximum temperature of 25.0 ℃ ± 1.6) and a minimum and maximum humidity of 54.7% ± 5.4 and 77.5% ± 6.1 respectively. The survival and molts (number of adults formed) were noted daily for 168 h post-injection and three independent tests were performed between August and October 2018.

2.4. Priming of cockroaches with S. aureus

Two priming suspensions of *S. aureus* were prepared: one preserved on NA and the other on BHI broth. They were grown on NA plate at 37 ℃ for 24 h. The solutions were prepared according to the same protocol described above. In the first week, two groups of nymphs of 15–30 nymphs were primed with 20 μL of saline or a bacterial suspension (corresponding to OD_{600} 0.8, i.e., 10^9 CFU/ml or 2×10^7 CFU/cockroach). The sham nymphs (not infected) were called naïve. Although, after the first week, both groups (sham and infected) were injected with 20 μL of a more concentrated bacterial suspension (containing 10^{12} CFU/ml or a total of 2×10^{10} CFU/cockroach).

Nymphs primed and challenged with the bacteria stored on NA were maintained at a minimum temperature of 20.9 ℃ ± 1.0 and a maximum temperature of 25.4 ℃ ± 1.9, and a minimum and maximum humidity of 45.6% ± 10 and 65.9% ± 11.78 respectively. The survival and the number of adults formed were observed daily, until 528 h post-injection. The test and replicates were performed in September 2017.

Nymphs primed and challenged with the bacteria stored on BHI broth were maintained at a minimum temperature of 21.3 ℃ ± 0.6, and a maximum temperature of 23.8 ℃ ± 0.5 and the minimum and

maximum humidity were maintained at 57.2% ± 5.0 and 77.6% ± 4.0 respectively. The survival and the number of adults were observed daily, until 312 h post-injection. The test and replicates were performed between September and October 2018.

2.5. Biochemical parameters

Biochemical tests were performed with nymphs injected with 10^{10} CFU/cockroach and 10^{7} CFU/cockroach as described above. The *S. aureus* used in the biochemical tests were stored on NA. Intact nymphs, and those injected with sterile saline represented the negative controls. The hemolymph and fat body were collected after 24 h, 48 h, and 72 h post-injection. The replicates at each of these periods (N = 7) represent a pool of tissues from three nymphs. We did the collections of tissues in August 2017.

The nymphs were anesthetized on ice for about 10 min, cleaned with distilled water and the hemolymph was collected after removing only one of the back legs. The hemolymph was collected in a microtube and immediately stored at -10 °C. The abdomen was cut open with a pair of surgical scissors, and a part of the fat body was carefully collected, using a pair of forceps and stored at -10 °C.

Glucose, cholesterol, and protein levels were measured with Labtest kits, according to the manufacturer's protocol, using 10 µL of hemolymph. Free amino acids were measured according to Spies [34], using 10 µL of hemolymph that was incubated with 1.5 mL of Ninhydrin 0.5% (diluted in isopropyl alcohol) and 490 µL of distilled water, for 15 min at 40 °C. The system was centrifuged at 1372 *g* for 10 min. After centrifuge, the supernatant was measured spectrophotometrically at 570 nm. The protein concentration of the fat body was determined by the method of Bradford [35], using 10 µL of the sample mixed with 40 µL distilled H2O and 2.5 mL Coomassie Brilliant Blue reagent. The system was centrifuged at 1372 *g* for 10 min, and the supernatant obtained was measured at 595 nm.

Lipid peroxidation (TBARS) was measured according to Buege and Aust [36]. The tissue samples were obtained by homogenizing the fat body of the cockroaches in 20 mM phosphate buffer (pH 7.5) in a ratio 1:2 (mg fat body: ml buffer), 0.5 g of the fat body sample was homogenized in 1 mL phosphate buffer (20 mM, pH 7.5). 500 µL of homogenate was mixed with trichloroacetic acid (TCA 10%) and thiobarbituric acid (TBA 0.67%) prepared in a water solvent. Samples were heated at 100 ^{0}C for 30 min, cooled and then centrifuged at 1372 *g* for 10 min, and the supernatant was measured at 535 nm. Data are expressed as nmol of malondialdehyde formed/mg of the fat body.

2.6. Measurement of bacterial burden in the hemolymph

For quantification of bacteria in the hemolymph, nymphs were injected with 20 µl of 10^{10} CFU/cockroach or 10^{7} CFU/cockroach. Bacteria were preserved on BHI broth and grown in NA as described previously in this study. The hemolymph of each nymph was collected after 24 h, 48 h, 72 h, and one-week post-injection. Here, 10 µL of the hemolymph was serially diluted in 1% sterile saline and spread on NA for 24 h at 37 °C. Sterility of the hemolymph from the control groups was confirmed by spreading them on the plate without dilution, and no growth was observed. The results are expressed in CFU/mL. The tests were performed between March and May 2018.

2.7. Data analysis

Statistical tests were performed using GraphPad Prism 6. The survival data were analyzed using the Kaplan–Meier method, and comparisons between groups were made using the log-rank test. Fisher's exact probability test was applied to compare the percentages of adults between intact, sham, and the other injected groups. Biochemical tests and CFU determination with the hemolymph were tested for normality D'Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov. Since the data had no normal distribution, Kruskal-Wallis test and Dunn's multiple post hoc comparisons were used. In all cases, $p < 0.05$ was considered significant. The data are expressed as mean ± standard error of the mean.

3. Results

3.1. N. cinerea is sensitive to bacterial conditions

Nymphs infected with different concentrations of *S. aureus* (2×10^{5}, 2×10^{6} and 2×10^{7} CFU/cockroach) maintained in NA did not present significant changes in survival when compared to the sham and control groups after one-week post-infection (Fig. 1A). In contrast, the treatment with 2×10^{10} CFU/cockroach caused a significant decrease in survival, which was evident from 96 to 216 h post-infection (Fig. 1A). Fig. 1B shows the percentage of nymphs that became adults at the end of the period of observation. New adults from the groups of nymphs infected with 2×10^{8} and 2×10^{10} CFU/cockroach presented different phenotype after molting, which encompassed morphological alteration in the wings and variation in pigmentation (Fig. 2). The adults of control, sham, and other lower bacterial concentrations groups did not present any abnormal phenotypes.

The survival curves of the second set of nymphs injected with bacterial solutions (2×10^{8} and 2×10^{10} CFU/cockroach), preserved on BHI broth, showed an increase in mortality when compared to the nymphs injected with bacterial solutions preserved on NA (Fig. 3A). The infection with 2×10^{8} CFU/cockroach killed 60% of the nymphs after 72 h post-infection (Fig. 3A). In this group, there were only nymphs at the end of one week (Fig. 3B). The concentration 2×10^{10} CFU/cockroach killed 80% of nymphs after 48 h, and after 96 h post-infection, there was no survival (Fig. 3A-B). Here, one of the nymphs molted to an adult; however, this adult did not survive.

It was possible to observe that the nymphs injected with the highest concentration of bacteria (2×10^{10} CFU/cockroach), stored on both culture mediums, displayed alterations in their behavior. The cockroaches avoided contact with each other, consumed less food, and remained isolated until their death. Consequently, they did not aggregate as the control and cockroaches injected with lower concentrations of bacteria (authors observations).

3.2. Immunization of N. cinerea is affected by bacterial conditions

Priming test showed that the nymphs immunized with the 2×10^{7} CFU/cockroach of *S. aureus* preserved on NA were able to tolerate a second and stronger challenge (2×10^{10} CFU/cockroach) when compared to the naive group (Fig. 1C). The sham group had a 60% decrease in survival at 72 h post-infection, whereas in the primed group with a low quantity of bacteria the mortality decreased to 30%. After 15 days of the second challenge, the survival of primed nymphs was higher (40%, n = 32) than the naive group (11%, n = 9). At the end of this experiment, the number of newly formed adults after molting was significantly lower for the primed cockroaches, but the naive group had more newly formed adults (Fig. 1D).

This protection did not occur in the nymphs primed with bacteria preserved on BHI broth. It was possible to observe a delay in the mortality of the primed group, although all the cockroaches died in one week post-infection (Fig. 3C).

3.3. Changes in the metabolites of nymphs infected with S. aureus stored on NA

The analysis done with hemolymph of the nymphs indicated that all biochemical parameters tended to decrease in the nymphs infected with the concentrations 2×10^{7} and 2×10^{10} CFU/cockroach when compared to the control nymphs. Free amino acid levels significantly decreased after 24 h and 72 h post-infection in the nymphs infected with

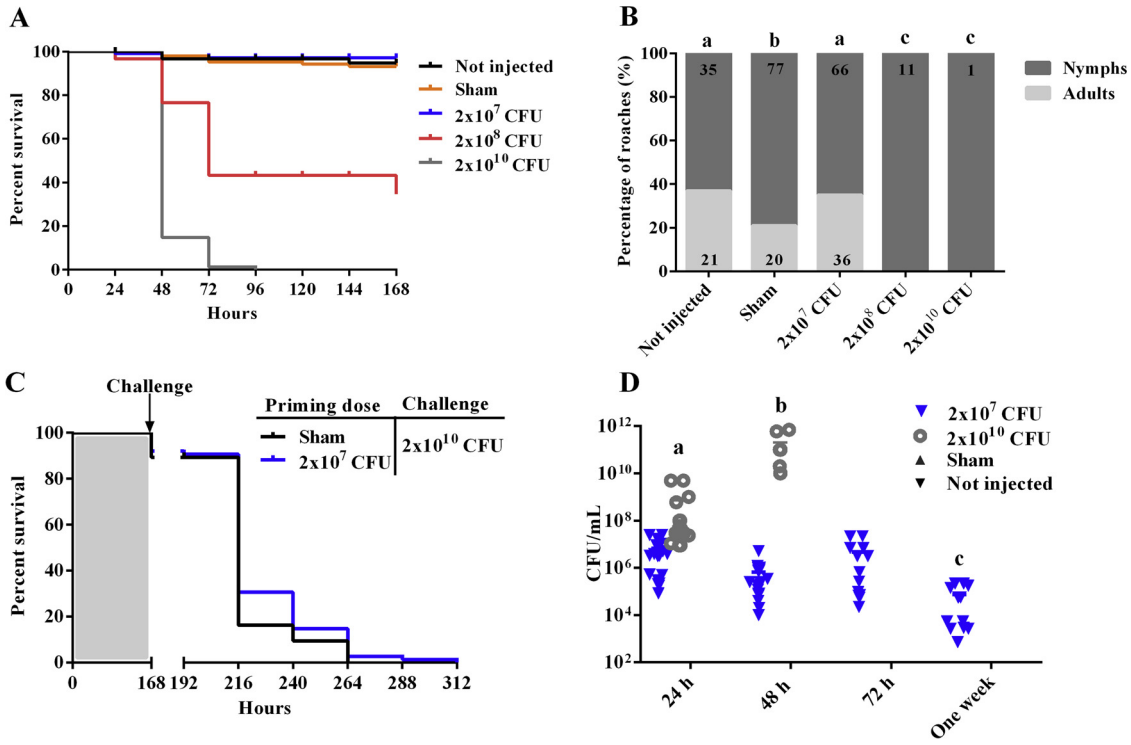

Fig. 3. The sensitivity of *N. cinerea* after infection with *S. aureus* preserved on BHI broth. (A) Survival curves of nymphs infected with 2×10^7 CFU/cockroach (N = 105 nymphs), 2×10^8 CFU/cockroach (N = 30 nymphs), 2×10^{10} CFU/cockroach (N = 75 nymphs), sham (N = 105 nymphs) or not injected (N = 60 nymphs). The Log-rank (Mantel-Cox) test indicates significant differences in the survival of groups infected with higher concentrations ($p < 0.05$). (B) Percentage of nymphs and adults after 168 h (7 days) post-infection. The absolute number of cockroaches alive is inside the bars. Statistical differences ($p < 0.05$) were determined by Fisher's exact test (letters). (C) Survival curves of *N. cinerea* primed with 2×10^7 CFU/cockroach (N = 75 nymphs) or sham (N = 75 nymphs). Primed nymphs with bacteria were not protected when challenged with a higher concentration of the same bacteria (2×10^{10} CFU/cockroach) after one week. The Log-rank (Mantel-Cox) test indicates that concentrations tested varied significantly in the survival of groups ($p < 0.05$). (D) Viable *S. aureus* in the hemolymph of nymphs infected with 2×10^7 CFU/cockroach or 2×10^{10} CFU/cockroach was assessed by serial dilution and spread on the plate. The bacteria load was verified within the first three days and on the 7th day post-infection. The sterility of the hemolymph of the sham and nymphs that were not injected were confirmed. Statistical differences ($p < 0.05$) were determined by Kruskal-Wallis test (< 0,0001) and Dunn's multiple comparisons post hoc test (letters). Data represent mean and standard error of mean of nymphs infected with 2×10^7: 24 h (N = 19 nymphs), 48 h (N = 20 nymphs), 72 h (N = 14 nymphs), and one week (N = 12 nymphs); Bacterial solution 2×10^{10}: 24 h (N = 17 nymphs), and 48 h (N = 8 nymphs).

2×10^{10} CFU/cockroach, while the nymphs injected with 2×10^7 (Fig. 4A) showed a decrease in free amino acids only after 48 h post-injection. The cholesterol and glucose levels decreased significantly in the nymphs infected with 2×10^{10} CFU/cockroach along the time (Fig. 4B-C). The total proteins in hemolymph were also considerably reduced in the infected nymphs until 48 h post-infection (Fig. 4D). The levels of proteins in the fat body significantly increased in the infected groups when compared to the control during the first 24 h, and still increased at 72 h post-infection for the nymphs infected with 2×10^7 (Fig. 5A). The malondialdehyde levels in the fat body were higher in the infected groups until 48 h, when compared to control; however, this was not statistically significant (Fig. 5B). There was no significant difference in the biochemical parameters assayed for using the hemolymph and the fat body between the control and sham groups (data not shown).

3.4. Determination of CFU in the hemolymph of nymphs infected with S. aureus stored on BHI broth

The nymphs injected with 2×10^7 CFU/cockroach displayed a significant decrease in the microbial content along the time. However, those injected with 2×10^{10} CFU/cockroach had a significant increase in CFU until death (Fig. 3D). After 48 h, some nymphs infected with the highest concentration of bacteria started the decomposition of tissues (with a bad smell), and the collection of hemolymph was compromised due to decay.

4. Discussion

The present study demonstrated that thoracic injection of *S. aureus* killed nymphs of *N. cinerea* depending on the bacteria culture medium preservation, caused morphological abnormalities in newly formed adults, decreased the levels of metabolites in the hemolymph, altered the locomotion and social behaviors. The bacterial load in the hemolymph of infected nymphs increased which could have resulted in the mortality of the cockroaches that received the highest concentration (2×10^{10} CFU/cockroach), or decreased in the cockroaches that received 2×10^7 CFU/cockroach. Similar results were reported in silkworm larvae infected with *S. aureus* [7] and *D. melanogaster* infected with *S. pneumoniae* [37]. According to literature, living *S. aureus* can multiply locally and then gradually spread to the entire body of the organism [6]. However, no significant alterations in survival were observed in insect models that received dead *S. aureus* orally (1.9×10^9 CFU) [38], injected into the thorax (10^8 and 10^{5-7} CFU) [7,39], abdomen (8×10^6 CFU) [14] and injected with exoproteins formed in the log phase of growth [40] (Supplementary Table 2).

Nymphs primed with a sublethal concentration of bacteria (2×10^7 CFU/cockroach), preserved on NA, were protected against a second and stronger challenge with the same bacteria (2×10^{10} CFU/cockroach) administered one week later (Fig. 1C). Some plausible explanations on how *S. aureus*–primed nymphs were protected from a stronger challenge are: 1) primed nymphs acquired the ability to recognize and kill the bacteria faster than sham, increasing the survival; 2) primed

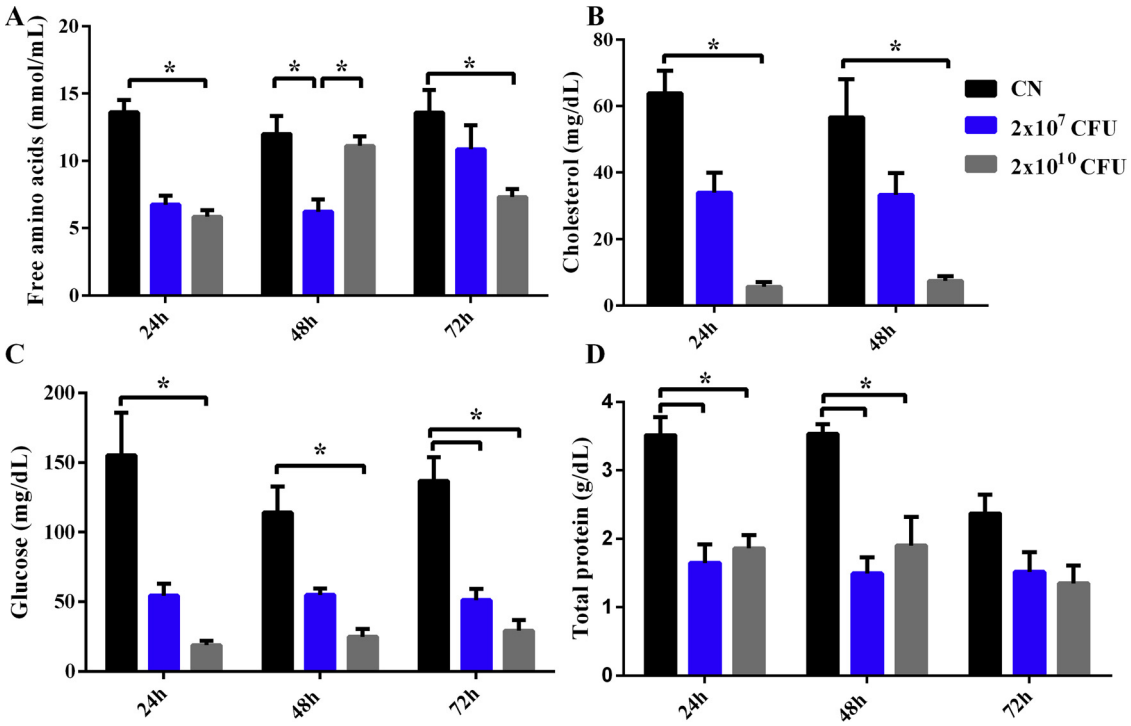

Fig. 4. Levels of metabolites in the hemolymph of nymphs injected with two concentrations of *S. aureus*, stored on NA, 2×10^7 CFU/cockroach (N = 63 nymphs) and 2×10^{10} CFU/cockroach (N = 63 nymphs), or not injected (control or CN) (N = 63 nymphs). (A) Free amino acids, (B) Cholesterol, (C) Glucose, and (D) Total proteins were measured after 24 h, 48 h, and 72 h post-infection. Statistical differences ($p < 0.05$) were determined by Kruskal-Wallis and Dunn's multiple comparisons test (*). Data are representative of 7 samples (pool of 3 nymphs) for each time and treatment.

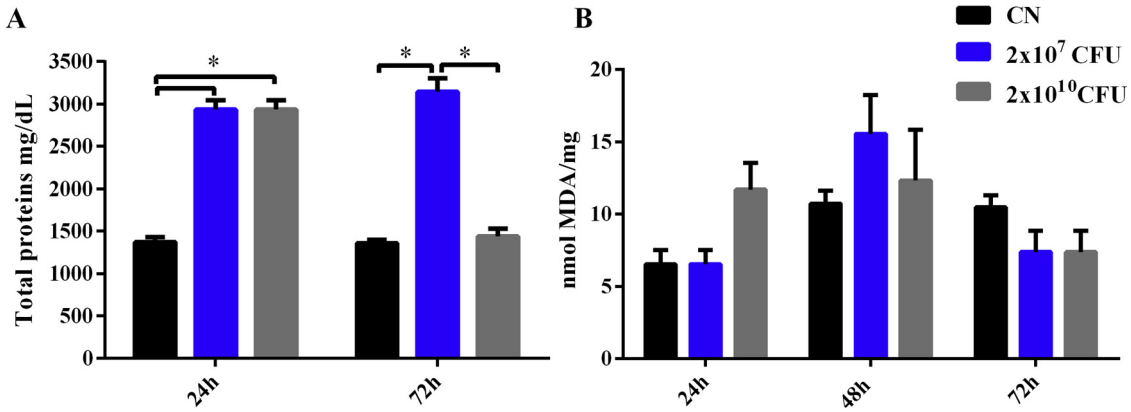

Fig. 5. Levels of metabolites in the fat body of nymphs injected with two concentrations of *S. aureus*, stored on NA, 2×10^7 CFU/cockroach (N = 63 nymphs) and 2×10^{10} CFU/cockroach (N = 63 nymphs), or not injected (control or CN) (N = 63 nymphs). (A) Total proteins and (B) Malondialdehyde (MDA) were measured after 24 h, 48 h, and 72 h post-infection. Statistical differences ($p < 0.05$) were determined by Kruskal-Wallis and Dunn's multiple comparisons test (*). Data are representative of 7 samples (pool of 3 nymphs) for each time and treatment.

nymphs could be able to tolerate a higher load of *S. aureus* after a second injection that was highly lethal to sham nymphs [41]; and, 3) the presence of CFU in the hemolymph of nymphs, one week after the injection of 2×10^7 CFU/cockroach (Fig. 3D), may indicate a persistent production of immune effectors to combat the infection, contributing to enhancing their survival. However, nymphs primed with *S. aureus*, preserved on BHI broth (2×10^7 CFU/cockroach) or saline, and challenged with a second and stronger concentration (2×10^{10} CFU/cockroach) after one week were not protected. We suggest that the variation in the composition of bacterial preservation media could have affected the metabolism of *S. aureus*, contributing to the up-regulation or suppression of virulence genes expression, as observed in other studies [42–46]. In this case, immune molecules produced by nymphs after priming were not able to increase the tolerance. Further, the combination of high quantities of bacteria and the virulence of *S. aureus* led to a decrease in resistance, the ability to reduce the parasite load, and consequently a reduction in the tolerance. Since the only difference in the *S. aureus*-induced sepsis was the bacterial preservation medium, *N. cinerea* appears to be sensitive to the variations in the bacteria metabolism in both medium utilized.

The decrease in the metabolites (glucose, cholesterol, amino acids, and proteins) in the hemolymph were similar in the infected groups until 72 h post-infection. In opposite, *Drosophila* infected with *S. aureus* had no alteration in glucose levels, and a significant increase in the protein levels after 18 h, measured in the body [47]. The protein levels in the hemolymph were also high for the *B. mori* between 6 h and 24 h after the infection with *S. aureus* [48]. Although, the immune-metabolic demand usually increases the catabolism of glucose, amino acid, and

protein [49], which might lead to the depletion of the metabolites in the hemolymph of the cockroaches. The fat body is the primary producer of proteins [50], so the high levels of proteins in this tissue indicate intensive protein synthesis, which could be essential to guarantee the survival of the nymphs injected with the sublethal concentration of bacteria. Of note, the catabolism of amino acids and proteins are essential sources of substrates for carbohydrate synthesis and antimicrobial peptides production, in the fat body [50,51].

The considerable decrease in cholesterol levels in the hemolymph of infected groups can be related to cholesterol utilization by the host and the bacteria. Insects do not synthesize cholesterol but obtain it from the diet [52–55]. Several bacteria and pathogenic microorganisms also lack cholesterol synthesis, and they are cholesterol-host-dependent [56]. Besides, the nymphs infected with the highest concentration of *S. aureus* had a reduction in food consumption (personal observation), which could have contributed to the decrease in glucose and cholesterol availability.

Cholesterol is involved in the synthesis of several hormones, especially those involved in the molting process, maintenance of the integrity of cell membranes and tissues of the entire organism [55]. The malformation of newly formed adults may also be a consequence of the decrease in cholesterol levels, deprivation of food and the metabolic dysregulation, food deprivation led to reduced immune response and lower activity of phenoloxidase (PO), in *T. molitor* [57]. The enzyme PO participates in the production of melanin, a pigment substance used in the immune response (encapsulation of pathogens), wound healing, hardening of the cuticle, and exoskeletal pigmentation [58]. The injection of high quantities of bacteria in the cockroaches, as well as the possible production of virulence molecules by bacteria, could inhibit PO activity, preventing the melanization [59], which might compromise the integrity of the new cuticle synthesis.

The advance in ecdysis was observed in the crustacean *Daphnia magna* [60] and *Gammarus pulex* [61], in response to the high risk of infection caused by wounding, as an adaptation to different environmental conditions. Here, we could not conclude a clear relation between infection and the number of new adults formed in the groups of infected nymphs when compared to sham or not injected nymphs.

Immune response in insects against gram-positive bacteria involves the classical signaling Toll pathway and NF-κB–induced systemic production of antimicrobial peptides [19,47,62]. Humoral and cellular mechanisms need to be explored in the cockroach *N. cinerea*. Genes related to stress and detoxification in the head and the fat body of cockroaches *N. cinerea* specimens were evaluated using a Transcriptomic analysis developed by Segatto *et al.* [63]. The molecular information about the transcriptome can be a powerful tool to identify the expression of the immune elements in the cockroach.

5. Conclusion

Several issues about the immune response in insects remain unknown. Most studies were conducted using holometabolous insects (Diptera and Lepdoptera), but we try to explore the immune response of the hemimetabolous cockroach *Nauphoeta cinerea*. In essence, we demonstrated that this cockroach is an attractive alternative for researches in immunology. The characterization of molecular and cellular mechanisms are necessary to underlie the mechanisms of host-pathogen interaction, involved in bacterial infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101343.

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