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Original article

Assessing *Galleria mellonella* as a preliminary model for systemic *Staphylococcus aureus* infection: Evaluating the efficacy and impact of vancomycin and *Nigella sativa* oil on gut microbiota

Faris S. Alnezary [a], Masaad Saeed Almutairi [b,*], Abdullah A. Alhifany [c], Thamer A. Almangour [d]

[a] Department of Pharmacy Practice, College of Pharmacy, Taibah University, Madinah 41477, Saudi Arabia
[b] Department of Pharmacy Practice, College of Pharmacy, Qassim University, Qassim 51452, Saudi Arabia
[c] Department of Clinical Pharmacy, College of Pharmacy, Umm Al-Qura University, Makkah, Saudi Arabia
[d] Department of Clinical Pharmacy, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia

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ABSTRACT

Background: Staphylococcus aureus is a Gram-positive bacterium that can cause various infections. The *Galleria mellonella* has been used as a preliminary test for infection model. The study aimed to evaluate the effectiveness of *G. mellonella* as a microbiome model and compare the efficacy of vancomycin and antimicrobial activity of *Nigella sativa* (NS) on the gut flora.

Methods: G. mellonella larvae were subjected to metagenomic analysis. The larvae's guts were collected, homogenized in phosphate-buffered saline (PBS), and the gut contents isolated for bacterial DNA extraction. Larvae were assigned into the following groups: negative control (PBS only); positive control (MRSA only); vancomycin treated group; NS oil treated group and combination (vancomycin and NS oil) treated group. Larvae were cultured, inoculated with *S. aureus*, and treated with vancomycin and NS oil. Larval activity, cocoon formation, growth, melanization, and survival were monitored. The toxicity of vancomycin and NS oil was tested, and *S. aureus* burden and natural microbiota were determined. Hemocyte density was measured. Statistical analysis was conducted using R.

Results: Enterococcus related species dominated approximately 90 % of the gastrointestinal tract of the larvae. The survival rate following treatment was 85 % with vancomycin, 64 % with NS oil, and 73 % with a combination of both. The count of *Enterococcus* Colony Forming Units (CFUs) was significantly lower in the vancomycin treatment group (8.14E+04) compared to those treated with NS oil (1.97E+06) and the combination treatment (8.95E+05). Furthermore, the *S. aureus* burden was found to be lower in the NS oil (1.04E+06) and combination treatment groups (9.02E+05) compared to the vancomycin treatment group (3.38E+06). Hemocyte densities were significantly higher in the NS oil (8.29E+06) and combination treatment groups (8.18E+06) compared to the vancomycin treatment group (4.89E+06).

Conclusions: The study supported the use of *G. mellonella* model as a preliminary test to assess the effect of different antimicrobials against *S. aureus* and gut microbiota. NS oil showed more selectivity against *S. aureus* and protectiveness for the natural *Enterococcus* gut flora.

1. Introduction

Staphylococcus aureus is a facultative anaerobic, Gram-positive bacterium, usually colonizes the nose, skin and gastrointestinal (GI) tract without causing symptoms, which is also infamous for causing medical device-related infections (Brown et al., 2014; Otto, 2018). Moreover, *S. aureus* can cause a wide range of infectious diseases, including boils and furuncles as well as more severe infections such as endocarditis and pneumonia (Tong et al., 2015). It has been proven that *S. aureus* can facilitate interactions with the host, induce tissue damage, and mutate to

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* Corresponding author.
E-mail addresses: fnezary@taibahu.edu.sa (F.S. Alnezary), mas.almutairi@qu.edu.sa (M.S. Almutairi), aahifany@uqu.edu.sa (A.A. Alhifany), talmangour@ksu.edu.sa (T.A. Almangour).

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a resistant strain (Methicillin-resistant *Staphylococcus aureus* (MRSA)) via several mechanisms (Lowy, 1998). Therefore, there is a continuous need to identify and develop novel antimicrobials to combat infections caused by such pathogen (Almangour et al., 2019; Almangour et al., 2020).

Despite the urgent need for novel antibiotics, yet, many roles and regulations have to be fulfilled prior to their approvals (Savoia, 2012). After passing *in vitro* identification of potential antimicrobial compounds, further *in vivo* tests are needed to determine their efficacy and safety (Yang et al., 2018). Typically, the experiments at this stage are conducted in a murine infection model, however these are time consuming and expensive, and they require careful ethical consideration. There is therefore a need to develop a preliminary infection model that can generate *in vivo* data in a quick and cost-effective manner without raising ethical concerns (Savoia, 2012; Yang et al., 2018). Such new models would reduce animal experimentation for new antibiotic development by highlighting compounds that are ineffective or highly toxic for the gut microbiome. Therefore, the introduction of the *G. mellonella* infection model may help to this end. The invertebrate model, *G. mellonella*, is being used as a preliminary and alternative test to rodent models in a wide range of research areas, such as the study of bacterial, fungal, and viral infections, as well as the study of toxins and the screening of antimicrobial drugs. The popularity of *G. mellonella* is attributed to the lack of ethical regulations, its short life cycle, ability to survive at 37 °C, and simplicity regarding handling in the laboratory (Kavanagh and Fallon, 2010; Champion et al., 2016; Tsai et al., 2016). Furthermore, *G. mellonella* has already been used to study microbial virulence and showed a similar response to mammals (Jander et al., 2000; Peleg et al., 2009; Yang et al., 2018).

Nigella sativa, a natural antimicrobial, Family *Ranunculaceae*, is also known as black cumin or black seed. Traditionally, it has been used to treat many medical conditions in the Mediterranean region as well as southern and north African countries (Gholamnezhad et al., 2016). It is composed of oils, proteins, carbohydrates, fibers, and contains various pharmacologically active compounds, such as thymoquinone (TQ), thymol, thymohydroquinone, dithymoquinone, carvacrol, p-cymene, 4-terpineol, sesquiterpene longifolene, t-anethol, and α-pinene (Gholamnezhad et al., 2016). Anggon and Kuswandari evaluated antibacterial activity of NS oil against *S aureus* of necrotic pulp of deciduous teeth isolates. The study showed that the inhibitory concentration of the NS oil was 0.8 %, which created an inhibitory zone of 7.9 ± 0.02 mm that lasted for 7 days (Anggono and Kuswandari, 2017). Interestingly, the NS oil maintained its antimicrobial activity over time compared to other antibiotics in the study. Hannan *et al.* showed that NS oil at a concentration of 4 mg/disc has an inhibitory effect against MRSA (zone size > 12 mm was considered to be significant) (Hannan et al., 2008). Furthermore, Mouwakeh et al. found that NS oil and its bioactive compounds can be applied as resistance modifiers of MRSA strains (Mouwakeh et al., 2019). However, Emeka et al. found a variable susciptability profile of MRSA isolates from diabetic patients' wounds to NS oils (Emeka et al., 2015). On the other hand, Kumar *et al.*, supplemented the diet of broiler chickens with NS seeds which significantly ($P < 0.04$) suppressed intestinal *Salmonella* spp. counts while numerically lowered *Escherichia coli* counts without significant impact on the beneficial bacterium such as *Lactobacillus* sp. (Kumar et al., 2017). These findings encouraged us further to investigate the utility of *G. mellonella* as a microbiome model and to evaluate the antimicrobial activity of NS oil in comparison to standard antimicrobials.

In this study, we exposed *G. mellonella* to *S. aureus* and treated the larvae with natural (NS oil) and synthetic (vancomycin) antimicrobials. Our primary objective was to assess the suitability of *G. mellonella* as a model for systemic *S. aureus* infection and for studying the impacts of these antimicrobials on the gut microbiota. We also aimed to compare the efficacy of vancomycin and NS oil in clearing *S. aureus*, with the ultimate goal of establishing *G. mellonella* as a valuable model for studying gut microbiota-pathogen interactions.

2. Methods

2.1. Reagents, microorganisms, and insects

The reagents and culture media were all purchased from Sigma-Aldrich (St. Louis, MO, USA), except *Nigella sativa*, which was ordered from Amazing Herbs (Bedford, GA, USA). Batches of *G. mellonella* larvae were purchased from (Timberline, USA). Bacterial strains were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The NS oil was prepared as follows: 500 mg of NS oil, extracted from capsules, was initially dissolved in 1 mL of Dimethyl Sulfoxide (DMSO). This was followed by the addition of 9 mL of normal saline to the mixture, ultimately yielding a solution with a concentration of 50 mg of volatile oil per 1 mL. This procedure aligns with the methodology described by Fararh et al. in 2002.

2.2. Bacterial strains

MRSA strains 600799, 700699 BA, and 700699 LB were utilized in this study. The strains were routinely cultured in Blood Agar and were incubated overnight at 37 °C. After confirming the purity of the bacteria, cells were inoculated into Brain heart infusion (BHI) broth and were incubated for another night at 37 °C. Prior to injection into the larvae, cells were centrifuged (5000 g for 5 min) and washed three times in phosphate-buffered saline (PBS).

2.3. Metagenomic analysis of G. mellonella larvae

Intact guts were collected from sterile last instar larvae and homogenized in PBS. The PBS containing gut contents were enriched in 20 mL BHI broth by incubation at 37 °C for overnight. Enriched microbiota was sedimented by centrifugation at 4000 rpm for 10 min, washed with PBS and processed for bacterial DNA extraction.

2.4. DNA extraction and sequencing

The AnaPrep automated DNA extractor (BioChain) was used to extract bacterial DNA, as previously mentioned (Endres et al., 2019). Thermo Fisher's NanoDrop and Qubit were used to measure the amount of DNA, and a BioAnalyzer (Agilent) was used to determine the quality of the DNA. Following Illumina's instructions, DNA libraries were created, multiplexed on a flow cell, and run on an Illumina NextSeq using paired-end sequencing. Trimmomatic was used to trim the produced fastq files (Bolger et al., 2014), and FastQC was used to evaluate the sequencing quality.

2.5. Larval culture, inoculation, and treatment

During the sixth instar, larvae of the greater wax moth G. *mellonella* (Timberline, USA) were maintained on wood chips at 15 °C in the dark. Larvae weighing 250–350 mg were selected and used within 2 weeks of receipt. In each arm of the study, ten healthy larvae were placed in sterile 9 cm Petri dishes. Larvae were inoculated with 10 mL of *S. aureus* suspension (containing 1.0E+05–1.0E+07 CFU total) through the last left proleg into the hemocoel with a U-100 insulin syringe (UltiCare, USA).

Larvae were moved out of the refrigerator to a 30 °C incubator for 1 day prior to all experiments to make sure they were not sick. The larvae were then incubated at 37 °C for 120 h after inoculation and monitored daily to assess activity, cocoon formation and growth, melanization and survival. A total of ten larvae were used in each experiment, and each experiment was repeated twice. A negative control group was included in each experiment; one group received no injection, and the other received PBS only to test the effect of physical injury.

Larvae were assigned into the following arms: 1) Negative control (PBS only), n = 10, 2) Positive control (MRSA only, three groups for the

three strains, *S. aureus* 600799, *S. aureus* 700699 BA, and *S. aureus* 700699 LB), n = 30, 3) Vancomycin treated group (20 mg/kg) (three groups for the three strains), n = 30, 4) NS oil (70 mg/kg) treated group (three groups for the three strains), n = 30, 5) and Combination (vancomycin, 20 mg/kg + NS oil, 70 mg/kg) treated group (three groups for the three strains), n = 30.

2.6. Toxicity of antimicrobial agents to larvae

The toxicities of the antimicrobial agents (NS oil and vancomycin) were tested by administering repeat doses of 70 mg/kg and 20 mg/kg, respectively, at 0, 24, and 48 h to groups of 10 larvae. In the treated group, larval survival was not significantly different from the control group (treated with PBS only), suggesting that these regimens are not toxic.

2.7. S. aureus strains and culture conditions

An inoculum of 1.0E+6-8 *S. aureus* cells was injected into each larva with 10 μL of the bacterial suspension. To ensure consistent count of *S. aureus,* serial dilution was made with every experiment and viable colony forming units (CFUs) were counted.

2.8. Determination of natural microbiota (Enterococcus) in G. mellonella larvae

After 48 h of inoculation, one infected larva was chosen randomly. Then, this larva homogenized in a pellet pestle and micro-centrifuge tube with 1 mL of PBS, serially diluted, plated onto m-Enterococcus agar plates and incubated at 37 °C for 48 h. The bacterial burden was calculated based on the number of *Enterococcus* CFU that grew at specific dilutions.

2.9. Determination of S. aureus burden in G. mellonella larvae

After 48 h of inoculation, one infected larva was chosen randomly. A pellet pestle was used to homogenize this larva with 1 mL of PBS, diluted serially, plated onto nutrient agar plates, and then incubated at 37 °C for 24 h. The bacterial burden was calculated based on the number of *S. aureus* CFU that grew at specific dilutions. To confirm the identity of *S. aureus,* CHROMagar plates were used to plate samples.

2.10. Determination of hemocyte density

The larval hemocyte density was determined by the inoculation of viable *S. aureus* ($\sim 1 \times 10^{5\text{-}7}$ larva^{-1}) (Sheehan et al., 2018; Sheehan and Kavanagh, 2018). In order to determine changes in hemocyte density, 30–40 μL of larval hemolymph (n = 4) was extracted into a micro-centrifuge tube and placed on ice to prevent melanization. The hemolymph was diluted in 0.37 % (v/v) mercaptoethanol-supplemented PBS and the cell density was determined under light microscope using a hemocytometer without attempting to differentiate between hemocyte subtypes. Hemocyte density was written in terms of hemocyte density/larva. Experiments were performed for the three MRSA strains (mentioned above), and the means ± standard deviation (SD) were calculated.

2.11. Statistical analysis

All experiments duplicated, and the results were expressed as the mean ± SD. All statistical analyses were performed using R version 3.6.1 and figures were produced using the package ggplot2 (Wickham, 2009). Differences were considered significant at $P < 0.05$.

3. Results

3.1. The microbiome of G. mellonella

The gut contents of the *G. mellonella* larvae were determined by conventional culture-dependent and advanced culture-independent methods. A culture-dependent method was performed by plating the gut content on blood agar and selective media (m-Enterococcus agar) under aerobic and anaerobic conditions and showed that the gut is dominated by *Enterococcus* species.

Furthermore, the culture-independent method (metagenomics analysis through shotgun metagenomic sequencing) showed a predominance of close-related *Enterococcus* species in the larvae of *G. mellonella*, including *Enterococcus casseliflavus, Enterococcus gallinarum*, and other *Enterococcus* species (Fig. 1). These results from culture-dependent and culture-independent methods for *G. mellonella* microbiome indicate that the larva has a few numbers of strains and a sparse microbiome.

3.2. Survival of G. mellonella larvae after S. aureus infection

Overall, the antimicrobial treatment improved survival in MRSA infection for all strains. The total survival rate was 85 %, 64 %, 73 % for vancomycin, NS oil, and combination of both antimicrobials, respectively. Both vancomycin (20 mg/kg) and NS oil (70 mg/kg) showed a significant ($P < 0.001$, and $P = 0.003$) effect on larval survival compared with the untreated control group. For individual strains, the survival rate for *S. aureus* 600799 was 80 %, 80 %, and 75 % for vancomycin, NS oil, and combination of both antimicrobials, respectively. For *S. aureus* 700699 BA, the survival rate was 90 %, 50 %, and 60 % for vancomycin, NS oil, and combination of both antimicrobials, respectively. Moreover, the survival rate for *S. aureus* 700699 LB was 85 %, 60 %, and 85 % for vancomycin, NS oil, and combination of both antimicrobials, respectively (Fig. 2).

3.3. Natural microbiota (Enterococcus) count of G. mellonella larvae after treatment

After 48 h of antimicrobials exposure, mean *Enterococcus* CFUs were significantly lower in the vancomycin arm (8.14E+04) than NS oil (1.97E+06) and combination of both antimicrobials (8.95E+05), $P = 0.014$, and $P = 0.005$, respectively. Furthermore, the CFU counts in the vancomycin arm were significantly lower than those in the untreated PBS arm (8.14E+04 vs. 1.51E+06, $P = 0.0008$) (Fig. 3). For individual strains, mean *Enterococcus* CFU for *S. aureus* 600799 were 3.48E+04, 5.75E+05, and 5.97E+05 for vancomycin, NS oil, and combination of both antimicrobials, respectively. For *S. aureus* 700699 BA, mean *Enterococcus* CFU 3.16E+04, 2.92E+06, and 1.00E+06 for vancomycin, NS oil, and combination of both antimicrobials, respectively. Moreover, mean *Enterococcus* CFUs for *S. aureus* 700699 LB were 1.78E+05, 2.40E+06, and 1.09E+06 for vancomycin, NS oil, and combination of both antimicrobials, respectively. Overall, this data indicates that the addition of NS oil leads to higher *Enterococcus* CFU counts, suggesting that it may have a role in protecting the gut microbiota.

3.4. S. aureus burden in G. mellonella larvae after treatment

Overall, CFU counts showed lower burden and better clearance of *S. aureus* in the NS oil (1.04E+06) and combination (9.02E+05) arms than in vancomycin arm (3.38E+06), $P = 0.02$, and $P = 0.022$, respectively (Fig. 4). Individual strains showed similar data, mean *S. aureus* CFUs for *S. aureus* 600799 were 1.68E+06, 5.05E+05, and 3.44E+05 for vancomycin, NS oil, and combination of both antimicrobials, respectively. For *S. aureus* 700699 BA, mean *S. aureus* CFU 2.03E+06, 1.23E+06, and 5.57E+05 for vancomycin, NS oil, and combination of both antimicrobials respectively. Moreover, mean *S. aureus* CFUs for *S. aureus* 700699 LB were 6.43E+06, 1.40E+06, and

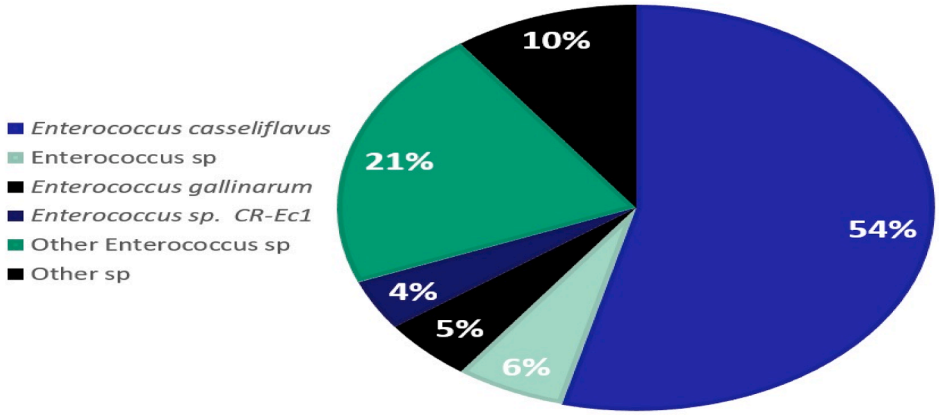

Fig. 1. Metagenomic analysis of *G. mellonella* larvae.

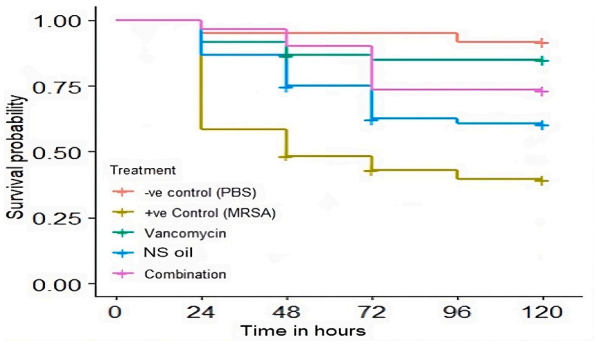

Fig. 2. Larvae overall survival (n = 300).

1.81E+06 for vancomycin, NS oil, and combination of both antimicrobials, respectively. All in all, this data clearly indicates that the addition of NS oil helps in *S. aureus* clearance.

3.5. Immune response of G. mellonella larvae to S. aureus infection and treatment

After 24 h of exposure, changes in hemocyte count were assessed. Mean hemocyte densities were significantly lower in the control (MRSA) arm (3.38E+06) than vancomycin (4.89E+06) arm (P = 0.005). However, the NS oil (8.29E+06) arm (P = 0.002) and the combination arm (8.18E+06) (P = 0.019) were significantly higher than vancomycin (4.89E+06) arm. Furthermore, there was no significant difference in the mean hemocyte density between the vancomycin (4.89E+06) arm and the untreated PBS (6.40E+06) arm (P = 0.26) (Fig. 5). For individual strains, the mean hemocyte density for *S. aureus* 600799 was 3.45E+06, 5.45E+06, 9.63E+06, and 7.93E+06 for MRSA, vancomycin, NS oil, and combination of both antimicrobials, respectively. For *S. aureus*

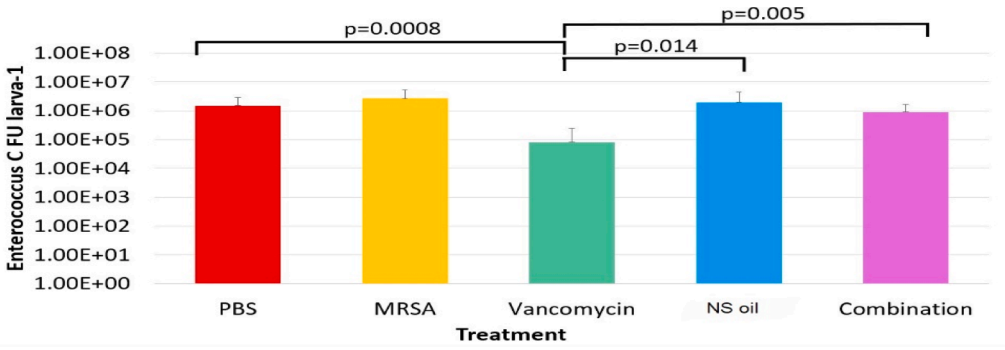

Fig. 3. Mean *Enterococcus* CFU per larva.

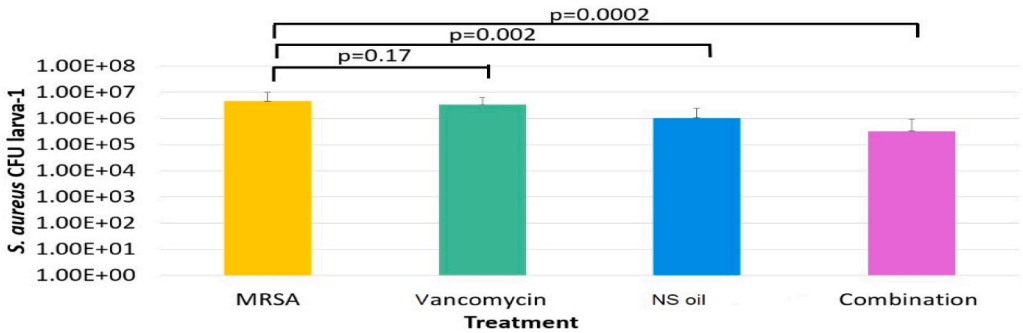

Fig. 4. Mean *S. aureus* CFU per larva.

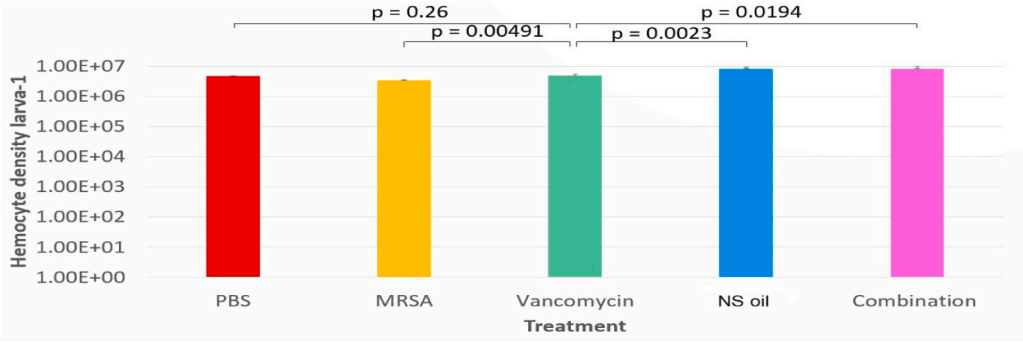

Fig. 5. Mean Hemocyte density per larva.

700699 BA, mean hemocyte densities were 3.02E+06, 4.25E+06, 7.25E+06, and 6.14E+06 for MRSA, vancomycin, NS oil, and combination of both antimicrobials, respectively. Moreover, mean hemocyte densities for S. aureus 700699 LB were 3.68E+06, 4.98E+06, 8.00E+06, and 1.05E+07 for MRSA, vancomycin, NS oil, and combination of both antimicrobials, respectively. Overall, this data indicates that the addition of NS oil leads to an increase in hemocyte density, suggesting that NS oil may have an immune-stimulant effect in *G. mellonella*. This data also shows the immunosuppressive effect of *S. aureus* on the *G. mellonella*.

4. Discussion

The invertebrate model, *G. mellonella,* is composed of a very simple microbiome containing mainly the *Enterococcus* species. In this study, we were able to utilize the *G. mellonella* larvae as a non-mammalian model system to compare the antistaphylococcal activity of vancomycin, NS oil, and a combination of both. The *G. mellonella* model demonstrated a selective antistaphylococcal activity of the NS oil while vancomycin showed a non-selective killing of both beneficial and pathogenic bacteria. Previously, *G. mellonella* model has been used to evaluate either the efficacy of antimicrobial agents or virulence microorganisms (Desbois and Coote, 2012; Gibreel and Upton, 2013; Sheehan et al., 2019). However, this study is the first to show that this model can be used to assess the effect of different antimicrobials on survival, immune response, microbiome, and the clearance of the pathogenic organism entirely.

Previous studies sequenced *G. mellonella* and identified its gut microbiome. Allonsius *et al.* characterized the microbiome present at different body sites of the *G. mellonella* larvae and found that this animal model has a very low diversity microbiome that is mostly dominated by one *Enterococcus* taxon (Allonsius et al., 2019). Krams *et al.* evaluated the taxonomic composition of the midgut of the larvae of the *G. mellonella* using 16S rRNAV3 region, and showed that enterococci are the dominating group of bacteria (Krams et al., 2017). Our metagenomic analysis confirms that the gut of *G. mellonella* microbiome has a low number of strains and a sparse microbiome, which is consistent with previous findings.

The *G. mellonella* model has been shown to be a suitable preliminary screening for efficacy assessment of antistaphylococcal agents (Desbois and Coote, 2012; Gibreel and Upton, 2013). Gibreel and Upton used *G. mellonella* larvae to compare a synthetic version of epidermicin with vancomycin in the treatment of *S. aureus.* However, the outcome measures of this study were mainly on the survival and immune response of the larvae from this treatment (Gibreel and Upton, 2013). Furthermore, Sheehan et al. described how *S. aureus* activates the immune response of *G. mellonella* larvae, grows, disseminates throughout the tissues, and ultimately kills the host (Sheehan et al., 2019). This study showed that inoculum of *S. aureus* (2.0E+06 larva per larva) decreased larval viability by about 50 % at 48 h, which was similar to our results. The study also showed that *S. aureus* infection increased hemocyte densities (Sheehan et al., 2019). However, in our study, we observed that *S. aureus* had a significant reduction in the hemocyte densities of the infected larvae compared with the control arm. These results confirm that *G. mellonella* larvae could be utilized as a *S. aureus* infection model to study the effects of different antimicrobials on the larval immune response, nodules formation, which are similar to soft tissue infections in humans.

It was suggested in the literature that losing natural microbiota is associated with a reduction in the immune response (Alhifany et al., 2019). Krams *et al.* found that the number of CFUs of *Enterococci* and expressions of certain immunity-related antimicrobial peptide (AMP) genes such as Gallerimycin, Gloverin, 6- tox, Cecropin-D, and Galiomicin are significantly lowered after antibiotic treatment (Krams et al., 2017). However, our results were not correlated with the previous findings (Krams et al., 2017). In our study, the vancomycin arm has the lowest *Enterococcus* CFU counts, but the hemocyte density was similar to the control arm (PBS), which has normal *Enterococcus* CFU counts. On the other hand, we saw the correlation between immune response and microbiota (*Enterococcus* count) in the NS oil and combination arms, which confirms the previous observation that maintaining microbiota is associated with an enhancement in the immune response (Krams et al., 2017). In our study, we found that NS oil preserves *Enterococcus*, thereby contributing to the protection of gut microbiota. This aligns with the findings of Akinrinde AS and colleagues, who investigated the effect of NS oil on gut microbiota integrity. They similarly concluded that NS oil helps preserve gut microbiota, thereby safeguarding the gut (Akinrinde et al., 2022).

The protective effect of the NS oil was previously reported in the literature. Mousa *et al.* conducted a clinical trial to evaluate the efficacy of NS in preventing febrile neutropenia in children with brain tumors. NS showed not only a decrease in the incidence of febrile neutropenia and the length of hospitalization but also an improvement in nutritional status. It was noticed that body mass index (BMI) in the intervention group was not as negatively affected as the control group, which was attributed to the protective effect of NS on the GI tract from the chemotherapy. This protective effect has been assumed to support food intake, thereby improving nutritional status in general (Mousa et al., 2017). Even though this may show that NS could protect the gut microbiome, the exact effect on specific bacterial species has not been determined yet. Therefore, further clinical research is necessary to assess the beneficial GI effects of NS oil and to support its use in clinical practice. Furthermore, Kapan *et al.* conducted an *in vivo* study and proved that NS is able not only to preserve the intestinal epithelial barrier function but also to prevent bacterial translocation through the barrier to the blood (Kapan et al., 2012).

Some factors may limit the findings of our study. First, during the treatment course, only one dose of all antimicrobials was given to treat the *S. aureus* infection. We now assume that if more than one dose is given, a better understanding of the effect on the microbiome would be

shown. Therefore, future studies may consider multiple dosing. Second, the NS oil showed a lack of pronounced activity in terms of survival rate, and the investigators suggested that the formulation and the bioavailability of the NS oil could be responsible for that issue. NS oil was used as crude oil and dissolved in dimethyl sulfoxide (DMSO). Although this formulation was tested alone and showed to be safe for the larva, it might have affected the survival in the NS oil and the combination arms. Nevertheless, *S. aureus* burden was lower in the presence of the NS oil, but that was not correlated with the larva survival. Therefore, we suggest that NS oil formulation may have some pharmacokinetic limitations that need to be identified and eliminated in order to improve its effectiveness. Furthermore, even though the NS oil was not comparable with vancomycin, we do not consider this a significant limitation. Vancomycin is a well-known antistaphylococcal agent. Moreover, this study did not aim to demonstrate superiority over the current standard treatment; we only used vancomycin as a positive control. Other limitations should be taken into account that we used 'pet food' instead of 'research grade' larvae, which may have grown on hormones or antibiotics. It is known that feeding the larvae with antibiotics can increase the pool of microbial resistance, which can also affect our results. Nevertheless, our larvae were purchased from a well-known company (Timberline, USA), which has high quality worms with standard amounts of protein, fat, and fibers. Therefore, we hope that would improve the reproducibility of our study. Moreover, Allonsius *et al.* compared the microbiome of bait larvae to the research grade larvae and found that the microbial communities in their gut, haemolymph and fat bodies of both types appeared to have the same bacterial taxa (Allonsius et al., 2019). Finally, we counted hemocytes manually under light microscope, which gives us qualitative findings than quantitative ones. However, we thought that would be enough for the purpose of our study.

5. Conclusion

This study supports the use of *G. mellonella* model as a preliminary test to assess the *in vivo* antistaphylococcal activity of vancomycin and NS oil and the effect on the gut microbiota. NS oil appeared to be more selective than vancomycin against *S. aureus* and protective for the natural *Enterococcus* gut flora of *G. mellonella*. We highlight the need for evaluation of this new model, which is a useful preliminary screening tool for assessing not only the *in vivo* efficacy of new antimicrobials but also to study the effect of new compounds on the microbiome before proceeding to mammalian studies, which may help to reduce animal experiments and costs.

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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References

Akinrinde, A.S., Adekanmbi, A.O., Olojo, F.O., 2022. Nigella sativa oil protects against cadmium-induced intestinal toxicity via promotion of anti-inflammatory mechanisms, mucin expression and microbiota integrity. Avicenna J. Phytomed. 12 (3), 241–256.

Alhifany, A.A., Almutairi, A.R., Almangour, T.A., Shahbar, A.N., Abraham, I., Alessa, M., Alnezary, F.S., Cheema, E., 2019. Comparing the efficacy and safety of faecal microbiota transplantation with bezlotoxumab in reducing the risk of recurrent Clostridium difficile infections: a systematic review and Bayesian network meta-analysis of randomised controlled trials. BMJ Open 9 (11), e031145.

Allonsius, C.N., Van Beeck, W., De Boeck, I., Wittouck, S., Lebeer, S., 2019. The microbiome of the invertebrate model host Galleria mellonella is dominated by Enterococcus. Animal Microbiome 1 (1), 7.

Almangour, T.A., Perry, G.K., Terriff, C.M., Alhifany, A.A., Kaye, K.S., 2019. Dalbavancin for the management of gram-positive osteomyelitis: Effectiveness and potential utility. Diagn. Microbiol. Infect. Dis. 93 (3), 213–218.

Almangour, T.A., Perry, G.K., Alhifany, A.A., 2020. Dalbavancin versus standard of care for the treatment of osteomyelitis in adults: A retrospective matched cohort study. Saudi Pharmaceut. J.

Anggono, F.D., Kuswandari, S., 2017. Comparison of antibacterial activity inhibitory of black cumin (Nigella sativa) oil, Cresophene®, and Calcium hydroxide. Padjadjaran J. Dentis. 29 (1).

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120.

Brown, A.F., Leech, J.M., Rogers, T.R., McLoughlin, R.M., 2014. Staphylococcus aureus colonization: modulation of host immune response and impact on human vaccine design. Front. Immunol. 4, 507.

Champion, O.L., Wagley, S., Titball, R.W., 2016. Galleria Mellonella as a Model Host for Microbiological and Toxin Research. Virulence 7 (7), 840–845.

Desbois, A.P., Coote, P.J., 2012. Utility of greater wax moth larva (Galleria mellonella) for evaluating the toxicity and efficacy of new antimicrobial agents. In: Advances in Applied Microbiology, vol. 78. Elsevier, pp. 25–53.

Emeka, L.B., Emeka, P.M., Khan, T.M., 2015. Antimicrobial activity of Nigella sativa L. seed oil against multi-drug resistant Staphylococcus aureus isolated from diabetic wounds. Pak. J. Pharm. Sci. 28 (6), 1985–1990.

Endres, B.T., Begum, K., Sun, H., Walk, S.T., Memariani, A., Lancaster, C., Gonzales-Luna, A.J., Dotson, K.M., Bassères, E., Offiong, C., 2019. Epidemic Clostridioides difficile ribotype 027 lineages: comparisons of Texas versus worldwide strains. Open Forum Infectious Diseases. Oxford University Press US.

Fararh, K.M., Atoji, Y., Shimizu, Y., Takewaki, T., 2002. Isulinotropic properties of Nigella sativa oil in Streptozotocin plus Nicotinamide diabetic hamster. Res. Vet. Sci. 73 (3), 279–282.

Gholamnezhad, Z., Havakhah, S., Boskabady, M.H., 2016. Preclinical and clinical effects of Nigella sativa and its constituent, thymoquinone: A Review. J. Ethnopharmacol. 190, 372–386.

Gibreel, T.M., Upton, M.J., 2013. Synthetic epidermicin NI01 Can protect *Galleria mellonella* larvae from infection with *Staphylococcus aureus*. J. Antimicr. Chemother. 68 (10), 2269–2273.

Hannan, A., Saleem, S., Chaudhary, S., Barkaat, M., Arshad, M.U., 2008. Anti bacterial activity of Nigella sativa against clinical isolates of methicillin resistant Staphylococcus aureus. J. Ayub Med. Coll. Abbottabad. 20 (3), 72–74.

Jander, G., Rahme, L.G., Ausubel, F.M., 2000. Positive correlation between virulence ofPseudomonas aeruginosa mutants in mice and insects. J. Bacteriol. 182 (13), 3843–3845.

Kapan, M., Tekin, R., Onder, A., Firat, U., Evliyaoglu, O., Taskesen, F., Arikanoglu, Z., 2012. Thymoquinone ameliorates bacterial translocation and inflammatory response in rats with intestinal obstruction. Int. J. Surg. 10 (9), 484–488.

Kavanagh, K., Fallon, J.P., 2010. *Galleria mellonella* larvae as models for studying fungal virulence. Fungal Biol. Rev. 24 (1–2), 79–83.

Krams, I.A., Kecko, S., Jõers, P., Trakimas, G., Elferts, D., Krams, R., Luoto, S., Rantala, M.J., Inashkina, I., Gudrā, D., 2017. Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae. J. Exp. Biol. 220 (22), 4204–4212.

Kumar, P., Patra, A.K., Mandal, G.P., Samanta, I., Pradhan, S., 2017. Effect of black cumin seeds on growth performance, nutrient utilization, immunity, gut health and nitrogen excretion in broiler chickens. J. Sci. Food Agric. 97 (11), 3742–3751.

Lowy, F.D., 1998. Staphylococcus aureus infections. N. Engl. J. Med. 339 (8), 520–532.

Mousa, H.F.M., Abd-El-Fatah, N.K., Darwish, O.A.-H., Shehata, S.F., Fadel, S.H., 2017. Effect of Nigella sativa seed administration on prevention of febrile neutropenia during chemotherapy among children with brain tumors. Child's Nervous Syst. 33 (5), 793–800.

Mouwakeh, A., Kincses, A., Nove, M., Mosolygo, T., Mohacsi-Farkas, C., Kisko, G., Spengler, G., 2019. Nigella sativa essential oil and its bioactive compounds as resistance modifiers against Staphylococcus aureus. Phytotherapy Res. 33 (4), 1010–1018.

Otto, M., 2018. Staphylococcal Biofilms. Microbiol. Spectr. 6 (4).

Peleg, A.Y., Monga, D., Pillai, S., Mylonakis, E., Moellering Jr, R.C., Eliopoulos, G.M., 2009. Reduced susceptibility to vancomycin influences pathogenicity in Staphylococcus aureus infection. J. Infect. Dis. 199 (4), 532–536.

Savoia, D., 2012. Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiol. 7 (8), 979–990.

Sheehan, G., Clarke, G., Kavanagh, K., 2018. Characterisation of the cellular and proteomic response of Galleria mellonella larvae to the development of invasive aspergillosis. BMC Microbiol. 18 (1), 63.

Sheehan, G., Dixon, A., Kavanagh, K., 2019. Utilization of Galleria mellonella larvae to characterize the development of Staphylococcus aureus infection. Microbiology 165 (8), 863–875.

Sheehan, G., Kavanagh, K., 2018. Analysis of the early cellular and humoral responses of *Galleria mellonella* Larvae to infection by Candida albicans. Virulence 9 (1), 163–172.

Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L., Fowler Jr., V.G., 2015. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin. Microbiol. Rev. 28 (3), 603–661.

Tsai, C.J.-Y., Loh, J.M.S., Proft, T., 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence 7 (3), 214–229.
Wickham, H., 2009. Elegant Graphics for Data Analysis (ggplot2). Springer-Verlag, New York, NY.
Yang, H., Chen, G., Hu, L., Liu, Y., Cheng, J., Ye, Y., Li, J., 2018. Enhanced efficacy of imipenem-colistin combination therapy against multiple-drug-resistant Enterobacter cloacae: in vitro activity and a Galleria mellonella model. J. Microbiol. Immunol. Infect. 51 (1), 70–75.