



## Research article

Microbiota aggravates the pathogenesis of *Drosophila* acutely exposed to vehicle exhaustYujuan Li<sup>1</sup>, Lei Pan<sup>1</sup>, Pengcheng Li, Gaole Yu, Zhichao Li, Shaokang Dang, Faguang Jin<sup>\*\*</sup>, Yandong Nan<sup>\*</sup>

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## ARTICLE INFO

## Keywords:

Vehicle exhaust  
Intestinal microbiota  
Intestinal epithelium

## ABSTRACT

Vehicle exhaust (VE) is the primary cause of urban air pollution, which adversely affects the respiratory system, exacerbates lung diseases, and results in high mortality rates. However, the underlying mechanism of the pathogenesis is largely unclear. Here, we developed a *Drosophila* model to systematically investigate the effects of VE on their health and physiology. We found that VE significantly impaired life span and locomotion in *Drosophila*. Interestingly, there was an increase in bacterial load in the guts upon VE exposure, suggesting VE is able to induce dysbiosis in the guts. Microbiota depletion can ameliorate the impairment of life span and locomotion. VE causes permeability of intestinal epithelial cells and increases proliferation of intestinal cells, suggesting VE disrupts intestinal homeostasis. We elucidate the underlying mechanism by which VE triggers Imd and DUOX gene expression. Taken together, this *Drosophila* model provides insight into the pathogenesis of *Drosophila* exposure to VE, enabling us to better understand the specific role of microbiota.

## 1. Introduction

Air pollution in the environment is a long-term major public health threat. Vehicle exhaust (VE) is mainly composed of compound matter include nitrogen monoxide, nitrogen dioxide, oxidized hydrocarbons, solid particles, and nitrogen oxides (R. Westerholm and K. E. Egeback 1994). Once these particles are inhaled, they cannot be cleared, resulting in irreversible damage (R. K. Robinson et al., 2018), and it often carries microbes. In addition, VE causes diseases of the brain, heart, nerves, liver, and urogenital tract, as well as psychological damage, and diseases of other systems (A. Salvi et al., 2017; C. Habert and R. Garnier 2015; J. Krauskopf et al., 2018; M. Hullmann et al., 2017). However, the effects of VE on the gastrointestinal tract are unknown. Metazoans harbor complex communities of microbes that are collectively referred to as the microbiota or microbiome. It is well-known that the microbiota pivots to adjust to human physiologies and disease (D. N. Lesperance and N. A. Broderick 2020). Change in the composition and function of the microbiota has been linked to different neuropsychiatric disorders, such as social behavior, stress, and anxiety-related responses in humans (K. Mathee et al., 2020).

The microbiota provides beneficial effects, including decomposition of indigestible element from food; synthesis of vitamins; removal of harmful exogenous organisms that influence host metabolism; establishing a strong systemic and enteric immune system; production of signals for epithelial cells to renew and maintain enteric integrity; secretion of antibacterial goods; and promoting the development of colonization resistance to pathogenic bacteria. An imbalance in intestinal symbiotic homeostasis can result in detrimental effects (J. Sun and E. B. Chang 2014). We used *Drosophila* as a model, which is a strong genetic model of innate immune system responses to bacterial, fungal, and viral infections; it is also an ideal model for studying host-microbial interactions in the gut (N. Buchon et al., 2013). When bacteria and fungi enter the *Drosophila* gut, they activate a variety of defense mechanisms referred to as systemic immune responses (J. A. Hoffmann 1995a).

There is no immune cell in insect body like that of mammals, and insect antibacterial response has no high specificity of antigen-targeting, but insects respond differential accordingly to the kinds of microbes they recognize (A. Takehana et al., 2004; B. Lemaitre et al., 1997; F. Leulier et al., 2003b; V. Bischoff et al., 2004). The immunodeficiency (Imd) pathway is important for intestinal infection responses in *Drosophila*.

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Intestinal homeostasis disorder in *Drosophila* can activate the intestinal Imd pathway primarily by intestinal microbiota and ingested microbes, and forcefully caused by microbial infection (D. Hultmark 1993a). Activation of this pathway causes the generation of antimicrobial peptides (AMPs) in *Drosophila* (A. Takehana et al., 2002; F. Leulier et al., 2003; T. Kaneko et al., 2004). AMPs are micro, positively-charged peptides that conduce to inherent defenses in view the negatively-charged membranes of microbes (M. A. Hanson and B. Lemaitre 2020). When encountered with a microbial cell envelope, AMP will embed in the hydrophobic region of the lipid membrane, resulting in instability of the membrane and ultimately cell death (H. S. Joo et al., 2016). These AMPs are regulated by Toll and Imd NF- $\kappa$ B signaling pathways in systemic response to microbial recognition. Thus, AMPs are usually used as readouts to display the activity of these immune pathways (M. A. Hanson and B. Lemaitre 2020b).

Reactive oxygen species (ROS) kill bacteria as a defensive mechanism that contributes to the innate immune response of insects. Like AMP, ROS capture microorganisms by means of melanin cascades deposited by melanin (T. Tanji and Y. T. Ip 2005). DUOX mediates generation of ROS, which is the primary intestinal immune response to sustain intestinal homeostasis of hexapoda. Simultaneously, intestinal health offers basic conditions for expression and trigger of DUOX, and is necessary to sustain homeostasis of symbiotic microbes (E. M. Ha et al., 2009; E. M. Ha et al., 2009; H. Diaz-Albiter et al., 2012; J. H. Oliveira et al., 2011; Z. Yao et al., 2016). DUOX, a member of the intestinal nicotinamide adenine dinucleotide phosphate oxidase (NADPH), is involved in many aspects of gut interactions with microorganisms, such as microbial clearance, intestinal epithelial cell renewal (ECR), redox-dependent signaling pathway regulation, biomolecular crosslinking, and symbiotic and pathogen differentiation (S. H. Kim and W. J. Lee 2014).

Since responses to many stressors are highly conserved among species, the toxic effects of VE on *Drosophila* can be used as a representative model. In this study, *Drosophila* flies were used to investigate whether VE had negative physiological and physical effects. When intestinal Imd and ROS-related genes were expressed, gastrointestinal microbiota dysregulation and physiological changes occurred, causing death after intestinal injury in *Drosophila*. The *Drosophila* model allowed analysis of the contribution of VE to the intestinal pathogenesis of *Drosophila*. However, the effect of VE exposure on host gut microbes is still unknown. A series of functional tests were carried out after VE exposure to determine its effects on intestinal microbes and the immune mechanism in *Drosophila*.

## 2. Materials and methods

### 2.1. Stocks and bacterial culture

All *Drosophila* stocks (Oregon R) were gifted by the institute of genetic and developmental biology, Chinese Academy of Sciences (CG12051), and were cultured at 25 °C and 60% humidity with 12 L:12 D light cycles in standard yeast culture medium (W. Liu et al., 2012). Adult *Drosophila* were used for experiments at 2–4 days after emergence. The germ free (GF) model was described with modification (S. C. Shin et al., 2011). In short, we collected the eggs of fruit flies within 8 h and washed them twice with ddH<sub>2</sub>O. We use 1:30 diluted disinfectant (Procter & Gamble; Gamble Co., Cincinnati, OH, USA), followed by 2.5% sodium chloride (Sigma Aldrich, St. Louis, MO, USA), 70% ethanol, and 0.01% TritonX-100T PBS were cleaned once, respectively. The GF fly system was added with known bacteria to form conventionally reared (CR) or gnotobiotic flies (W. J. Lee and P. T. Brey 2013). The NP3084 and DUOX RNAi *Drosophila* strain was purchased from Prof. Gao Guanjun (Shanghai Technology University) (X. Xiao et al., 2017).

Bacteria were obtained from General Microbiological Culture Collection Center in China, and extracted from *Drosophila* using specific media (L. Guo et al., 2014) (M. Lopez-Siles et al., 2012), identified based on the strength of the 16S rRNA sequence with PCR primers (F: 5'-AAAGATGGCATCATCAAC-3',

R:5'-TACCGTCATTATCTTCCGCAA-3'), as previously described (W. Liu et al., 2017). To obtain commensal bacteria, specific media were used to culture strains of *E. coli* and *L. plantarum* (M. Lopez-Siles et al., 2012b).

### 2.2. Vehicle exhaust collection method and drosophila exposure

Vehicle exhaust was collected by using a transparent 50L plastic bag in the exhaust pipe after starting the car, which is filled and tied up. *Drosophila* were placed in a transparent bag with a capacity of 10 L and the air inside the bag was drained. A drainage tube was left at the mouth of the bag to avoid gas reflux, and a 250 mL syringe was used to inject VE and air into the bag through the drainage tube. To control the concentration of VE, the volume was controlled (250 ml/time) by injecting air and VE 10 times to reach a VE concentration of 50%.

### 2.3. Survival rate of drosophila after VE exposure

According to the number flies/tube (10 females and 10 males), the experimental group and the control group were treated for 3 h every day for a total of 6 days. The number of deaths was recorded.

### 2.4. Two bacteria make up the three proportions of medium for fruit flies

*L. plantarum* and *E. coli* were combined for a total of 1 ml. The proportions [1:1 (0.5 ml: 0.5 ml), 1:100 (0.01 ml: 0.99 ml), and 1:10,000 (0.0001 ml: 0.9999 ml)] were mixed with the medium.

### 2.5. Measuring running and climbing ability

Flies were treated with VE for 3 h and then transferred to a glass cylinder (5 ml). Locomotion tests were carried out as previously described (M. J. Palladino et al., 2002). To test climbing behavior, all flies dislodged to the bottom of the tube. Climbing was observed using a video cassette recorder (VCR), and creeping-in distance at the 6th second was recorded. To test running speed, six flies were gently vibrated to the bottom of a glass cylinder in the dark. This equipment was parallel and vertical to the photo-source 15 cm away. Running was observed using a VCR, and the time to cross to the other end of the glass cylinder was counted.

### 2.6. Smurf assay

The blue dye no. 1 (Sigma-Aldrich, 2.5% wt/vol) was mixed with *Drosophila* standard medium. *Drosophila* were exposed to VE after 4 days after being reared on the medium with dye for 12 h. A fly was deemed a smurf when dye coloration was detected outside the digestive tract. The dye was used to observe the intestinal integrity of *Drosophila* (K. Chen et al., 2019). Dissemination of stains was observed in the fed *Drosophila* bodies. Calculation of Smurf % has been described previously (W. Liu et al., 2017; M. Rera et al., 2011).

### 2.7. Feces index

Fruit flies were placed inside their food tubes covered by tinfoil, which was placed close to the tube wall without gaps, and the blue dye was placed in them for 4 h. The tinfoil was removed and the blue dots were counted (P. Cognigni et al., 2011).

### 2.8. PH3 dyeing

The guts were dissected in phosphate-buffered saline (PBS) and fixed organic components with 4% paraformaldehyde for 30 min. Swatches were blocked with special solution for 30 min in a mixture of 0.3% Triton X-100, 0.2% goat serum, and 0.1% fetal calf serum. Samples were incubated in rabbit-derived antibodies against phospho-histone 3 (Millipore, H0412; 1:1500 dilution) overnight at 4 °C, washed three times with PBS

with 0.3% Triton X-100, and incubated with anti-rabbit in PBS (Invitrogen, WP20007; 1:1000 dilution) and DAPI dye (Invitrogen, 1:1000 dilution) for 2 h. The treated intestinal samples were observed under a fluorescence microscope (Leica DM4000).

### 2.9. Intestinal CFUs of fruit flies were measured

After exposure to VE for 4 days, 12 *Drosophila* flies were selected, and the guts were dissected. After grinding and gradient dilution with aseptic PBS, the solution was placed in NA agar plates, which were incubated at 37 °C for 48 h, and the number of CFUs was counted (Y. Jia et al., 2021).

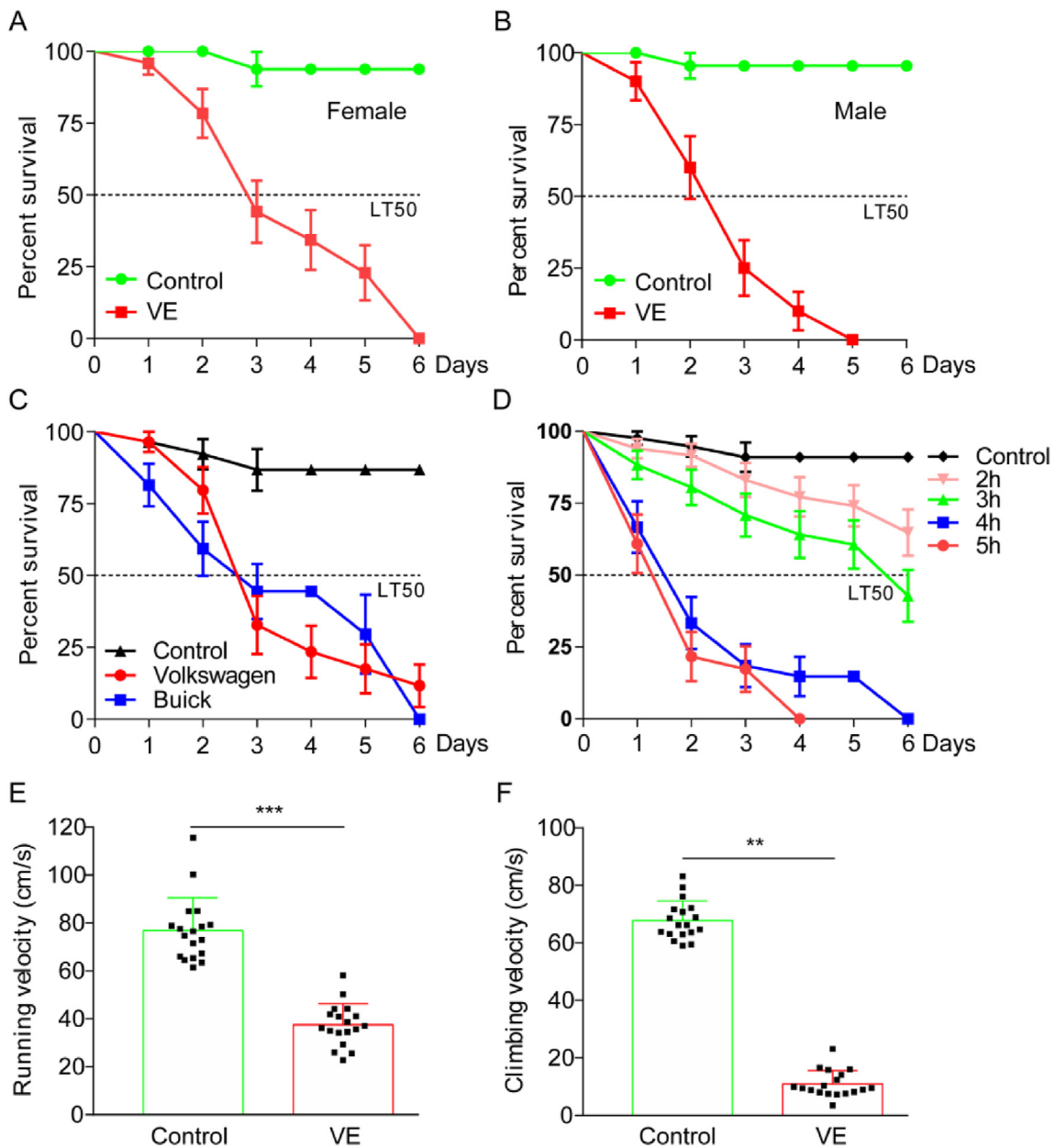
### 2.10. Antibiotic experiment

Antibiotics (penicillin 0.07 g/50 ml glucose, streptomycin 0.1 g/50 ml glucose) were dissolved in distilled water and mixed with the medium

(C. Zhou et al., 2008). Conventionally Reared *Drosophila* (CR) exposed in VE were collected and was placed in the medium tubule containing antibiotics. Fly survival and behavioral abilities were recorded.

### 2.11. Quantitative real-time PCR

For CR treated with VE for 4 days, total RNA was extracted from the intestines of 30–40 *Drosophila* with TRIzol, and cDNA was synthesized with Prime Script RT reagent Kit (TakaRa). Reverse transcription was performed with 0.5 µg total RNA using Oligo dT, and 1StcNDA was diluted 10–20 times with sterile water. The experiment was performed at least in triplicate using SYBR Green (Roche) in a LightCycler 480 System (Roche). The Ct value was normalized to 1 in the control sample using the  $\Delta\Delta Ct$  method and the related expression of the standardized gene rp49 (Y. Jia et al., 2021). Calculation of  $2^{-\Delta\Delta Ct}$  has been described previously (W. Liu et al., 2017). The amplification machine was obtained from Bio-Rad



**Figure 1.** VE reduced the longevity and behavioral abilities of *Drosophila*. (A–B) Survival rates of *Drosophila* of both sexes exposed to VE. (C) The survival rate of *Drosophila* exposed to two types of VE. (D) The survival rate of *Drosophila* exposed for different times to VE. (E–F) Running and climbing speeds of flies after VE exposure. Experiments were repeated more than 10 times (means  $\pm$  SEM, n=10), control served as negative control. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Laboratories (cat no. 172–5121). The primer sequences were as follows: *Attacin*: 5'-CTGCACTGGACTACTCCACATCA, 3'-CGATCTGCGACTGC-CAAAGATTG; *Diptericin*: 5'-CTGCACTGGACTACTCCACATCA, 3'-CGATCTGCGACTGCCAAAGATTG; *DUOX*: 5'-GCGGAGATTGCA-CACATTG, 3'-ATGCCCTTTTGCCTTGCTG; and *rp49*: 5'-GACGCTT-CAAGGGACAGTATCTG, 3'-AAACGCGGTTTCAGCATGA.

## 2.12. Statistical analysis

Flies were randomly divided into groups, and descriptive analysis is provided in the caption. According to the experimental nature and sample type, the Student's t-test was adopted. All analyses were performed using GraphPad Prism statistical software. The number of independent experiments was greater than three times. ANOVA tests were used to analyze the data. Figures show mean standard error of the mean (S.E.M.). NS,  $p > 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## 3. Results

### 3.1. VE compromises life span and locomotion of flies

We first examined the effect of VE on the survival of fruit flies. We found that the life span of females exposed to VE was significantly shortened compared with the control group (Figure 1A). Similarly, the median survival time of VE-treated males was dramatically shorter than that of control group (Figure 1B). These results indicate that both female and male flies are susceptible to VE. Of note, female flies were more resistant to VE than males; this may be partially explained by the fact that body sizes of females are bigger than that of males. We mainly used male flies in our experiments, because they are easier to manipulate. To further determine the effects of VE, we varied the time length of VE exposure. The longer flies were exposed to VE daily, the higher the mortality of *Drosophila* was with a peak at 4 h and 5 h exposure (Figure 1D). We determined the lifespan of flies after exposure to VE that was produced by different cars, Volkswagen and Buick. Buick Regal and Volkswagen Santana are the two models commonly used by people, and the exhaust produced by them is representative. Analogously, reduced life span was observed following VE exposure from different cars (Figure 1C). Taken together, these findings demonstrated that VE causes a reduction in lifespan of flies.

For any organism, coordinated locomotion plays an important role in fundamental activities of life. We thus examined the locomotion ability of flies challenged with VE. As expected, the running speed of *Drosophila* was significantly impaired after exposure to VE (Figure 1E), suggesting that VE has adverse effects on *Drosophila* locomotion. VE also decreased the climbing speed of flies treated with VE (Figure 1F). Collectively, our results demonstrated that VE is toxic to flies, and reduces the fitness of *Drosophila*.

### 3.2. VE changed the number of intestinal colonies and decreased the behavioral ability of drosophila

Given that the level of animal health are tightly associated with microbial communities, we asked whether VE altered the composition of intestinal microbiota. We thus generated germ-free (GF) flies as in previous studies (W. Liu et al., 2017). We assessed live bacterial loads of gut of CR exposed VE by plating fly homogenates on nutrition agar plates. Our data showed that VE-treated flies exhibited an increase in the total bacterial load compared with controls (Figure 2A), indicating that VE can cause intestinal dysbiosis. We then measured the number of intestinal symbiotic bacteria in the flies after VE exposure. Our results showed that there was no statistical difference in the number of intestinal CFUs between *L. plantarum* and *E. coli* in the control group. Following VE exposure, CFUs of *E. coli* in the intestinal tract of *Drosophila* were significantly increased compared with that of *L. plantarum* (Figure 2B). The survival rate of *Drosophila* challenged with VE was significantly reduced

compared with conventionally reared (CR) flies (Figure 2C), while GF flies exhibited comparable survival as the CR control in normal conditions. These results indicate that the microbiota aggravates the pathogenesis of VE-treated flies. We further examined whether microbial depletion could ameliorate the locomotion of flies following VE exposure. Indeed, we found that there was no difference in climbing and running abilities between the CR and GF groups before VE intervention, as shown in Figures 2D and 2E. Following VE exposure, running and climbing speeds of the CR group were significantly decreased compared with the GF group; this may be related to the decrease in motility of *Drosophila* after VE exposure.

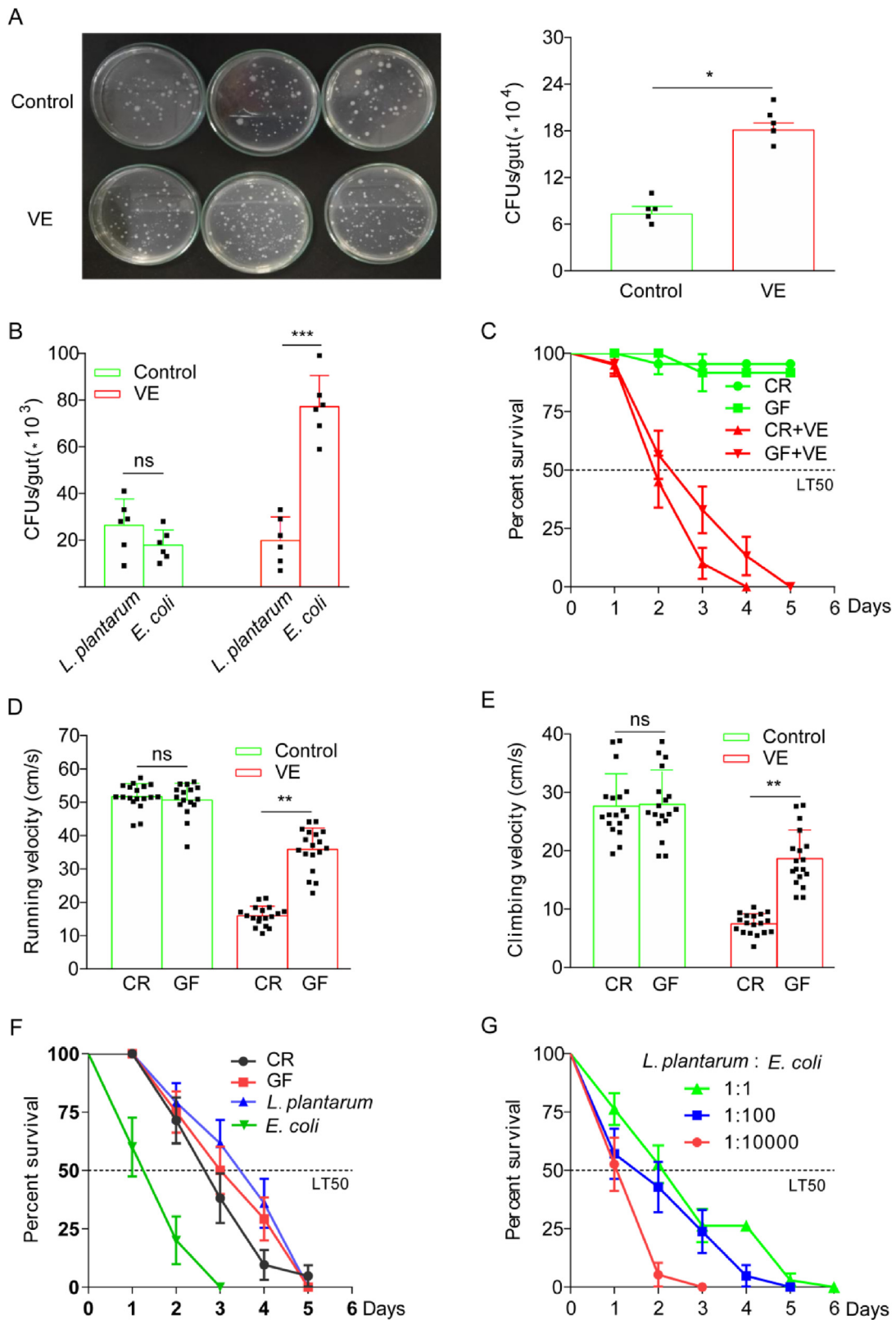
To further explore the role of bacterial species in the response to VE, we generated mono-associated flies with *L. plantarum* and *E. coli*. We found that *L. plantarum*-associated flies exhibited a similar life span compared to GF flies, while *E. coli*-associated flies showed a significantly shorter life span (Figure 2F). These results suggested that potential pathogens, rather than commensal bacteria, exacerbate the pathogenesis of *Drosophila* exposed to VE. To verify this, we simultaneously incubated *L. plantarum* and *E. coli* in a dose-dependent manner. We found that the higher the rate of *E. coli*, the shorter the life span of treated flies (Figure 2G). Given that the microbiota triggers stress-related issues in the body, we evaluated the possible role of VE-treated flies in mortality. Our results showed that the intestinal flora of *Drosophila* treated with VE was dysregulated, resulting in a significant increase in the number of harmful bacteria such as *E. coli*.

### 3.3. VE causes intestinal dysplasia

Intestinal dysbiosis has a propensity to disrupt epithelial lining and integrity of guts, in which bacteria escape from the intestine to promote systemic inflammation in the body (S. H. Kim and W. J. Lee 2014). We further examined whether VE could induce intestinal dysplasia. Intestinal permeability was assessed using Smurf assay as previously described (K. Chen et al., 2019). Our data showed that the smurf rates of the CR and GF flies were low in the absence of VE (Figure 3A). However, the proportion of smurf in CR flies following VE treatment was increased, indicating VE can cause intestinal dysplasia. More importantly, the proportion of smurf in GF flies treated with VE was significantly lower than in CR flies, suggesting that the microbiota mediates the disruption of intestinal integrity of flies. We then used a non-invasive method to assess excretory physiology through analysis of graphical features of fecal output in dye-fed flies as previously described (R. I. Clark et al., 2015). Representative images of the fecal output from untreated, VE-treated CR, and VE-treated GF flies are shown in Figure 3B. VE-treated CR flies showed a significant increase in the number of fecal deposits, significant changes in the size and shape of deposits, and significantly increased lightness (a measure of water content, Figure 3B) compared to untreated flies. Moreover, microbial depletion attenuated the morbidities of feces, including the number of fecal deposits, the size and shape of deposits, and lightness and hue. Under normal conditions, aged and/or damaged intestinal epithelium is rapidly replaced or repaired through the proliferation of intestinal stem cells, making it a marker of intestinal dysregulation. Mitotic cells can be labelled using phospho-histone 3 (PH3) antibody. An increased number of mitotic cells were observed in VE-treated intestines, suggesting that VE gives rise to intestinal damage and cellular proliferation (Figure 3C).

### 3.4. Microbial depletion improves the impaired locomotion and mortality

We set out to determine whether microbiota depletion attenuates the morbidity and mortality of flies with VE exposure. Flies were fed with a diet containing mixed antibiotics from day 10 of adulthood to remove intestinal bacteria. Indeed, flies fed with antibiotics showed an increase in survival compared to control flies exposed to VE (Figure 4A), indicating that the microbiota can affect mortality in *Drosophila*. Moreover, microbiota depletion attenuated the impairment of locomotion flies



**Figure 2.** VE increase the bacterial load in *Drosophila*, and bacterial species differentially affect mortality of VE-treated flies. Measurement of CFUs in the gut of *Drosophila* after VE exposure . (B) VE induced changes of intestinal microbes in *Drosophila*. (C) VE treated the survival rate of CR and GF groups. (CR+VE vs GF+VE,  $P < 0.05$ ). (D-E) VE-induced the running and climbing velocity of *Drosophila* (means  $\pm$  SEM,  $n = 10$  in each group, respectively). (F) Survival rate of *Drosophila* treated with VE in CR, GF, *L. plantarum* and *E. coli* groups. (CR+VE vs *E. coli*+VE,  $P < 0.05$ , GF+VE vs *E. coli*+VE,  $P < 0.05$ , *L. plantarum*+VE vs *E. coli*+VE,  $P < 0.05$ ). (G) The different mortality of *Drosophila* with three kinds of proportion microbial (1:1 VS 1:100,  $P > 0.05$ ; 1:1 VS 1:10000,  $P < 0.01$ ) (means  $\pm$  SEM,  $n = 10$  in each groups). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

challenged with VE (Figures 4B, 4C). Taken together, these results suggest that microbial depletion ameliorates the compromised locomotion and mortality of flies exposed to VE.

### 3.5. VE-induced flies exhibited higher expression of genes involved in innate immunity, and DUOX down regulation increased mortality in *Drosophila*

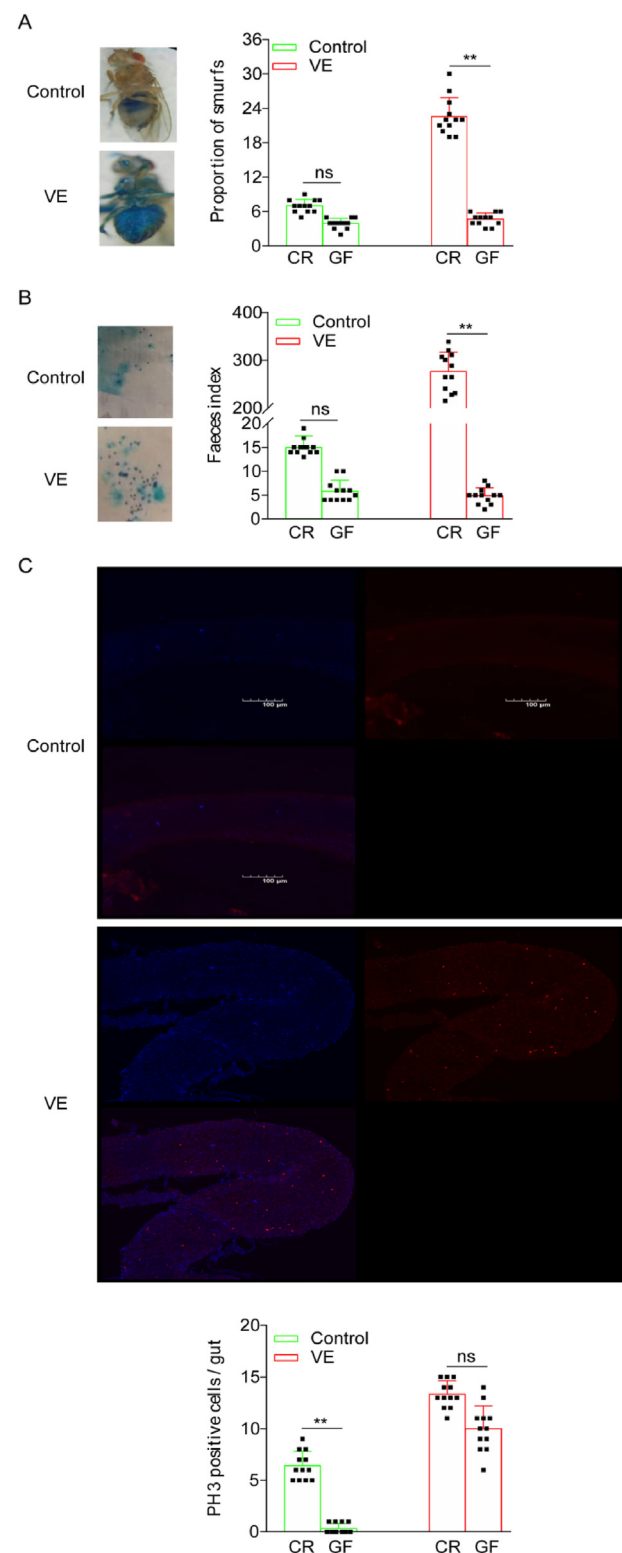
We investigated the immune mechanisms involved in the VE intervention triggering or inducing these results. Expression of the AMPs *Attacin* and *Diptericin* was elevated in CR and GF flies after VE treatment for 5 days (Figures 5A, 5B). It is noteworthy that the gene expression level in the GF group was higher than that in the CR group. These results suggest that VE exposure activates the Imd pathway in the CR and GF groups and promotes AMP gene expression. The intestinal homeostasis of *Drosophila* was disturbed, and expression of *Attacin C* and *Diptericin B* encoding Imd was elevated, especially in the GF group. Thus, in *Drosophila* exposed to VE, the immune deficiency pathway was activated. Germ-free flies exposed to VE were more likely to activate the immune deficiency pathway. Dual oxidase (DUOX) is a ROS-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; it is important for maintaining the ROS pathways and intestinal homeostasis in *Drosophila* (Z. Yao et al., 2016). As expected, the expression of DUOX was significantly lower in the CR VE-treated flies for 5 days than control flies (Figure 5C). Moreover, ROS levels were no different in VE-treated GF flies than control GF flies. To validate the role of ROS in mediating the response to VE for 5 days, we knocked down DUOX in CR with the GAL4 UAS system. DUOX silencing markedly increased VE toxicity to flies (Figure 5D). Taken together, these results suggest that VE triggers oxidative innate immunity in *Drosophila*.

## 4. Discussion

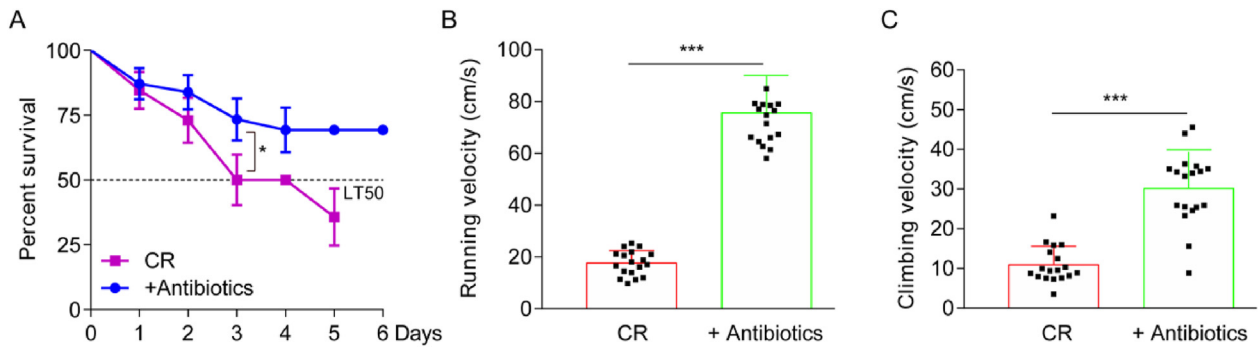
In general, VE enters the body through the respiratory system into the lungs, and causes a series of pathophysiological changes in the respiratory system. However, it can also enter the body by different routes. In addition to being absorbed through the skin, there are many ways for exhaust particulate matter to enter the gut. It can enter the intestine through intake of particulate matter via contaminated water or food (K. De Brouwere et al., 2012). Exhaust particulate can also stay in the oropharynx and enter the intestine by deglutition saliva and mucus sedimentation in the throat.

Particulate matter remains in the nasopharynx and oropharynx, while particulate matter deposited indirectly by nasal cilia can be swallowed, allowing particulate matter to enter the stomach and the rest of the intestine (L. A. Beamish et al., 2011). Gut microbes have a range of functions, and the flora is dynamic and impressionable to the host and biotic environment. Intestinal epithelial cells are required to activate the immune system to generate an effective immune response in response to flora alterations in a seasonable and appropriate manner (X. Xiao et al., 2017).

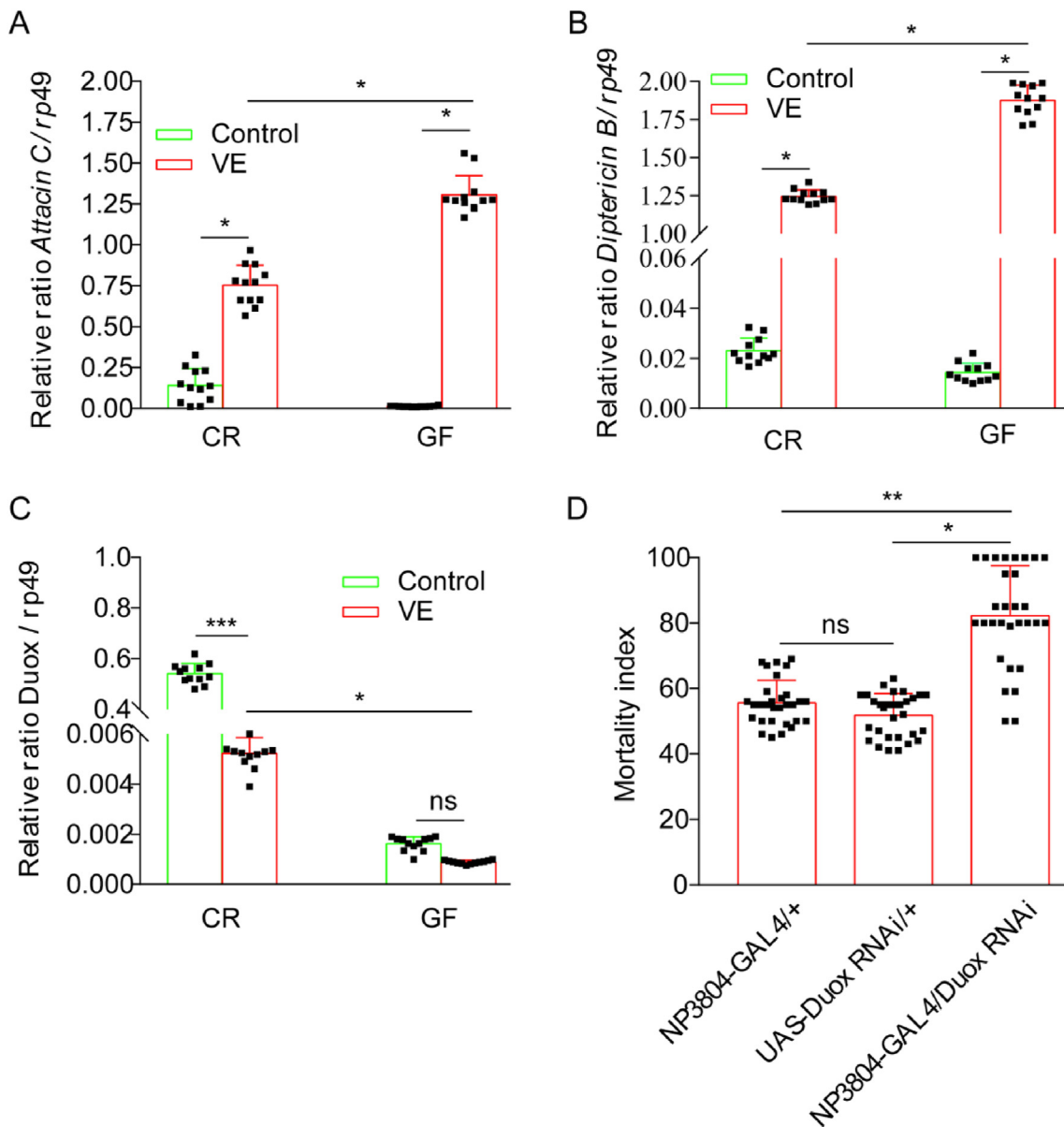
An intact animal intestinal epithelium is key to defense against pathogenic microbes (F. Bonnay et al., 2013). Exposure to pathogenic microbes causes aggravated intestinal permeability and disruption interrelated with decreased tight junction protein expression in the intestinal epithelium (E. A. Mutlu et al., 2011). Studies have shown that ingestion of pathogenic bacteria can damage epithelial cells and lead to death of host intestinal cells (N. Buchon et al., 2013). If the infection is catastrophic, intestinal epithelial damage is irreversible and cannot be renewed or repaired, resulting in host death (S. Y. Salim et al., 2014). In this case, the host must trigger an immune defense to defend against the pathogen, as well as launch mechanisms to maintain tissue integrity. However, if these mechanisms are not functioning properly, intestinal



**Figure 3.** VE disrupts the integrity of intestines. (A) The representative images of smurf flies and the percentage of smurf in flies. Smurf flies displayed intestinal barrier dysfunction by blue dye permeation throughout the body. (n = 15). (B) Shown are intestinal contents in Blue dye-fed intact flies. Blue dye-labeled deposits in their color and concentration in flies. (n = 15). (C) VE stimulated the proliferation of intestinal stem cells in the gut of flies. Representative images in which mitosis is indicated by red staining with PH3, and nuclei are stained blue with DAPI. (n = 15). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 4.** Microbiota removal improved the impaired locomotion and mortality. (A) Antibiotic impaired the survival of flies. (n = 10). (B-C) Antibiotic improved the locomotion of flies. Flies were treated with antibiotic, and the velocity of climbing and running was assayed. (n = 15) \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.



**Figure 5.** VE triggers oxidative innate immunity. (A-C) VE activated the expression of target genes associated with oxidative innate immunity. *DUOX*, *Attacin*, and *Diptericin* mRNA levels for VE associated wild-type flies (n = 15). (D) Mortality index of *DUOX*-silenced adult flies challenged with VE (n = 15). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

homeostasis is disrupted and the host ultimately dies. Therefore, determining the mechanism of intestinal epithelial injury caused by exhaust media and effective intervention is an important issue.

Flies in nature live about 60–100 days. Fruit flies exposed to VE had a significantly shorter life span (6 days), especially for males (Figure 1B). Under normal conditions, the life span of CR flies is longer than GF flies, but that of CR flies VE was shorter than GF flies exposed to VE (Figure 2C). Bacteria and other microbes usually enter a fly's body through exposed wounds in the cuticular layer or via the barrier epithelium of the respiratory and reproductive pattern in the digestive tract (A. Kleino and N. Silverman 2014). This may be related to the destruction of intestinal microbial homeostasis by VE. Furthermore, VE increased the number of harmful bacteria in the intestinal tract of *Drosophila*, and the intestinal barrier was destroyed, increasing intestinal permeability and resulting in intestinal barrier dysfunction. The higher the proportion of harmful bacteria, the greater the negative impact on the life span of *Drosophila*. Interestingly, with *L. plantarum* and *E. coli*, as the concentration of *E. coli* increased, the life span of *Drosophila* was significantly shortened, confirming that harmful bacteria adversely affect the life span of flies (Figure 2G).

A study of infection showed that beyond influencing the immune system, bacteria have other impacts such as changing host behavior and food intake (C. E. Schretter 2020). These results are consistent with the literature, which reports that gut homeostasis disharmony in *Drosophila* resulted in decreased social behavior (K. Chen et al., 2019). VE significantly decreased running and climbing abilities of *Drosophila*. Antibiotics can inhibit the growth of pathogenic bacteria, and improve the running and climbing, as well as prolong the life span of *Drosophila* exposed to VE. The GF group, however, were unaffected and were connected to a sterile intestinal environment (Figure 4).

The innate immune system plays a crucial role in host defence against foreign pathogenic microbes (Romo M. Riera et al., 2016). Immune homeostasis is a precondition for protecting the immune system defense gastrointestinal infection. In *Drosophila*, the immune system primary uses the Imd signaling pathway to defend against Gram-negative bacteria infection (H. Myllymaki et al., 2014), and the Imd pathway is activated by DAP-type peptidoglycan (DAP-PGN), which is universally found in Gram-negative bacteria, but has also been found in some Gram-positive species, such as *Bacillus* spp. and *Listeria* (B. Lemaitre and J. Hoffmann 2007; C. R. Stenbak et al., 2004). Triggering the receptors launches a signaling cascade that induces the production of AMPs (A. Takehana et al., 2002; F. Leulier et al., 2003; T. Kaneko et al., 2004). *Drosophila* generates AMPs via cellular and humoral mechanisms to defend against the invasion of outside pathogens via innate immune responses (D. Hultmark 1993b; J. A. Hoffmann 1995b). Epithelial cells in these tissues generate AMPs and their immune response relies on the Imd pathway (D. Ferrandon et al., 1998; P. Tzou et al., 2000). We selected *Attacin-C* and *Diptericin B* from the AMP gene that mainly showed antibacterial activity to design in this experiment (R. I. Clark et al., 2015), and found that in flies exposed to VE, intestinal homeostasis was disturbed, and expression levels of AMP-encoding genes such as *Attacin-C* and *Diptericin B* were increased, and the immune deficiency (Imd) pathway was activated (Figures 5A, 5B).

Studies in *Drosophila* and other insects have shown that gut epithelia relies on the basal production of DUOX-dependent ROS to maintain gut microbe homeostasis (H. Diaz-Albiter et al., 2012; K. A. Lee et al., 2013). DUOX is a ROS-producing NADPH oxidase. Several lines of evidence have shown that the bacterial-modulated DUOX system is important for microbial clearance, gut epithelial cell renewal (ECR), redox-dependent adjustment of signaling pathways, cross-linking of biomolecules, and distinguishing between symbiotic bacteria and pathogens (E. M. Ha et al., 2009). Appropriate ROS levels are reached by triggering basal DUOX expression by intracellular Ca<sup>2+</sup> mobilization (E. M. Ha et al., 2009; Y. S. Bae et al., 2010). Beside its antimicrobial response, it is clear that DUOX plays a key role in gut permeability and

adjustment of signal transduction refer to immune gene expression, vulnus healing, and stem cell adjustment (S. H. Kim and W. J. Lee 2014). DUOX silencing resulting in an increase in the mortality rate of flies (Figure 5D). However, the Imd pathway has other immune impacts in the gut such as adjusting enterocyte shedding (Z. Zhai et al., 2018), digestive enzymes (B. Erkosar et al., 2014), and DUOX-dependent and Nox-dependent generation of ROS (S. H. Kim and W. J. Lee 2014). The relationship between Imd and ROS-mediated dependence on DUOX was not shown in our data, but this could be an interesting new pathway to explore in the future.

## 5. Conclusion

We found that VE affects the composition of normal intestinal colony count in *Drosophila*, ultimately leading to a shortened lifespan, increased intestinal permeability, and progressive motor deficits. Silencing of the DUOX gene disrupted intestinal homeostasis, accelerated intestinal microbiota dysregulation, and shortened the life span of fruit flies. Our study provides new insight into the role of the microbiota in the pathogenesis of VE-induced intestinal injury in *Drosophila*, such as the Imd and ROS pathways, which could be used to guide treatment of intestinal system diseases.

## Declarations

### Author contribution statement

Yandong Nan and Faguang Jin: Conceived and designed the experiments; Wrote the paper.

Yujuan Li and Lei Pan: Performed the experiments; Wrote the paper. Shaokang Dang and Zhichao Li: Analyzed and interpreted the data; Wrote the paper.

Pengcheng Li and Gaole Yu: Contributed reagents, materials, analysis tools or data; Wrote the paper.

### Funding statement

Dr Yandong Nan was supported by Key R & D Program of Shaanxi Province (No. 2019SF-009).

### Data availability statement

Data will be made available on request.

### Declaration of interest's statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

## Acknowledgements

We would like to thank all members of Department of Respiratory and Critical Care Medicine of Tangdu Hospital of Air Force military Medical University lab for helpful discussions. We thank International Science Editing (<http://www.internationalscienceediting.com>) for editing this manuscript.

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