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RESEARCH REPORT

Pathological changes in the main immune organs of silkworm infected with *Staphylococcus aureus*

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Abstract

Silkworm *Bombyx mori* (*B. mori*), a lepidopteran model organism, has become an important model for molecular biology. It also offers an excellent model to study the innate immunity because of its similarity to human beings. *Staphylococcus aureus* (*S. aureus*) is a typical gram-positive pathogenic microorganism that causes serious pneumonia, meningitis, endocarditis and septicemia. In this study, silkworm was used as animal model to study the innate immune responses against the pathogenic bacterial infections. We investigated the median lethal dose (LD_{50}) of *S. aureus* infection in the silkworm, and the pathological changes in their hemolymph and midgut after infection. The LD_{50} of *S. aureus* infecting the silkworms was 4.39×10^4 colony-forming units per milliliter (CFU/mL) after 72 h. The peritrophic membrane of the silkworm showed severe damage after 36 h. Insect hemocytes can participate in various innate immune responses, such as encapsulation and nodule formation. Our results imply plasmacytes of hemocytes can adhere to and spread over *S. aureus* in the hemolymph and may play an important role in the resistance of the silkworms to *S. aureus* infection.

Key Words: *Bombyx mori; Staphylococcus aureus;* hemolymph; midgut

Introduction

Silkworm (*Bombyx mori*) breeding has a long history, and silk is an indispensable textile, with an important part of the commodity trade. The silkworm not only has economic value, but is also used as a model organism in biological research. For instance, the toxicity of pesticides to silkworms is often used as an indicator of the effects of chemical pesticides on the ecological environment (Wang *et al*., 2011). The growth of silkworms fed mulberry leaves steeped in a liquid suspension of genetically modified pollen has been used as a reference in studies of the effects of transgenic foods (Li *et al*., 2002).

The silkworm is an ideal insect for immune studies due to its well-characterized innate immune system in which many genes are known to have a role in controlling the immune response (Tanaka *et al*., 2008). The immune system of the silkworm mainly involves humoral and cellular immunity. Humoral immunity refers to the recognition of pathogens in the insect hemolymph, the subsequent cascade of proteases, the antibacterial peptides induced, phenoloxidase, the intermediate products of melanization, and other immune factors present in the body fluid. In insects, 'cellular immunity' refers to phagocytosis, nodulation, encapsulation, and other functions mediated by hemocytes (Stokes *et al*., 2015; Wang *et al*., 2017). There are 21 immune-related gene families or superfamilies in the silkworm genome, including 218 associated with pattern recognition, signal regulation, or effector molecules, which provide important references in the study of the immune systems of lepidopteran insects (Tanaka *et al*., 2011; Tanaka *et al*., 2018).

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that is widely distributed in nature and is commonly present in air, soil, and water. It is an important pathogen in bacterial food poisoning and one of the pathogens that cause bacterial diseases in the silkworm. When the silkworm consumes mulberry leaves, microorganisms enter the intestine through the mouth with the food, causing intestinal epithelial cells to contact a large number of microorganisms, often causing infection (Kurokawa *et al*., 2007; Hiroki *et al*., 2019). When silkworm was infected with *S. aureus*, the expression of genes related to immunity was altered, including genes encoding peptidoglycan recognition proteins and the class C scavenger receptor BmSR-C (Wang *et al*., 2016); the

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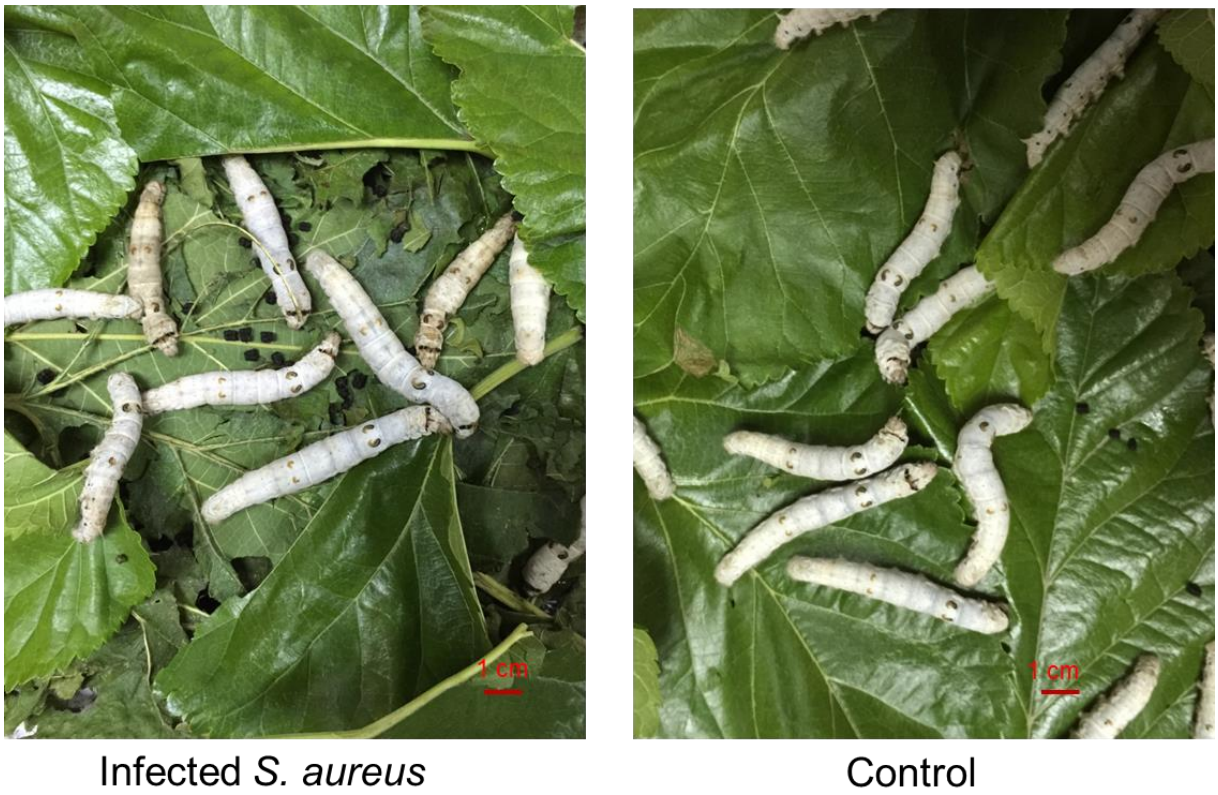

Fig. 1 State of silkworm infected with *S. aureus*

expression of antimicrobial peptides in the midgut and fat body was increased (Wu *et al*., 2010); and the concentration of nitric oxide (NO) in the hemolymph was changed (Zhang *et al*., 2015).

In this study, after silkworms were fed *S. aureus*, we determined the median lethal dose (LD_{50}) of *S. aureus*, and detected the pathological changes in the midgut and hemocytes of the silkworms after infection.

Methods

Silkworm rearing

The silkworm strain Qingsong × Haoyue was used in this study. Fertilized silkworm eggs were maintained in containers at 25 °C. Hatched larvae were reared to the 5th instar on mulberry leaves at 25 °C and 80% humidity, under 12 h light/12 h dark cycle for their entire lifespans (Pan *et al*., 2017).

Silkworm infection

Two-day-old 5th instar larvae (L5D2) were randomly divided into two groups (30 larvae per group, three experimental replicates), the first of which was injected with 5 μL of 1×10^{10} CFU/mL *S. aureus* (ATCC 26085) and the second with phosphate-buffered saline (PBS), as the control.

Determination of LD_{50}

The LD_{50} of *S. aureus* in the silkworm was determined by counting the number of deaths in each group of 15 silkworms 72 h after they were injected intraperitoneally with a serial l0-fold dilution of *S. aureus* suspension ($l0^2$-$l0^{10}$ CFU/mL). The LD_{50} was calculated with the Reed–Muench method.

Hemolymph smear

Hemolymph was collected from the larvae at 12, 24, and 36 h after infection with *S. aureus* and quickly spread on a glass slide. The hemolymph was allowed to dry naturally and was stained with Wright-Giemsa stain for 1 min. Phosphate buffer solution (pH 6.4) was added, and the slide was shaken gently and left at room temperature for 5-10 min. The slide was washed, dried and observed with interference fluorescence microscopy.

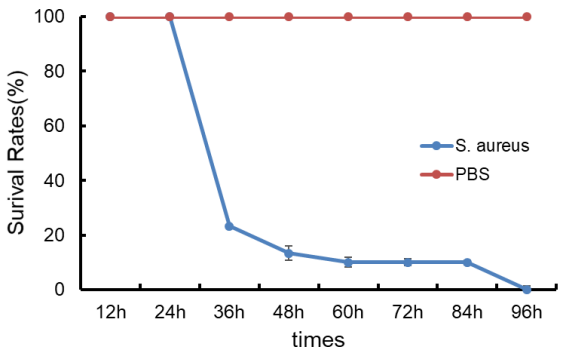

Fig. 2 Survival curve of silkworms infected with *S. aureus*

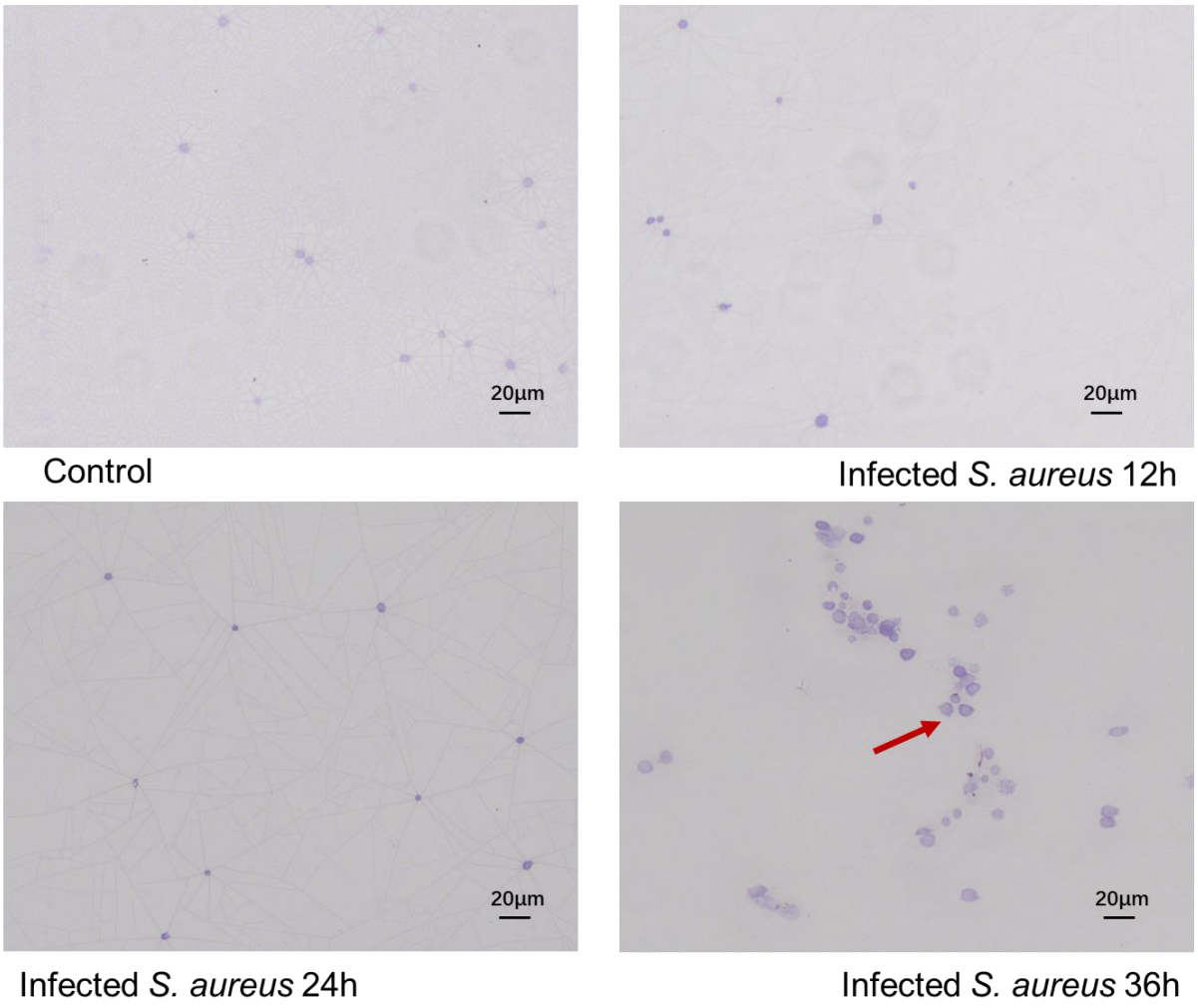

Fig. 3 Cell smears of silkworm hemolymph infected with *S. aureus*. There were several large cells in the silkworm hemolymph 36 h after infection with *S. aureus*

Electron microscopic analysis of silkworm hemolymph cells

Hemolymph (10 μL) was collected from the larvae at 12 h after infection with *S. aureus* and quickly spread on a glass slide. Fixing solution (2.5% glutaraldehyde, pH > 7.2) was added dropwise and the slide incubated for 30 min. The slide was washed three times with 0.1 M phosphoric acid buffer, dehydrated with a graded series of alcohol, and placed in a vacuum dryer to dry the sample. The dried sample was then sprayed with gold, and observed and photographed with a Hitachi S-3400N scanning electron microscope (Hitachi, Japan).

Paraffin sectioning

At 12, 24, and 36 h after *S. aureus* infection, the midgut and fat body tissues of the silkworms were dissected, and fixed in 10% neutral-buffered formalin for 2 weeks. Sections (6 μm) were cut, mounted on glass slides, dewaxed in xylene, rehydrated through a graded alcohol series, washed in distilled water, and stained with hematoxylin and eosin. All slides were examined and photographed under a Leica DMI4000 B microscope (Leica, Wetzlar, Germany).

Results

LD_{50} of S. aureus *infection in* B. mori

As the period of silkworm infection with *S. aureus* increased, the crawling speed of the silkworms slowed, their leaf consumption gradually decreased, and the body wall became grayish brown (Fig. 1). When the survival rate after infection with *S. aureus* was determined, the results indicated that the silkworms began to die 24 h after infection, only 23.3% of silkworms were alive at 36 h after infection, and all the silkworms had died within 96 h of infection (Fig. 2). The LD_{50} of *S. aureus* in silkworms at 72 h was calculated with the Reed–Muench method to be 4.39×10^4 CFU/mL.

Effect of S. aureus *infection on* B. mori *hemocytes*

When the hemolymph of silkworms infected with *S. aureus* was subjected to a smear test, we found the number of large cells in the hemolymph had increased at 36 h after infection (Fig. 3). The large cells were then identified by electron microscopy as plasmatocytes (account for 10% of total cells), which adhered to *S. aureus* with many pseudopodia (Fig. 4).

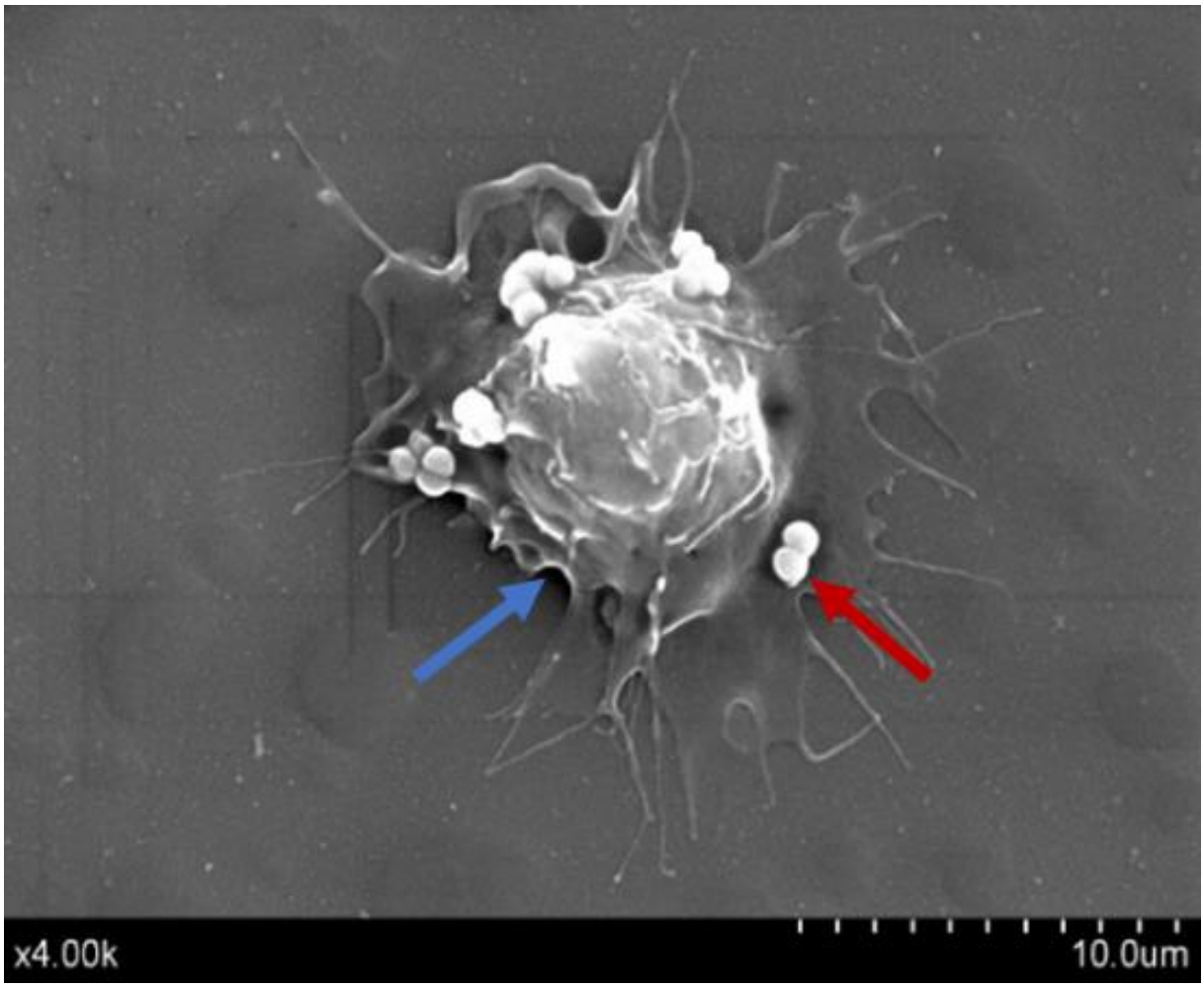

Fig. 4 Electron micrograph of phagocytosis of *S. aureus* by a silkworm plasmatocyte. *S. aureus* indicated by red arrow, plasmatocytes indicated by blue arrow

Plasmatocytes adhere to and spread over foreign bodies and are the main capsule-forming hemocytes. We speculated that these plasmatocytes play an important role in the cellular immunity of silkworms infected with *S. aureus*.

Effect of S. aureus *infection on* B. mori *midgut*

The peritrophic membrane of the silkworm midgut plays an important role in protecting the midgut and in the immune defense of the larva, and is an effective physical barrier in the silkworm. After the larvae had been infected with *S. aureus* for 36 h, the peritrophic membrane of the midgut was broken and dispersed, indicating that its protective function was abolished (Fig. 5).

Discussion

Wound infection is one of the ways that *S. aureus* invades the body, and can cause skin infection, suppuration, and even sepsis. We prepared the infection model by the way of silkworm epidermal puncture to simulate the invasion of *S. aureus* into the body due to trauma, and the silkworm has a high mortality rate and an LD_{50} of 4.39×10^4 CFU/mL at 72 h.

The hemolymph is an important component of the cellular immune system of the silkworm (Garsin *et al*., 2003; Tan *et al*., 2013). There are five kinds of cells in the hemolymph: prohemocytes, granulocytes, plasmatocytes, oenocytoids, and spherulocytes (Liu *et al*. 2013; Zhang *et al.* 2022.). Many genes may be involved in the immune reaction process of silkworm hemolymph against the invasion of bacteria. Such as BmSR-C could bind to both Gram-positive bacteria in hemocytes (Zhang *et al*. 2021), BmHDD1 could induced after injected with different types of bacteria, it was mainly generated by hemocytes and secreted into hemolymph (Zhang *et al*. 2017).

Plasmatocytes is one of the most abundant cell types in hemolymph, can participate in various innate immune responses, especially in encapsulation and node formation. *Bmintegrin β3* of plasmatocytes may triggered by *S. aureus* infection of silkworm, it may relate to the extensibility and adhesion of plasmatocytes cells (Zhang *et al*. 2017;

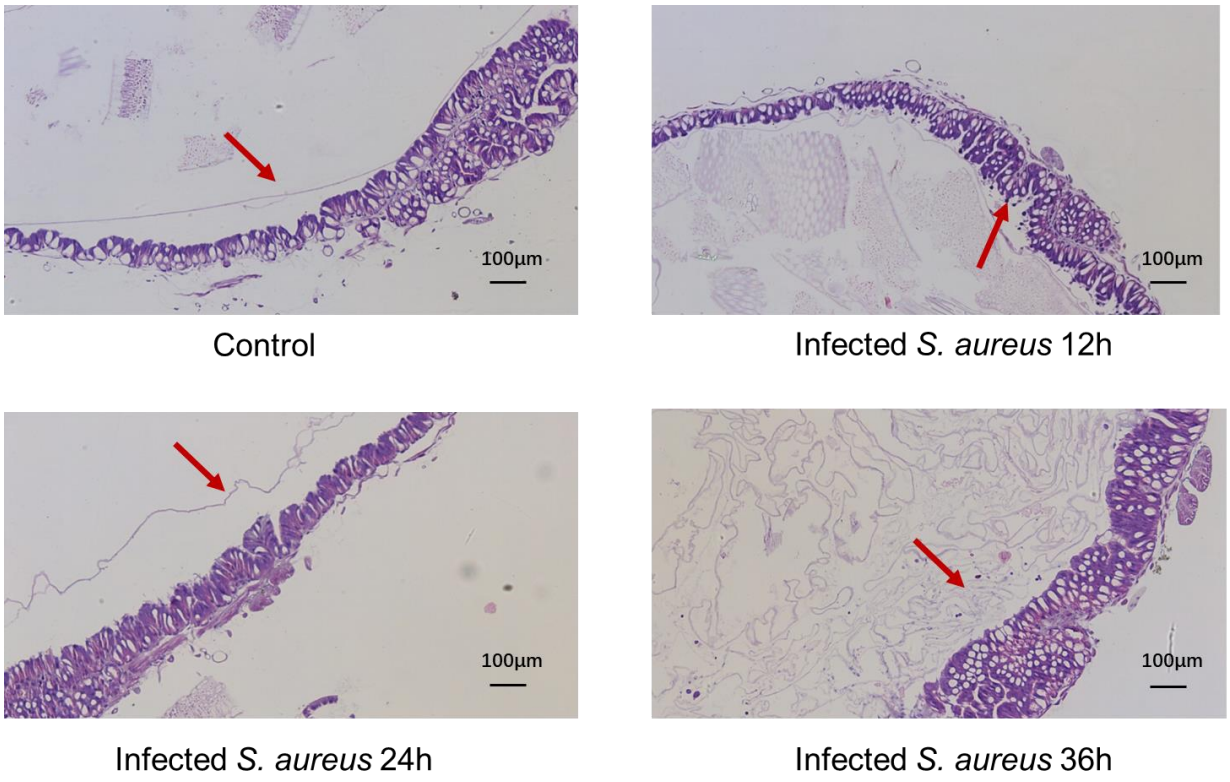

Fig. 5 Tissue sections of silkworm midgut after infection with *S. aureus*. Red arrows indicate the peritrophic membranes. Peritrophic membrane was cracked at 36 h after *S. aureus* infection. In the control group injected with PBS, the peritrophic membrane was intact

Zhang *et al.* 2022). By scanning electron microscopy, the plasmatocytes were identified to play an important role in the resistance of silkworms against *S. aureus* infection. They are large, with a diameter of 10-25 μm, and have filamentous and lamellar pseudopodia, it also showed more resistant than other hemocyte morphotypes to *B. mori* nucleopolyhedrovirus infection (Hori *et al*., 2013).

The midgut is an important immune organ of insects, and the peritrophic membrane of the silkworm larva is located in the midgut. It is a colorless, transparent tubular structure composed of protein, chitin, and acid mucopolysaccharide (Mltsuhashi *et al*., 2007; Yang *et al*., 2010). Its main function is to protect the cells of the intestinal wall. When silkworm was infected with *S. aureus* for 36 h, the peritrophic membrane showed severe damage, and the larval survival rate was <20%, it indicates that *S. aureus* has infected midgut tissue.

In conclusion, the silkworm is susceptible to *S. aureus* infection, with a low survival rate at 72 h after infection. *S. aureus* infection damages the silkworm midgut and causes peritrophic membrane rupture. The plasmacytes in the silkworm hemolymph can capture *S. aureus*, and may play an important immune role in *B. mori* against *S. aureus* infection.

Acknowledgements

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