Original Article

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Bombyx mori as a model for *Niallia circulans* pathogenicity

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SUMMARY Increasing incidences of resistance to antibiotics by pathogenic bacteria is a worldwide concern and isolation of antibiotic-resistant strains of *Niallia circulans* (formerly known as *Bacillus circulans*), an opportunistic human pathogen, has been reported. Due to their lack of ethical constraints as well as their cost-effective rearing, invertebrates have been commonly used to study infection by bacteria pathogenic to humans. In this study, we demonstrate that a foodborne strain of *N. circulans* kills larvae of the silkworm, *Bombyx mori* within 48 h after hemolymph injection. The infected larvae turned black with an increase in the phenoloxidase (PO) activity in the hemolymph. Midgut injection of *N. circulans* resulted in the killing of larvae within 96 h. A significant increase in bacterial load was observed in the hemolymph 12 h after infection. The viable hemocyte number decreased to 48% within 12 h of injection. RT-qPCR analysis revealed that upon hemolymph infection with *N. circulans* the expression of the antimicrobial peptide (AMP) genes, *Bmdefensin-B* and *Bmgloverin-3*, were upregulated 2.5- and 1.8-fold, respectively, whereas 1.6-fold upregulation was observed for *BmToll-2* in the larval fat body. Therapeutic effects of antibiotics like tetracycline, imipenem, ceftriaxone, ampicillin, and clindamycin were observed against *N. circulans* in the *Bombyx* larvae with varying efficacies. Results from this study suggest that larvae of *B. mori* can be used as infection models for screening therapeutics that are effective against *N. circulans*.

Keywords *Niallia circulans*, *Bombyx mori*, *Galleria mellonella*, infection model, antibiotic resistance

1. Introduction

Niallia circulans (formerly known as *Bacillus circulans*) is a Gram-positive, spore-forming, rod-shaped species (*1*). It is usually found in soil and the non-pathogenic *N. circulans* and other *Bacillus* spp. are used for the industrial production of enzymes, especially proteinases (*2*). The pathogenic *N. circulans* strains have been implicated in multiple human infections such as septicemia, and wound and abscess infections, especially in immunocompromised individuals (*3-11*). In some instances, *N. circulans* infection resulted in the death of the immunocompromised patient (*12*). A recent report has shown that *N. circulans* can also infect immunocompetent patients, which in this incident leads to spondylodiscitis (*13*). Since *N. circulans* is an endospore-forming bacteria, its spores can be resistant to UV radiation and certain disinfectants which makes it a source of a contaminant in operating rooms in hospitals and it can cause pseudo-epidemics in hospitals and clinic.

N. circulans strains resistant to common antibiotics pose a threat as they can lead to the formation of more dangerous multidrug-resistant (MDR) strains. The increased rate of the appearance of antibiotic-resistant bacterial strains is a major concern worldwide. The unregulated prescriptions of antibiotics, lack of government oversight on the self-use of antibiotics, poor hygiene along with the widespread use of antibiotics in livestock farming contributed to the dramatic rise in the appearance of antibiotic-resistant strains (*14-17*). It is imperative to have an infection model that can be used to evaluate antibiotic susceptibility of emerging human pathogenic bacteria that are resistant to antibiotics so that the patients can be treated efficiently.

The preferred infection model for bacteria that are pathogenic to humans is the house mouse, *Mus musculus*, however, it is cost-intensive. A major disadvantage of the large-scale use of mice as an animal model is the ethical issues. The zebrafish, *Danio rerio* has been used as an alternative vertebrate model to the house mouse for bacterial pathogenesis (*18*) but it has limitations in quantitative experiments where accurate injections of large volumes are required. A similar disadvantage exists in invertebrate animal models like the nematode,

Caenorhabditis elegans, and the fruit fly, *Drosophila* melanogaster (*19,20*). In this respect, the lepidopteran larvae can be an alternative infection model due to their relatively large body size making it easier for quantitative injection for antibiotic screening. Both the silk moth, *B. mori* (commonly called silkworm), and the wax moth, *G. mellonella* have been widely used as an infection model to study bacterial pathogenicity (*21,22*). These larvae can be very useful for rapid initial screening of compounds with the potential to be antimicrobials followed by confirmation in the mouse model.

Insects lack adaptive immunity but they possess innate immunity and there are remarkable similarities between insects and mammals with respect to innate immunity, antimicrobial peptide (AMP) gene expression, and Toll and Imd signaling pathways (*23*). When infected with a pathogen, insects mount a powerful immune response mediated by hemocytes, the fat body, the midgut, the salivary glands, and other tissues. A number of structural and functional similarities to the innate immune system have been found between insects and mammals. Because of these similarities, insects have become popular choices not only for evaluating the virulence of microbial pathogens but also for evaluating the efficacy of antimicrobial agents (*24*). Advantages of using insect larvae as an animal model include lack of ethical constraints, cost-effective rearing procedure, accurate injection of larger volumes to hemolymph or midgut, and easy pharmacological studies with isolated organs. Other than bacterial pathogenicity, silkworm larvae have been used as an animal model for fungal pathogenicity, exotoxin identifications, *etc*. (*25-27*). Using silkworm larvae, it has been shown that the therapeutic effect of antibiotics against *Staphylococcus aureus* can be studied (*28*). Recently, we have reported the therapeutic effects of antibiotics against *Escherichia coli* O157:H7 and *Klebsiella pneumoniae* in the *Bombyx* larvae infection model (*29,30*).

Pathogenic strains of *B. cereus* and *B. anthr*acis have been reported to kill *Bombyx* and *Galleria* larvae (*31-34*). It has been reported that both *Bombyx* and *Galleria* larvae harbor *N. circulans* in their gut under ordinary conditions (*35,36*). To our knowledge, there is no report on the pathogenicity of *N. circulans* in insects. In this study, we report that a food-borne *N. circulans* strain can kill *Bombyx* larvae opening up a new way of rapid screening for unknown compounds with antibacterial properties that might be effective against antibiotic-resistant strains of this pathogen.

2. Materials and Methods

2.1. *N. circulans* isolation and culture conditions

The *N. circulans* strain was isolated from street foods in Dhaka, Bangladesh. For characterization of the strain, standard biochemical tests were carried out (Supplementary Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*) and for examining susceptibility to antibiotics the antimicrobial resistance (AMR) profile (Supplementary Table S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*) was generated. 16*S*rRNA gene sequencing was carried out to confirm the identity of the isolate as *N. circulans*. Tryptic Soy Agar plates (TSA) or Tryptic Soy Broth medium (TSB) was used to culture *N. circulans* at 37°C. *E. coli* DH5α was cultured in Luria-Bertani (LB) medium at 37ºC.

2.2. Insect rearing conditions

B. mori (Nistari-M) larvae were maintained and reared in the Bangladesh Sericulture Research and Training Institute (BSRTI) using fresh mulberry leaves. As reported previously (*29*), larvae were kept at 25°C with ~75% relative humidity at a 16/8 hour of light/dark cycle. 5th instar feeding larvae (day 2-3) were used in all experiments. 6th instar feeding *Galleria* larvae were purchased from Ispahani Agro Ltd. (Gazipur, Dhaka, Bangladesh).

2.3. Infection of insect larvae with *N. circulans*

For injection experiments, bacteria were grown overnight, washed with phosphate-buffered saline (PBS), and suspended in PBS followed by injection into the blood (hemolymph) or midgut directly using a 1-0 mL insulin syringe (30G × 5/16"). Overnight culture of *N. circulans* was collected by centrifugation (6,000 rpm, 5 min) and washed with PBS followed by suspension at a density of 1.53×10^{10} colony forming unit (CFU)/mL in PBS. Fifty µL of PBS or bacteria (7.65×10^8 CFU) was injected into larvae (n = 10). The larvae were kept on tissue papers in Tupperware boxes with the lid partially opened. Upon prodding with tips if the larvae did not show any movement we considered them dead. For *Galleria* larvae, *N. circulans* (1.53×10^8 CFU) suspended in 10 µL of PBS or PBS only (control) were injected as described previously (3*0*).

2.4. PO activity assay

PO activity was measured following previously published reports (*37-39*) with minor modifications. Hemolymph was collected from infected larvae 12 h post-infection. One hundred µL of bleeding buffer was added to 500 µL of the hemolymph. Enzymatic assay was carried out at 490 nm using GloMax® Explorer Multimode Microplate Reader (Promega, Madison, WI, USA).

2.5. Bacterial load determination and hemocyte viability in *Bombyx* hemolymph

Bacterial load and hemocyte viability in the *Bombyx*

hemolymph after infection with *N. circulans* were determined as reported previously by our group (*29,30*).

2.6. RT-qPCR analysis of immune response genes

RNA was isolated from the fat body of the dorsolateral region of larvae as described previously (*40*) using TRIzol® according to the manufacturer's instructions (Invitrogen, Waltham, MA, USA). cDNA was prepared using ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. RT-qPCR was performed using Luna® Universal qPCR Master Mix (New England Biolabs) in a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The reference gene used for the normalization of RT-qPCR results was *Bm-rp49* (*41*). Primers used in this study are listed in Supplementary Table S3 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*). Primers for *BmToll-2* were designed with Primer 3 (*https://bioinfo.ut.ee/primer3/*) based on a previously published sequence (*42*). Gene expressions were quantified using the $2^{-\Delta\Delta CT}$ method.

2.7. Antibiotic susceptibility tests of *N. circulans in vitro* and *in vivo*

AMR profile of *N. circulans* strain (Supplementary Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*) showed that it is resistant to clindamycin (Incepta Pharmaceuticals, Dhaka, Bangladesh), moderately sensitive to tetracycline (Sigma-Aldrich, Saint Louis, MO, USA), and sensitive to imipenem (ACI Ltd., Dhaka, Bangladesh), ceftriaxone (Radiant Pharmaceuticals, Dhaka, Bangladesh), and ampicillin (Opsonin Pharma Ltd., Dhaka, Bangladesh). In accordance with Clinical and Laboratory Standards Institute (CLSI) standards, the MICs of these five antibiotics against *N. circulans* were determined through microdilution (*43*) in which absorbance at 600 nm was measured in a microplate reader (GloMax® Explorer Multimode Microplate Reader, Promega). In order to determine the therapeutic effects of antibiotics *in vivo* against *N. circulans*, *Bombyx* larvae (n = 10) were injected with bacteria or PBS and then injected with different antibiotic doses suspended in 50 µL of water. The viability of larvae was monitored up to 72 hours after injection. In accordance with Hamamoto *et al*. (*28*), the LD_{50} value for *N. circulans* and the ED_{50} of antibiotics were determined.

2.8. Statistical analyses

Student's *t*-test was used for statistical analyses using GraphPad Prism 9 software.

3. Results

3.1. *N. circulans* infection through hemolymph kills larvae of *B. mori*

We tested whether the food-borne *N. circulans* strain we isolated is pathogenic by injecting it into *Bombyx* larvae *via* hemolymph. Upon injection with 7.65×10^8 CFU of *N. circulans* ~80% of larvae died within 24 h and 100% died within 48 h and PBS- or *Escherichia coli* DH5α-injected larvae did not die (Figure 1A). Due to melanization, the larvae that died turned black (Figures 1B and 1C). One hundred-fold reduction of injected bacterial load resulted in the survival of 40% of larvae until 72 h and more dilution of bacteria reduced larval death even more (Supplementary Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*). The LD50 value of the *N. circulans* strain for silkworm larvae is 6.3×10^7 CFU. These results showed that the larvae of *B. mori* can be used as infection models for studying the pathogenicity of *N. circulans*. In another infection model, *Galleria mellonella*, larvae died upon injection with *N. circulans* (Supplementary Figure S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*).

3.2. *N. circulans* injection into the midgut kills *Bombyx* larvae within 96 h

In order to determine whether the oral introduction of

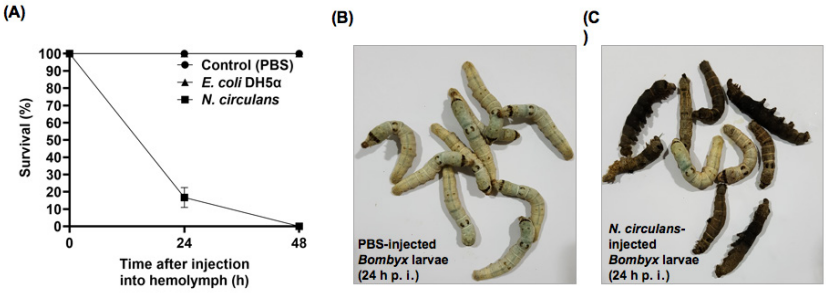

Figure 1. N. circulans kills larvae of B. mori. (A) Survival of *Bombyx* larvae (n = 10) upon injection with 7.65×10^8 CFU of *N. circulans* or *E. coli* DH5α into the hemolymph. PBS was injected into the control larvae. **(B, C)** Images of PBS- or bacteria-injected larvae after 24 h. Increased melanization caused the blackness of the dead larval bodies. Data shown in **(A)** are averages of three independent experiments.

N. circulans to *Bombyx* larvae would kill them or not, bacterial suspension was spread onto mulberry leaves. However, the larvae did not show any interest in eating leaves with bacteria (data not shown). To examine whether entry of *N. circulans* through the midgut can kill larvae, we injected 7.65×10^8 CFU bacteria suspended in PBS directly into the midgut. Within 96 h post-infection, 100% of larvae were dead (Figure 2). This result further confirmed that *N. circulans* infection kills silkworm larvae.

3.3. *N. circulans* infection activates phenoloxidase (PO) activity in *Bombyx* hemolymph

In order to confirm that the blackening of the body color of *N. circulans*-infected *Bombyx* larvae was due to melanization, we measured the PO activity in the hemolymph. After *N. circulans* infection *via* hemolymph, the PO activity in the hemolymph was increased ~7-fold compared with that of the PBS-injected larvae (Figure 3). This result indicates that *N. circulans* infection activates PO activity in *Bombyx* hemolymph.

3.4. *N. circulans* load in *Bombyx* hemolymph increases significantly 12 h post-infection

We collected hemolymph after 6, 12, and 18 h post-injection with 7.65×10^8 CFU of *N. circulans* in order to determine bacterial load. After 6 hours of infection, the hemolymph bacterial count slightly increased, and after 12 h the increase in bacterial load was very significant (Figure 4).

3.5. Reduction of hemocyte number after *N. circulans* infection of *Bombyx* larvae

The infection of the host by bacteria leads to cell death. Hemolymph samples were collected 6 and 12 hours after *N. circulans* injection to examine hemocyte viability in silkworm larvae. After 12 h of bacterial injection, ~52% of hemocytes died (Figure 5). These results indicate that *N. circulans* induces cell death in *Bombyx* larvae.

3.6. *N. circulans* upregulates *Bmdefensin-B*, *Bmgloverin-3*, and *BmToll-2* genes in *Bombyx* fat body

AMPs play important roles in host defense to fend off invading pathogens. Among the AMP genes whose expressions were examined by RT-qPCR, we found that the expression of *Bmdefensin-B* and *Bmgloverin-3* were increased by more than two-fold in the larval fat body upon infection with *N. circulans* compared with the PBS-

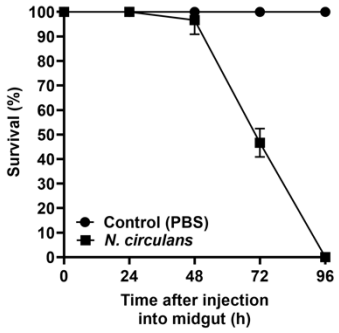

Figure 2. *N. circulans* infection through midgut kills *Bombyx* larvae. Survival of *Bombyx* larvae injected with *N. circulans* through the midgut. Larvae (n = 10) were injected with 7.65×10^8 CFU of *N. circulans* suspended in PBS or PBS only (control) into the midgut.

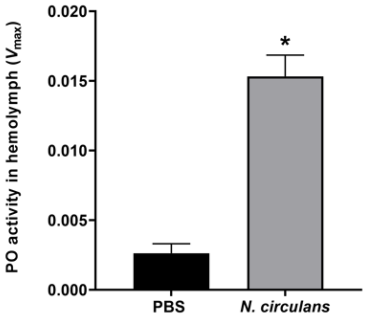

Figure 3. *N. circulans* infection of *Bombyx* larvae increases phenoloxidase (PO) activity in the hemolymph. PO activity after 12 h of injection of PBS or *N. circulans* into the hemolymph (* p-value: 0.0086).

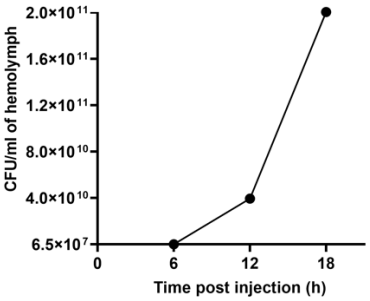

Figure 4. The proliferation of *N. circulans* in the hemolymph of *Bombyx* larvae after injection of 7.65×10^8 CFU. The data shown are averages of three independent experiments.

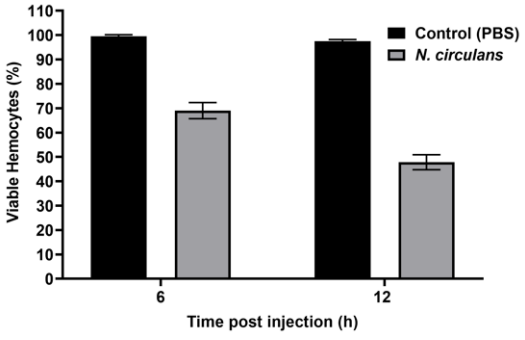

Figure 5. Reduction of hemocyte viability in the hemolymph *Bombyx* larvae upon injection with 7.65×10^8 CFU of *N. circulans*. PBS-injected larvae were used as a control. The data shown are averages of three independent experiments.

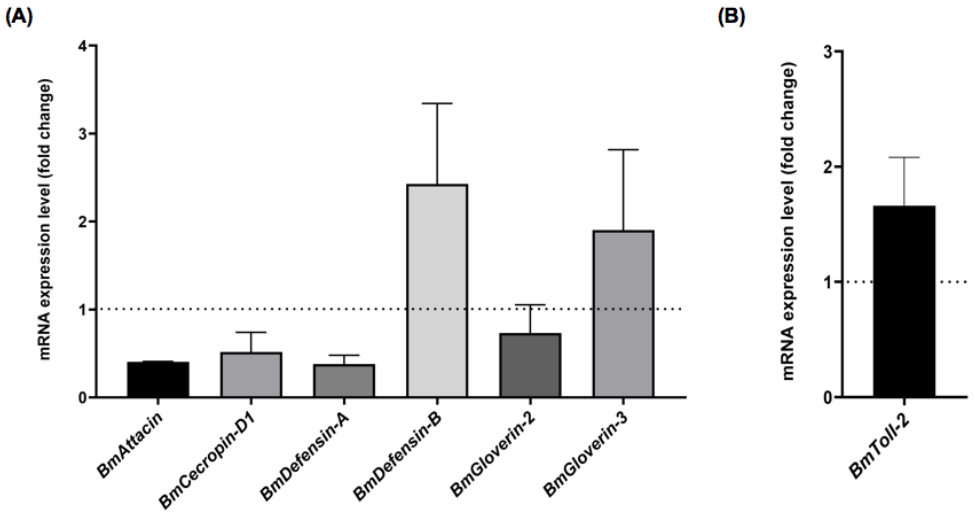

Figure 6. Upregulation of *Bmdefensin-B*, *Bmgloverin-3*, and *BmToll-2* in the *Bombyx* larval fat body. After injecting 7.65×10^8 CFU of *N. circulans* or PBS (control) fat body tissues were isolated after 6 h. The reference gene used for normalization was *Bmrp49*. The dashed line represents an expression level of 1.00. The data shown are from three independent experiments.

injected (control) larvae (Figure 6A). No upregulation was observed for *Bmattacin*, *Bmcecropin-D1*, *Bmdefensin-A*, *Bmgloverin-2*. Overexpression of *BmToll-2* in cultured *Bombyx* cells can upregulate *defensin* genes (*44*), and in this study, we examined *BmToll-2* expression after *N. circulans* infection. We observed ~1.6-fold upregulation in the larval fat body (Figure 6B). Our findings show that infection with *N. circulans* upregulates *Bmdefensin-B*, *Bmgloverin-3*, and *BmToll-2* in *Bombyx* larval fat body.

3.7. Antibiotics protect *Bombyx* larvae from *N. circulans*-induced death

In our next experiment, we examined the antibiotic susceptibility of the *N. circulans* strain. On the basis of the AMR profile (Supplementary Table S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*) and the minimum inhibitory concentrations (MICs) of the antibiotics tested (Table 1), the *N. circulans* strain is resistant to clindamycin, moderately sensitive to tetracycline, and sensitive to imipenem, ceftriaxone, and ampicillin in accordance with the CLSI standards (*43,45*). To determine the efficacy of antibiotics against *N. circulans* in *Bombyx* larvae, we injected antibiotics at different doses into larvae after *N. circulans* infection. We found that all five antibiotics showed varying degrees of therapeutic effect against *N. circulans*. For 90-100% survival, 10 µg per larva was sufficient for tetracycline and ceftriaxone whereas 100 µg per larva was required for ampicillin and imipenem to observe 100% rescue from *N. circulans*-induced death (Figure 7A). Table 1 shows the ED_{50} values of the antibiotics used. The antibiotics significantly reduced *N. circulans*-induced melanization of the larvae (Figure 7B). The results suggest that *Bombyx* larvae could be used to study the therapeutic effects of antibiotics against *N. circulans*.

Table 1. MIC of antibiotics against *N. circulans* and ED_{50} of antibiotics in *Bombyx* larvae against *N. circulans*

Antibiotics	MIC (µg/mL)	ED_{50} (µg per g of larva)
Ampicillin	0.781	1.29
Ceftriaxone	3.12	0.0131
Tetracycline	12.5	0.103
Imipenem	≤ 1.25	0.158
Clindamycin	> 1024	32.8

4. Discussion

Our study showed that both *B. mori* and *G. mellonella* larvae can be killed by *N. circulans* infection. Moreover, we have observed that antibiotics have a therapeutic effect on silk moth larvae. The results show that these larvae are an excellent animal model for studying the pathogenicity of *N. circulans* and could potentially be used for identifying novel antibiotics that are effective against strains of this bacterium that are highly resistant to known antibiotics, which can be confirmed by testing the candidate antibiotics on mice.

Our observations that an *N. circulans* strain isolated from street food can kill larvae of both silk moth and wax moth indicated that the strain is pathogenic to these insects. Similar to earlier studies about increased melanization after infection with bacteria (*21*), we found that the *N. circulans*-infected insect larvae also turned black (Figure 1). Our attempts to infect *Bombyx* larvae by feeding mulberry leaves with *N. circulans* failed (data not shown) whereas midgut-injection of *N. circulans* killed *Bombyx* larvae within 96 h (Figure 2). It is possible that the spreading of *N. circulans* on the mulberry

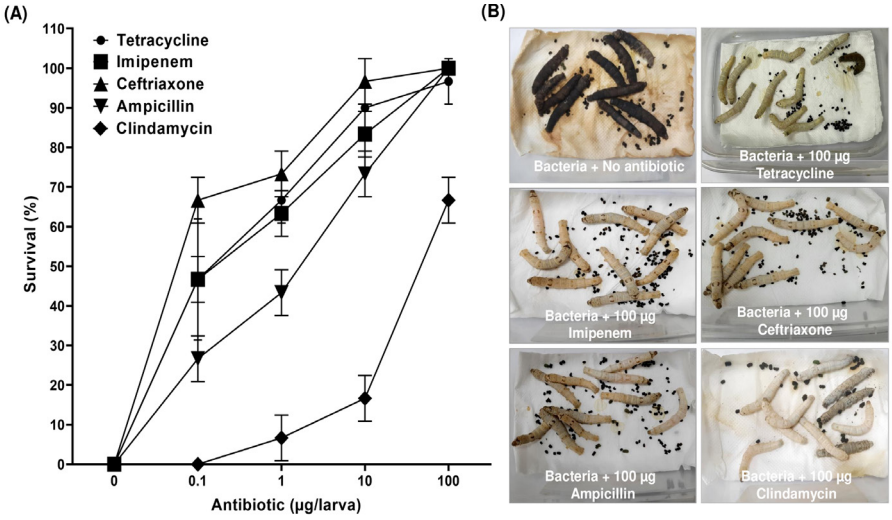

Figure 7. Antibiotics showed therapeutic effects against *N. circulans* in *Bombyx* larvae. (A) Larvae (n = 10) were injected with 7.65 × 10^8 CFU followed by injection of antibiotics at the indicated doses. Survival was counted after 48 h of injection. The data shown are averages of three independent experiments. **(B)** Images of bacteria-injected larvae with or without antibiotics after 48 h of injection.

leaves resulted in the production of an unpleasant smell that prevented the larvae from consuming it and we reported a similar observation for *E. coli* O157:H7 (*29*). It is known that larvae of Lepidoptera turned black in response to exposure to pathogens by producing melanin upon activation of hemolymph phenoloxidase to destroy it (*46*) and, as expected, the PO activity in *Bombyx* larvae was increased after *N. circulans* infection (Figure 3). Culture-filtrate of *S. aureus* or *P. aeruginosa* can kill silkworm larvae after hemolymph injection (*27*) but in this study, we found that the culture-filtrate of *N. circulans* did not kill any silkworm larvae within 24 h, although they appeared sick with very little movement upon touching (data not shown). It is possible that the food-borne *N. circulans* strain we used in this study is not a highly virulent strain or it did not produce enough exotoxins to kill larvae.

Our data showed that the number of circulating *N. circulans* in *Bombyx* hemolymph continued to increase until 18 h post-infection (Figure 4). It would be interesting to know whether there are any preferred silkworm organs that are infected by *N. circulans* which would shed light on its mechanism of action. Localization of a GFP-expressing *N. circulans* strain inside the larval body would be very useful. Similar to *Serratia marcescens*-induced hemocyte death in *Bombyx* larvae (*47*), we have also observed a reduction of viable hemocytes upon *N. circulans* infection (Figure 5), although it remains to be examined whether the cell death is due to apoptosis or not.

AMP genes are upregulated during bacterial infection to destroy the invader without affecting the host cells (*48*). Infection with *Bacillus bombyseptieus* resulted in the upregulation of a number of AMPs including gloverin, attacin, moricin *etc*. (*49*). After *N. circulans* infection of *Bombyx* larvae, we have observed upregulation of *Bmdefensin-B* and *Bmgloverin-3* among the AMP genes whose expressions were examined, although no increase of gene expression was observed for *Bmattacin*, *Bmcecropin-D1*, *Bmdefensin-A*, and *Bmgloverin-2* (Figure 6A). *BmToll-2* has been shown to upregulate AMP genes in *Bombyx* cultured cells *in vitro* (*44*) and we also found upregulation of *BmToll-2* after *N. circulans* infection of *Bombyx* larvae (Figure 6B). Loss-of-function mutant of *BmToll-2* would be useful to examine whether *N. circulans*-induced upregulation of *Bmdefensin-B* and *Bmgloverin-2* is *BmToll-2* dependent or not.

Therapeutic effects of antibiotics in *Bombyx* larvae against pathogenic bacteria like *S. aureus* have been reported (*28*). Recently, we have also reported such effects against *E. coli* O157:H7 and *K. pneumoniae* (*29,30*). Our data revealed that *Bombyx* larvae can also be used to examine the therapeutic effect of antibiotics against *N. circulans*. *In vitro*, *N. circulans* showed susceptibility to ampicillin, ceftriaxone, and imipenem, moderate sensitivity to tetracycline, and resistance to clindamycin (Table 1, Supplementary Table S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=138*). However, *in vivo* experiments revealed that all these five antibiotics protected silkworm larvae from *N. circulans*-induced death with varying efficacies (Figure 7). Interestingly, clindamycin, against which *N. circulans* showed resistance *in vitro*, rescued ~70% of larvae *in vivo*. Such a lack of correlation between *in vitro* and *in vivo* antibiotic susceptibility data of bacteria are common (*50*) and recently, we have also reported such discrepancies with *E. coli* O157:H7 and *K. pneumoniae* using *Bombyx* larvae infection model (*29,30*). These findings strengthen the notion that for testing antibiotic susceptibility tests of pathogenic bacteria appropriate infection models should be used rather than relying solely on *in vitro* data. The antimicrobial effect of medicinal plant extracts in silkworm larvae against *N. circulans* is currently under investigation. By rapidly

screening chemicals with antimicrobial properties against pathogens using *Bombyx* larvae as an animal model, we will be better equipped to combat the ever-increasing number of multidrug-resistant bacteria.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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