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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Is *Galleria mellonella* model a good alternative to study virulence in *Staphylococcus aureus* from bovine mastitis?

Juliana Rosa da Silva[1] · Joice Fátima Moreira Silva[1] · Monalessa Fábia Pereira[2] · Adalgisa Ribeiro Torres[3] · Maysa Serpa Gonçalves[1] · Márcia Cristina de Azevedo Prata[3] · Maria Aparecida Vasconcelos Paiva e Brito[3] · Geraldo Márcio da Costa[1] · João Batista Ribeiro[3]

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Abstract

Staphylococcus aureus is one of the agents of bovine mastitis of hardest control due to a complex pathogenesis comprising a variety of virulence factors, which ensures its persistence in the mammary gland, causing significant health and economic losses. Therefore, understanding the pathogenesis of this agent is imperative. *Galleria mellonella* has stood out as an invertebrate animal model for the study of infectious diseases that affect several hosts. This work aimed to evaluate *G. mellonella* larvae as an experimental model for the study of virulence phenotypes in an *S. aureus* population isolated from bovine mastitis. Thirty genetically divergent *S. aureus* strains were chosen based on PFGE analysis. After experimental infection, larvae survival rates, bacterial growth in hemolymph, melanization intensity of the dorsal vessel, and histological characteristics of the infected tissues were evaluated. The *G. mellonella* model showed a clear diversity in the *S. aureus* pathogenicity pattern, allowing the differentiation of strains with virulence phenotypes ranging from high to low degrees. Histological analysis confirmed that the strains tested were capable of inducing the formation of nodules and melanization spots in the dorsal vessels of the larvae in different magnitudes. The strains 16S-717, 19C-828, and 31S-1443 presented the highest virulence intensity among the bacteria tested and will be used further for the generation of *S. aureus* mutant populations to prospect genetic targets aimed to develop control strategies of bovine mastitis. Altogether, our results suggest that *G. mellonella* is an attractive and low-cost animal model for characterizing virulence phenotypes of large *S. aureus* populations.

Keywords Bovine diseases · Intramammary infection · Larvae model · Bacterial virulence · Invertebrate model

Responsible Editor: David Germano Gonçalves Schwarz

✉ Geraldo Márcio da Costa
gmcosta@ufla.br

✉ João Batista Ribeiro
joao-batista.ribeiro@embrapa.br; jbatistar@yahoo.com

Juliana Rosa da Silva
julianarosadasilva123456@gmail.com

Joice Fátima Moreira Silva
joicefmsbt@gmail.com

Monalessa Fábia Pereira
monalessa.pereira@uemg.br

Adalgisa Ribeiro Torres
artorres_2@hotmail.com

Maysa Serpa Gonçalves
MaysaSerpa@gmail.com

Márcia Cristina de Azevedo Prata
marcia.prata@embrapa.br

Maria Aparecida Vasconcelos Paiva e Brito
mavpaiva@gmail.com

[1] Department of Veterinay Medicine, Federal University of Lavras, Lavras, MG 37200-000, Brazil

[2] Department of Biological Sciences, State University of Minas Gerais, Carangola, MG 36800-000, Brazil

[3] Brazilian Agricultural Research Corporation, Juiz de Fora, MG 36038-330, Brazil

Introduction

Staphylococcus aureus is responsible for multiple pathologies in humans and animals. Bovine mastitis is one of the most frequent and economically important diseases in dairy cattle due to its large economic impact on dairy farms and industries [1]. The losses caused by this disease are related to the drop in milk production, milk disposal from animals under treatment, high drug costs, early replacement of cows, and eventually death. In dairy cows, losses result from changes in the physicochemical characteristics of milk, such as reduced fat and casein, in addition to an increase in the somatic cell count, leading to the loss of dairy product yield. Mastitis is also important from the aspect of impaired animal welfare [2].

S. aureus is among the most common etiological agents of mastitis in Brazil [3–5] and worldwide [6], being responsible for clinical, subclinical, and chronic cases with occasional difficult-to-control clinical manifestations [7], due to the complex pathogenesis, involving multiple virulence factors that favor its survival and multiplication in mammary gland [8]. A better understanding of the infection process by *S. aureus* in the udder microenvironment is needed in order to increase knowledge of the pathogen-host relationship, which will enable the development of more effective forms of control and prevention of the disease.

The severity of *S. aureus* infection generally depends on the expression of several virulence genes. At the outset, surface adhesins recognize host structures, facilitating colonization and subsequent multiplication of the microorganism [9]. Moreover, different enzymes (e.g., hyaluronidase, proteases, and nucleases), non-enzymatic activators (e.g., coagulase or staphylokinase), and exotoxins (e.g., cytolytic toxins, exfoliative toxins, leukocidins, enterotoxins, enterotoxin-like proteins, and toxin-1 toxic shock syndrome) promote bacterial escape from the host immune response [10]. This sequential expression of virulence factors seems to be crucial to the pathogenesis of mastitis caused by *S. aureus* [11].

Among the main virulence factors in *S. aureus*, the components of the microbial surface that recognize matrix adhesive molecules (MSCRAMMs) have been widely studied [12]. They are proteins that promote the adhesion of *S. aureus* to host tissues, including clumping factors A and B, encoded by the genes *clfA* and *clfB*, fibronectin-binding proteins (*fnbps*), collagen-binding adhesin (*cna*), and elastin-binding protein (*ebps*). Some staphylococcal toxins weaken the host response as they degrade host cells and manipulate the innate and adaptive immune responses, which allows pathogen escape and contributes to the proliferation of *S. aureus*, such as hemolysin (e.g., *hla hlb*), and leukotoxins (e.g., *luk- ED*) [13].

Studies on *S. aureus* virulence have been performed in several models, such as cell culture [14], mammary gland tissue (explant) [15], vertebrates, such as mice [16] and rabbits [17], and invertebrates, such as *Drosophila melanogaster* [18], *Tenebrio molitor* and *G. mellonella* [19]. Invertebrate models are cheaper and easier to establish and maintain in laboratories in comparison to vertebrate ones. Besides these benefits, invertebrates can be reared on a large scale and are not subject to the same ethical considerations [20].

G. mellonella larvae have been widely used to study pathogenesis and virulence for different microbial species because, in addition to the aforementioned advantages, they allow incubation at 37 °C (ideal growth temperature of various pathogens) and are relatively large, facilitating manipulation [21]. Additionally, the innate (cellular and humoral) immune response of *G. mellonella* shares many similarities with the mammalian innate immune response and, as a consequence, can be exploited to assess the virulence of microbial pathogens and produce results comparable to those obtained with mammalian systems [22]. In *G. mellonella*, the cellular response is mediated by hemocytes and involves processes such as phagocytosis, nodulation, and coagulation. Humoral defenses are composed of soluble effector molecules, such as antimicrobial peptides, complement-like proteins, and products created by proteolytic cascades, such as the phenoloxidase pathway, which are capable of immobilizing or destroying pathogens [23, 24]. This response can be measured and partially compared to the mammalian immune response because attenuated strains in mammalian models show lower virulence in *G. mellonella* and strains that cause severe infections are generally highly virulent for larvae [19, 25].

Several studies have evaluated *G. mellonella* as an experimental model to understand host–pathogen interactions [26], pathogenic characteristics in different strains [19, 27, 28], and the effectiveness of antimicrobial agents on *S. aureus* isolated from human diseases [29–31]. However, few investigations have been carried out with strains isolated from bovine mammary gland infections [32] that are generally genetically different from those isolated from human diseases [33]. Nevertheless, studies characterizing the virulence of different strains of *S. aureus*, isolated from cases of mastitis in cattle in the model *G. mellonella*, to the best of our knowledge do not yet exist.

The use of an alternative animal model allows the screening and study of *S. aureus*, through mass phenotyping, allowing the study of virulence in large bacterial populations. This knowledge is indispensable for the identification of genes and regulatory elements that contribute to specific phenotypes and influence the host–pathogen relationship. Herein we proposed this approach that aimed to evaluate the *G. mellonela* model to study virulence

phenotypes in genetically diverse strains isolated from bovine mastitis.

Materials and methods

Bacterial isolates and *G. mellonella* larvae cultivation

The *S. aureus* isolates ($n = 157$) used in this study are part of a collection of microorganisms maintained under freezing (− 70 ºC). All of them were previously isolated from clinical ($n = 53$) and subclinical mastitis ($n = 104$) cases from 22 dairy cattle herds located in the Minas Gerais state Brazil, and their identification and partial genotypic characterization were reported elsewhere [3].

The *G. mellonella* larvae were reared with an artificial diet at 25 °C according to Jorjao et al. [34] until the beginning of the infection experiments. All larvae used were at the last instar and weighed from 250 to 300 mg.

Molecular characterization of the *S. aureus* isolates by pulsed-field gel electrophoresis (PFGE)

The PFGE technique was used to evaluate the genetic diversity of the *S. aureus* isolates using a homogeneous electric field device (CHEF DRII, Bio-Rad®, USA). Bacteria were plated on BHI (brain heart infusion) agar (Sigma-Aldrich®, India) and incubated for 18 h at 37 °C. Hereafter, a colony of each isolate was transferred to tubes containing 3 ml of BHI broth (Sigma-Aldrich®, India) and incubated at 37 °C for 20 to 24 h. The pellets were obtained by centrifuging 650 µl of the cultures at 12,000 × *g* for three minutes and suspended in 350 µl of TE (10 mM Tris–HCl, 5 mM EDTA, pH 8.0). A 350-µl aliquot of each isolate suspension was added to 350 µl of 2% low-melting-point agarose (Bio-Rad®, USA) and kept melted at 55 °C until use; that mixture was used to fill the wells. After solidification, the plugs were incubated for at least 4 h at 37 °C in EC buffer (6 mM Tris–HCl, 1-M NaCl, 100 mM EDTA, 0.5% Brij-L23, 0.2% sodium deoxycholate, 0.5% lauroylsarcosine) and lysostaphin (1 mg/ml). The plugs were then washed five times in TE (10 mM Tris–HCl, 5 mM EDTA, pH 8.0) and stored in 3 mL of TE at 4 °C until use. A 3-mm slice from each plug was incubated in 100 µl of restriction endonuclease buffer (Sigma-Aldrich®, India) at 37 °C for 30 min. The buffer was discarded, and the plug was digested in 100 µl of restriction endonuclease solution containing 20 U of *SmaI* enzyme (Sigma-Aldrich®, India) for 24 h at 37 °C.

After restriction with *SmaI*, the plugs were placed in the wells of a 1.5% agarose gel in 0.5 × TBE (0.9 M Tris Base, 0.9 M Boric Acid, 1 mM EDTA pH 8.0) at 14 °C. The Lambda PFGE Marker (New England Biolabs®, USA) was included as a molecular size marker. The gel was run at 6 V/cm for 23 h with an initial pulse of 5 s and a final pulse of 40 s, and then stained with ethidium bromide (50 µg/mL) and photographed under ultraviolet light on an L-Pix Chemi Photo Digitizer (Loccus Biotecnologia®, Brazil). The band pattern was analyzed visually and using the software Bionumerics version 7.5 (Applied Maths®, Belgium). The bands were automatically assigned by the computer and manually corrected after the original images were visually checked and evaluated. Cluster analysis was performed based on the Dice coefficient. By the unweighted pair group method with arithmetic mean (UPGMA), a minimum spanning tree (MST) was generated to evaluate the association of the clusters of isolates and virulence profiles.

Evaluation of virulence phenotypes of *S. aureus* strains using *G. mellonella* larvae model

In order to select a subset of strains to evaluate the virulence in the *G. mellonella* model, preliminary tests were performed in a pilot experiment using infective doses ranging from 10^5 to 10^8 *S. aureus* CFU/larva for 30 strains previously identified by PFGE as genetically distinct. In this first step, 40 larvae were used to assess each strain (10 larvae per dilution) in the same conditions as described before in this study. This screening enabled us to identify 10 isolates presumptively presenting divergent virulence phenotypes and define the bacterial dose (*S. aureus* CFU/larva) to be inoculated in the subsequent experiments.

For a full examination of the *G. mellonella* model, the larvae experimental infection was carried out according to the conditions established in the present study. The following parameters were evaluated: larvae survival up to 72 h post-inoculation; development of *S. aureus* in the hemolymph at 1, 2, 4, 18, and 24 h after infection; immunological response of *G. mellonella* through macroscopic visualization of the melanization intensity of the dorsal vessel; and histological examination of larvae from each test 4 h after the experimental infection.

Preparation of *S. aureus* suspension and infections

The *S. aureus* strains were cultured in blood agar and incubated at 37 °C for 24 h, and then isolated colonies were transferred to 5 mL of Mueller Hinton broth (Himedia® France) until reaching an OD (optical density) of 0.1 at 620 nm. Afterwards, they were incubated in the same medium at 37 °C for 4 h to reach the exponential growth phase. Cultures (1 mL) were washed three times with sterile phosphate-buffered saline (PBS), and centrifugations were performed at 12,000 × *g* for 3 min between washes. The suspensions OD were measured to ensure inoculation of 10^6 *S. aureus* CFU/larva. A 10-µL aliquot containing 10^6 CFU of

each isolate was injected into the last left proleg of the larvae using BD insulin syringes (BD-Becton Dickinson®, USA). Groups of uninoculated and PBS-alone inoculated larvae were used as controls. Larvae inoculated with *S. aureus* ATCC29213, already described elsewhere as quite virulent for *G. mellonella* (Peleg et al. 2009), were used as controls. After inoculation, larval feeding was discontinued.

In all experiments, there was a positive control (larvae infected with *S. aureus* ATCC-29213); negative controls (uninoculated and PBS inoculated larvae); and 10 tests in which larvae were inoculated with *S. aureus* strains isolated from mastitis cases (4S-158, 16S-717, 16S-721, 17S-762, 31S-1443, 36S-1698, 12C-525, 13C-561, 19C-828 and 23C-999). After inoculations, the larvae were stored in Petri dishes lined with filter paper, protected from light, and incubated at 37 °C. The number of larvae used, the incubation time, and the periods of observation differed among the different experiments performed, and are described in the next sections. In all experimental infection tests, at the end of the inoculations, 50 µl of the inoculum was plated on Mueller Hinton Agar (Himedia® France) in triplicate for counting and confirmation of the inoculated dose (10^6 CFU/larva), by the microdrop technique.

Tests of larvae survival

After inoculations, groups of 10 larvae in each test were placed in Petri dishes lined with filter paper. The Petri dishes were also protected from light and incubated at 37 °C for 72 h. The Petri dishes were visually inspected every 24 h, dead larvae were removed, and the results were recorded. Larvae were considered dead if they did not exhibit any movement in response to the physical stimulus according to Pereira et al. [35]. The results were analyzed by SigmaPlot software version 14.0 (Systat Software, Inc.®, USA) using the Kaplan–Meier method. Differences in survival rates were calculated using the log-rank test, and multiple comparisons were performed using the Log-rank test, with a significance level of 5%. These analyzes enabled us to define isolates of high, medium, and low virulence and to establish survival curves. Experiments were performed in biological and experimental triplicates ($n = 10$ larvae per replicate, totaling 90 larvae per test).

Quantification of *S. aureus* in larvae hemolymph

The quantification of *S. aureus* in the larvae was performed at 1, 2, 4, 18, and 24 h after infection for each of the tests. Before hemolymph harvest, the larvae were kept for 5 min in ice, 5 min in 70% alcohol, and 5 min in sterile PBS. A puncture was performed on the posterior part of the larva, close to the last prolegs, with the aid of a needle. Slight compression was applied to the larval body, and 20 µl of hemolymph was collected from each larva using an automatic pipette. For each of the tests, samples from 3 larvae were collected at each time point. The collected hemolymph (20 µl) was serially diluted until 10^{-8}, and an aliquot of each dilution was plated on mannitol salt agar (Kasvi®, Brazil), incubated at 37 °C for 24 h. The CFU count was determined using the microdrop technique, determining the number of bacteria recovered at each time point in a volume of 10 µl (same injected volume). The results were analyzed in Sisvar Software (UFLA, Brazil) using Tukey's test, in which a p value < 0.05 was considered statistically significant.

Evaluation of the intensity of larvae dorsal melanization and histological exams

A group of 5 larvae for each test was kept in a bacteriological incubator at 37 °C for 4 h, following the methodology described before in this study. After this period, the melanization of the dorsal vessel was evaluated macroscopically and recorded photographically.

After photographing, 3 larvae in each test were sectioned in the posterior portion of the body by using scissors and fixed in PBS solution containing 10% formaldehyde to prepare the histological slides. The samples were dehydrated in an ethanol series (70–100%) and embedded in paraffin. The blocks were sectioned (3–4 mm) in a microtome (Leica Biosystems®), and the sections were mounted on microscope slides and stained with hematoxylin and eosin. The formation of nodules and melanization regions in the fat bodies and the pericardial cells were analyzed for the different isolates tested, and the integrity of the tissues was evaluated in the uninoculated and PBS-inoculated groups. Images were recorded under light microscopy.

Screening detection of virulence genes

The presence of virulence genes was evaluated by PCR in individual reactions of 25 ul according to conditions and primers indicated in Table 1. Ster et al. [36] (genes *clfA*, *clfB, fnbpA,* and *hla*), Tristan et al. [37] (*ebps* gene), and Jarraud et al. [38] (*luk-ED* gene*)* were used with some adaptations.

Individual reactions were performed for each of the target genes and contained 1X buffer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 0.25 mM of each primer, 1.5 U Taq polymerase, and 50 ng of template DNA. Amplification was performed in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems®, USA) programmed for initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The amplified DNA fragments were visualized after electrophoresis in agarose gel (1.5%, w/v), and stained with ethidium bromide solution (0.005%, w/v). A 100-bp DNA ladder (Ludwig®, Brazil) was used as

Table 1 Primers used in PCR tests to search for *S. aureus* virulence genes

Target genes	Products size	Sequence (5′-3′)	References
clfA	104 pb	F: TGCAACTACGGAAGAAACGCCG R: CCTCCGCATTTGTATTGCTTGATTG	Ster et al. (2005)
clfB	194 pb	F: TGCAAGTGCAGATTCCGAAAAAAAC R: CCGTCGGTTGAGGTGTTTCATTTG	Ster et al. (2005)
fnBPA	132 pb	F: CGACACAACCTCAAGACAATAGCGG R: CGTGGCTTACTTTCTGATGCCGTTC	Ster et al. (2005)
hla	195 pb	F: GCGAAGAAGGTGCTAACAAAAGTGG R: CGCCAATTTTTCCTGTATCATCACC	Ster et al. (2005)
ebps	186 pb	F: CATCCAGAACCAATCGAAGAC R: CTTAACAGTTACATCATCATGTTTATCTTTG	Tristan et al. (2003)
luk-ED	269 pb	F: TGAAAAAGGTTCAAAGTTGATACGAG R: TGTATTCGATAGCAAAAGCAGTGCA	Jarraud et al. (2002)

Genes encoding clumping factor proteins (*clfA*and*clfB*), fibronectin−binding protein (*fnbpA*), toxins (*hla*and*luk−ED*), and elastin−binding protein (*ebps*)

a size marker. The images were recorded in an L-Pix Chemi Photo Digitizer (Loccus Biotecnologia®, Brazil).

Correlation analysis

The correlation between the presence of virulence genes and virulence level in the *G. mellonella* model was assessed for all strains together. Pearson's correlation coefficients were calculated with 95% confidence intervals, with the software R, version 4.1.1.

Results

Screening of *S. aureus* strains

Based on DNA macro-restriction fragments by PFGE analysis, genetically distinct *S. aureus* strains ($n=30$) were chosen from 157 isolates for the pilot experiment with *G. mellonella* larvae. Then, strains ($n=10$) belonging to 9 herds with different virulence phenotypes were selected for the subsequent steps of the study (Fig. 1).

Characterization of *S. aureus* virulence in *G. mellonella* model

Pilot tests of larvae survival analyzes indicated that the best infective dose was 10^6 CFU/larva. Figure 2 shows the larval survival percentage at 24, 48, and 72 h after infection. The lethality rates of the strains tested showed variations in larval when 10^6 CFU/larva was used. Among the strains, 16S-717 caused the highest number of larval deaths 24 h after infection and was considered the most virulent. However, at the end of the experiment (72 h post-inoculation) there was no statistical difference ($p>0.05$) in the lethality rate compared to the strains 16S-717, 19C-828, 31S-1443, 17S-762, 4S-158, and ATCC-29213, which were classified in the high virulence group. The strains 12C-525, 36S-1698, 13C-561, and 23C-999 presented a medium lethality rate; however, did not differ statistically ($p>0.05$), and comprised the moderate virulence group. On the other hand, strain 16S-721 had the lowest lethality rate, statistically different from the others ($p<0.05$), being considered the isolate with the lowest virulence.

The ability of strains to infect, survive, and multiply in *G. mellonella* larvae was confirmed by determining the

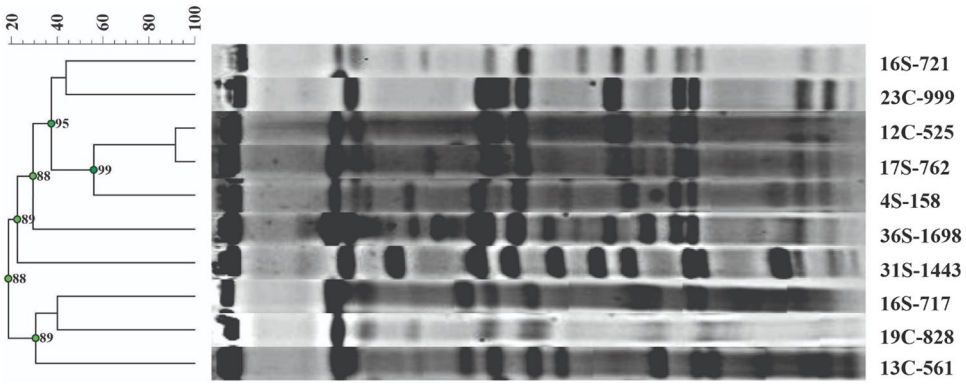

Fig. 1 Dendrogram by UPGMA clustering of PFGE fingerprints for *S. aureus* strains used in the *G. mellonella* infection. Clustering using the Dice coefficient generated a minimum spanning tree (MST)

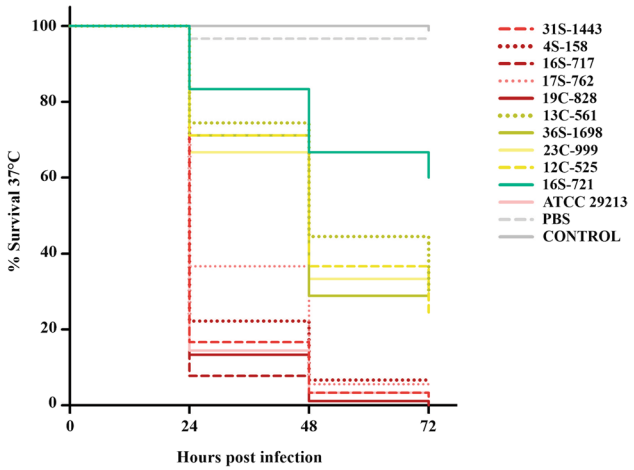

Fig. 2 Kaplan–Meier survival curves of *G. mellonella* larvae at 24, 48, and 72 h after infections with *S. aureus* strains (Log-rank test–$p < 0.05$). *G. mellonella* larvae were inoculated with 10 µl containing $10.^6$ CFU of *S. aureus* strains isolated from bovine mastitis and incubated at 37 °C. Negative controls: uninoculated (CONTROL) and PBS. Positive control: ATCC-29213. The difference between the high (reddish curves), moderated (yellowish curves) and low virulence (greenish curves) groups were strongly supported with $p = 0.000$

CFU/10 µl (same inoculated volume) in larval hemolymph at different post-infection times (Fig. 3). The only statistically significant difference was observed for isolate 16S-721 at 24 h, which at this time did not differ from the control and PBS tests ($p < 0.05$). There was a decrease in the number of bacteria recovered at the end of the observation period for tests in larvae challenged with ATCC-29213, 19C-828, 16S-717, and 13C-561. Therefore, the bacteria recovered in greater quantities at 18 and 24 h coincide with the strains identified with high virulence in the survival analysis.

The larvae inoculated with different isolates presented different melanization levels 4 h after experimental infection, and melanization spots in the dorsal vessel were observed in all tests, except in control and PBS (Fig. 4). Melanization was observed more intensively among isolates exhibiting higher virulence in the larvae survival analysis: 16S-717, 19C-828, 31S-1443, ATCC-29213, 17S-762, and 4S-158.

Histological analysis of infected tissue demonstrated that *S. aureus* strains were able to activate the cellular and humoral immune response in *G. mellonella* larvae, leading to the formation of nodules with adherent hemocytes and melanization spots. Histological evaluation of the larvae inoculated with PBS (Fig. 5a) revealed the integrity of tissues, with no melanization of the fat body or pericardial cells. On the other hand, the histology of the larvae infected with 12C-525 (Fig. 5b) and 19C-828 strains (Fig. 5c) pointed out immune response, represented by regions of melanization, nodule formation, and circulating hemocytes near the melanization spots.

Screening of virulence genes

A PCR to search for *S. aureus* virulence factors encoding genes into the genome of the isolates revealed 2 virulence profiles, which varied only in the presence or absence of the *ebps* gene. Strains 16S-721, 12C-525, 23C-999, and 36S-1698 were found to belong to the profile one, harboring the genes *clfA, clfB, fnbpA, hla, luk-ED*, and the isolates 16S-717, 19C-828, 31S-1443, 17S-762, 4S-158, and 13C-561 belonged to profile two, containing the genes *clfA, clfB, fnbpA, hla, luk-ED* and *ebps*. The strains belonging to profile one were genetically more related based on the similarity analysis of PFGE fingerprints (Fig. 6).

The number of virulence genes is positively correlated with the level of virulence in the *G. mellonella* model ($p = 0.005446$). Considering that the presence of the *ebps* gene was the only variable found in the profile of the virulence genes of the strains, it is possible to affirm that the presence of this gene is positively correlated with the greater virulence of strains in the *G. mellonella* model (Table 2).

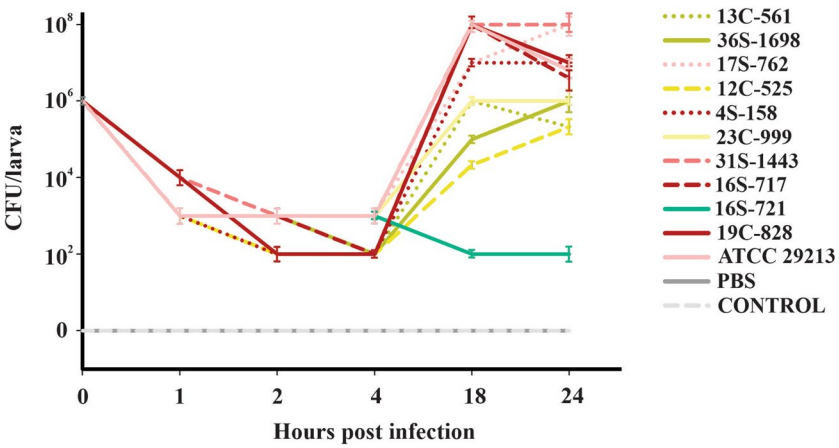

Fig. 3 *S. aureus* counts in hemolymph of *G. mellonella* larvae at 1, 2, 4, 18, and 24 h after infection. *G. mellonella* larvae were inoculated with 10 µl containing $10.^6$ CFU of *S. aureus* strains isolated from bovine mastitis and incubated at 37 °C. Negative controls: uninoculated (CONTROL) and PBS. Positive control: ATCC-29213

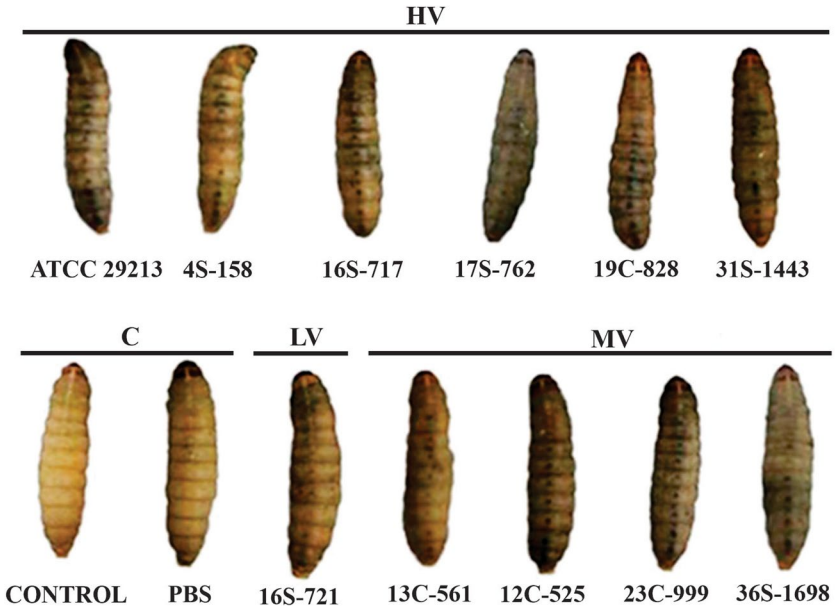

Fig. 4 Melanization of the dorsal vessel of *G. mellonella* larvae 4 h after the experimental infection by *S. aureus* strains isolated from mastitis cases. Larvae were injected with 10-µL aliquot containing $10.^6$ CFU of each strain into the last left proleg. High virulence (HV), moderate virulence (MV), low virulence (LV), and control (C)

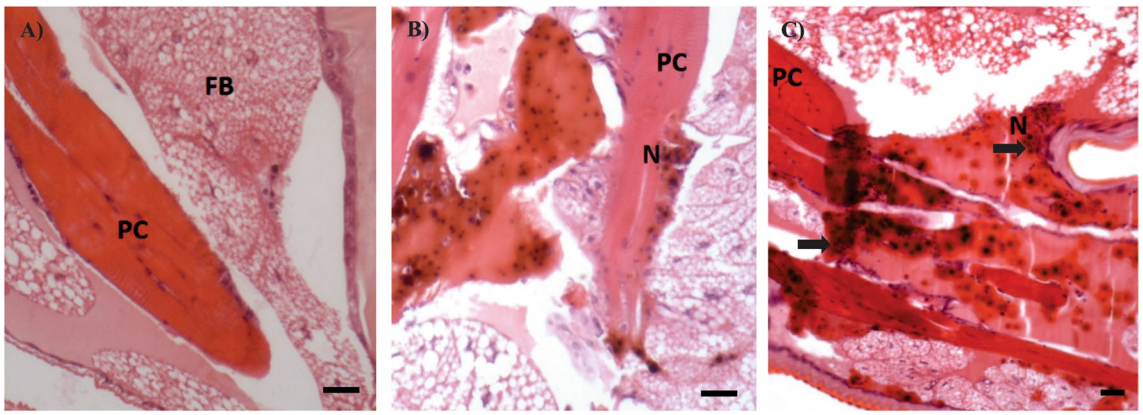

Fig. 5 Cellular and humoral immune responses of *G. mellonella* larvae 4 h after infection. **a** PBS group with healthy tissues, fatty body (FB), and pericardial cells (PC). **b** Group 12C-525 (moderated virulence) with the formation of nodules (N) and regions of low-intensity melanization, there is no melanization in the fatty body. **c** Group 19C-828 (high virulence) with intense melanization regions, melanization points in the pericardial cells, mature nodules with melanization in the center, and melanization points in the fatty body. Black arrows indicate bacterial cells 10 µm bars

Discussion

The use of invertebrate models for the study of in vivo virulence in several pathogens has been of interest to the scientific community. The *G. mellonella* larvae have been established as a screening model, allowing the study of pathogenesis by monitoring larval survival, bacterial count in hemolymph, immune response of infected larvae, and histological data [20]. To the best of our knowledge, this study describes for the first time the use of *G. mellonella* larva as an alternative host for studying virulence in genetically different *S. aureus* strains isolated from bovine mastitis.

The *S. aureus* population ($n = 10$) used in the last step of this study comprised 10 different pulsotypes, as determined by PFGE analysis. They were sufficient to identify a variation of virulence phenotypes, enabling the identification of strains with high (16S-717, 19C-828, 31S-1443, 17S-762, and 4S-158), moderated (12C-525, 36S-1698, 13C-561, and 23C-999) and low (16S-721) virulence in *G. mellonella*, mainly by the larvae survival analysis. Indeed, the use of ATCC-29213, previously described as highly virulent for *G. mellonella* [25], as a positive control, confirms the pathogenic potential of the strains evaluated in this work, since the strains belonging to the high virulence group, which includes ATCC-29213, did not differ statistically ($p > 0.05$) from each other.

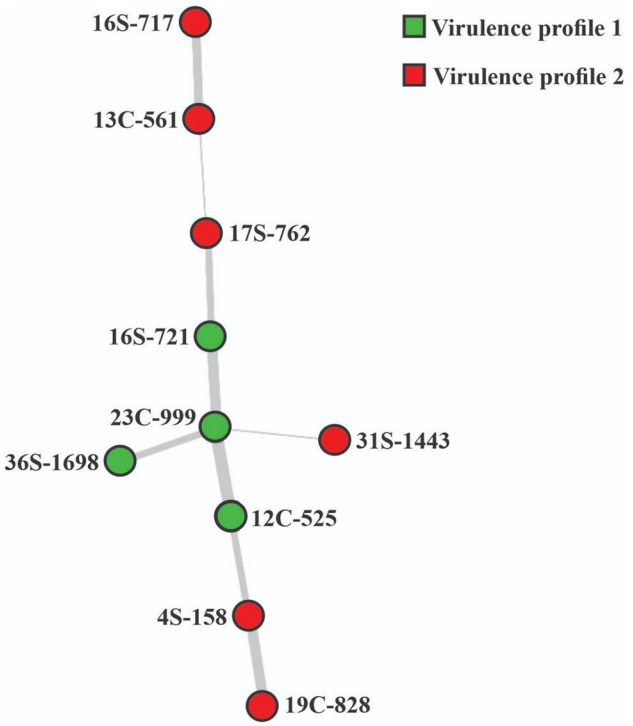

Fig. 6 PFGE minimum spanning tree (MST) of *S. aureus* used in *G. mellonella* larvae virulence study. The colors green and red represent virulence profiles one and two, respectively. The MST displays the highest overall score and reliability was calculated using UPGMA (unweighted pair method using arithmetic averages) associated with the priority rule and resampling permutation

Regarding staphylococci counts in larvae hemolymph, the initial decrease in the number of recovered bacteria occurred for all strains because the larval immune response is more intense in the first hours after infection, hindering bacterial multiplication [23]. Another aspect that may explain the drop in the initial count is the dilution of the inoculum in the total larval hemolymph. The bacterial recovery rate increased after the first 4 h after inoculation, indicating that *S. aureus* can adapt to the new host, possibly due to the expression of important virulence factors in the host/pathogen relationship, resisting the immune response of the larvae and multiplying itself. However, at the end of the period evaluated (24 h after infection), the recovery rate decreased for some strains (ATCC-29213, 19C-828, and 16S-717) of the high-virulence group. This may be a consequence of the intense melanization of the larvae, as the melanin barrier formed around the invading microorganisms, in addition to having toxic effects, immobilizes the microorganism and prevents it from reaching the nutrients necessary for its development [39].

In turn, for 13C-561, which showed moderated virulence, this observation must be due to the effectiveness of the immune response of the larva and the inability of these bacteria to continue multiplying in the face of host defenses.

G. mellonella, like other invertebrates, does not have an adaptive immune system, although its innate system shares many similarities with that of mammals. It includes a cellular response in which hemocytes (immune cells close to mammalian neutrophils) are responsible for cellular events and a humoral response with soluble effector molecules [20]. *G. mellonella* has a melanin-phenoloxidase system, a component of its humoral immune system, responsible for hemolymph coagulation, which can be observed by melanization and assists in the elimination of bacterial pathogens [40, 41]. In general, bacterial strains that were more virulent for larvae in survival analysis led to higher levels of melanization [35, 42]. As observed by Pereira and collaborators (2015), melanization began in the dorsal vessel region of the larva, and strains of high and moderate virulence provide more intense melanization in this region than those of low virulence (Fig. 4).

Histological analysis showed that all strains tested, including ATCC-29213, had the ability to activate the larval immune system, leading to the formation of nodules and melanization regions in different tissues. The most virulent strains, such as 19C-828, led to an intense immune response, characterized by greater melanization of the dorsal vessel, the presence of mature nodules, melanization

Table 2 Correlation between the virulence in *G. mellonella* and the virulence gene profile of *S. aureus* strains

Strains	Virulence genes profile	In vivo virulence	Correlation coefficient	*p* value
16S-721	*clfA, clfB, fnbpA, hla, luk-ED*	Low	0.8000947	0.005446
12C-525	*clfA, clfB, fnbpA, hla, luk-ED*	Moderate		
23C-999	*clfA, clfB, fnbpA, hla, luk-ED*	Moderate		
36S-1698	*clfA, clfB, fnbpA, hla, luk-ED*	Moderate		
16S-717	*clfA, clfB, fnbpA, hla, luk-ED, ebps*	High		
19C-828	*clfA, clfB, fnbpA, hla, luk-ED, ebps*	High		
31S-1443	*clfA, clfB, fnbpA, hla, luk-ED, ebps*	High		
17S-762	*clfA, clfB, fnbpA, hla, luk-ED, ebps*	High		
4S-158	*clfA, clfB, fnbpA, hla, luk-ED, ebps*	High		
13C-561	*clfA, clfB, fnbpA, hla, luk-ED, ebps*	Moderate		

spots close to pericardial cells, and melanization spots in the fat bodies. Less virulent strains also demonstrated the ability to activate the immune system, with the formation of nodules and melanization spots on a smaller scale.

The larvae survival rates, bacteria quantification in the hemolymph, and melanization 4 h after infection showed that the isolates with high virulence had a greater ability to multiply in larval tissues, being recovered in larger quantities 18 and 24 h later, resulting in greater melanization of the larval tissues. The results show that the parameters used in this study were efficient in evaluating the virulence of *S. aureus* strains from bovine mastitis by *G. mellonella* model.

Regarding the screening of virulence genes by PCR, the results identified 2 virulence profiles (1 and 2), which differed only with respect to the presence of the *ebps* gene, which encodes elastin-binding protein, and the profile 2 comprises strains that possessed this gene. The group of high virulence strains was composed only of profile 2, while among those with moderate and low virulence; only 13C-561 belonged to this profile. A positive correlation was observed ($p = 0.005446$) between the presence of the *ebps* gene and the virulence of staphylococci strains isolated from bovine mastitis. However, further investigations must be carried out to confirm the importance of this gene in the pathogenesis of the strains studied in *G. mellonella* larvae, given the variety of other virulence determinants that may be present in *S. aureus* [2].

The *ebps* gene has been detected in *S. aureus* strains from humans with endocarditis and osteomyelitis [37]. In cattle, previous studies reported the high presence of this gene in subclinical mastitis staphylococcal isolates from China, Iran, and Poland [43–45 respectively] and recent genomic studies using data from the National Center for Biotechnology Information database (NCBI) confirmed the high occurrence of this gene in clinical and subclinical mastitis isolates from several countries, including Brazil [46, 47]. The presence of *ebps* gene in bovine mastitis isolates is attributed to the fact that it mediates binding to surface proteins or soluble elastin peptides in mammary gland cells, and its importance is related to the first step of *S. aureus* infection: binding to the host [46].

In this study, the frequency of 6 virulence genes, 4 of them belonging to the virulence factors MSCRAMMs (*clfA, clfB, fnbpA*, and *ebps)* and 2 related to toxins activity (*hla* and *luk-ED)* were evaluated, which allowed the clusterization of virulence genotypes. Notably, the *S. aureus* strains studied were originally associated with intramammary infections in cattle, and therefore, the virulence determinants evaluated herein could be more relevant for infection in the mammary gland of this species [9, 48]. In fact, studies analyzing bovine and human isolates have shown that although there are common features between strains of both species, there are predominant genotypes and combinations of virulence genes for each host [33, 49].

It is not possible completely to extrapolate the results obtained in larvae to those expected in the mammary gland; nevertheless, other studies applying this model to inquire about *S. aureus* virulence and pathogenesis have shown a good correlation between *G. mellonella* and mammalian models [22]. The work performed by Peleg et al. [25] stands out, in which an association between virulence in larvae and virulence in a mammal model (mouse) was demonstrated. In that study, the authors used a knockout strain for the *agr* gene, a virulence regulator that was already shown to be important in the infection of several hosts. When comparing the virulence caused by the mutant strain and the wild strain, it was evident that the mutant strain caused lower lethality than the wild strain, both in *G. mellonella* and in mice.

A study conducted by Sharma-Kuinkel et al. [19] reaffirmed the association between *S. aureus* virulence results in mice and in *G. mellonella*. In both models, there was an increase in survival of the hosts infected by clonal complex 30 strains when compared to other strains used in the same experiment. In addition to the use of mice and larvae, the study evaluated the molecular characteristics of the strains through genome and transcriptome sequence analyses, showing that pathogenicity traits are related to the genomic profile and suggested that specific *S. aureus* genotypes are related to different types of infection [19].

A recent study reported a strong interaction of *S. aureus* strains with the humoral and cellular immune response of *G. mellonella*: the infection in the larvae resulted in nodule formation in insects with similar structures to those found in human abscesses [26]. This result reveals that *S. aureus* virulence factors are recognized by the larval immune system, and its elimination occurs through mechanisms similar to those of its natural mammalian host [12].

To date, there are no comparative studies involving bovines and invertebrate models to evaluate virulence for mastitis pathogens. A previous study using the *G. mellonella* model to test the virulence of *S. aureus* from bovine mastitis was performed by Silva [50], but only 2 genetically similar strains were used. The present study was the first to use *G. mellonella* larvae to evaluate a variety of molecularly characterized staphylococci strains that cause bovine mastitis. The results indicate a diversity of genotypes and phenotypes in the studied population, confirming the genetic diversity and complexity of the pathogenesis of mastitis caused by *S. aureus*. Indeed, the course of the disease depends on the pathogen and host features, especially regarding the intensity of the immune response. The mammary gland has a complex innate and adaptive response that leads to defense against invading organisms and prevents damage to host tissues, which may be partially associated with the immunity that *G. mellonella*

larvae triggered in response to infection, showing that its use may be interesting in the study of pathogens that cause mastitis.

As future prospects, the use of an alternative animal model also allows the study of large populations of *S. aureus*, as well as the screening of attenuated mutants by random mutagenesis, which is a powerful genetic tool for the identification of genes and regulatory elements that contribute to specific phenotypes [51]. In this scenario, the adoption of invertebrate models for virulence studies brings benefits, such as cost and time reduction, due to the shorter life cycle in relation to mammalian models (the cow for the study of the pathogenesis of mastitis, for example), greater/immunological uniformity of the animal due to greater control of environmental conditions, and mainly decreases the use of vertebrate animals that have ethical restrictions. Since these strains of *S. aureus* are genetically distinct, a broader characterization of their genomes (genomic sequencing, for example) would be interesting to better understand the genetic background of virulence in this bacterial population and to prospect genetic targets aimed to develop control strategies of bovine mastitis. Omics tools would also allow comparisons of these strains with others used in different studies.

Conclusion

Our study showed that *G. mellonella* larvae constitute a practical and attractive model to evaluate virulence phenotypes in *S. aureus* strains from bovine mastitis, allowing for the distinction of more and less virulent strains. This knowledge will allow identifying key factors for pathogenesis in larvae, which may help in understanding the *S. aureus* host–pathogen interaction.

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Declarations

Ethics approval and consent to participate Not applicable.

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