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The protective action of a novel Dinb protease against diarrhea infection in *Drosophila Melanogaster*

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ABSTRACT

The compounds of natural origin prevail to be of great importance in the identification of novel bioactive molecules and are less pernicious towards the ecosystem. Dinb family proteases of *Bacillus* species possess antimicrobial properties and are reported to inhibit the growth of many pathogenic bacteria, our study also focused on corroborating these effects. Five-day-old *Drosophila melanogaster* was exposed to *Salmonella enterica* for up to 96 hours. Once the disease was induced in flies they were shifted to the treatment vials containing 2 mg–10 mg of purified protease. The application of a 23.4 kDa purified protease improved the health of *D. melanogaster* when compared to the positive control. The protease treatment on the infected flies showed significant stability in cell survival (69%) as well as restoring normal cell functioning. The results indicated that purified protease extended fly life span, restored their locomotive (96.6%), and reproductive ability (38%–67.5%), and reduced oxidative stress in *D. melanogaster*. The study verified, the protease to be bactericidal against diarrhea pathogens and the significant recovery of the host immune system. Furthermore, the protein can be used to study host-pathogen immune interactions at the cellular level, followed by its testing against a variety of pathogens to explore its broad-spectrum application.

1. INTRODUCTION

Bacteria of the *Bacillus* genus have been explored for their use in medicine, healthcare, food, and the agriculture industry for decades [1]. One such probiotic bacteria is *Bacillus clausii,* which is used as a medicine to treat diarrhea. Spores of *B. clausii* are reported to improve gut health, restore healthy gut microbiota, and inhibit diarrhea pathogen and their toxins [2]. The ability of *B. clausii* to survive under harsh gut conditions comes with their ability to sporulate. Also, under physicochemical stress, *B. clausii* does not just sporulate but also releases a plethora of extracellular compounds, which contribute to the immunomodulatory properties of bacteria. The extracellular compounds include a vast range of proteins, peptides, and hydrolytic proteases with protective action [3]. Researchers have reported an increased release of proteases by normal gut microbiota, during diarrhea infection [4]. These proteases apprehend the pathogenic toxins and expel them out of the gut system [5,6]. Researchers have reported the isolation of serine proteases from various *B. clausii* strains and their role in counteracting bacterial and viral diarrhea pathogens [7].

Enteric infections have a serious health impact all around the globe, specifically in developing nations. Diarrhea or gastroenteritis, is a serious public health problem and is among the top four diseases causing maximum fatalities around the globe [8]. A broad range of bacteria and viruses have been reported as the causative agents of diarrhea this includes, *Giardia, Enterococcus, Staphylococcus, Clostridium difficile,* norovirus, astrovirus, and rotavirus [9,10]. The mechanism of infection varies according to the causal organism. It can be classified as acute, chronic, inflammatory, and noninflammatory diarrhea. The infection is mostly associated with poor hygiene and sanitary conditions. After entering through the oral route these pathogens attach themselves to the epithelial lining of the intestine, releasing toxins and compromising the host immune system. Gram-negative *Salmonella enterica,* a causative agent of typhoid is one such diarrhea pathogen [11]. *Salmonella enterica* infection or salmonellosis is the onset of high fever followed by gastric dysbiosis which infects humans and animals. The onset of gut disorders is associated with the release of reactive oxygen species (ROS) in the body, followed by oxidative stress [12,13]. Hence, finding a cure that could restore an organism's metabolic pathways and enzyme activities can significantly contribute to the health and well-being of diarrhea patients.

The model organism used in this study was *Drosophila melanogaster. Drosophila* has a very simple gut system, yet strikingly similar to the human gut in its anatomy and metabolism [14]. *Drosophila* shares 75% of its genes with humans making it an economic model to study

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various metabolic diseases [15]. With this in consideration, our present study focused on studying the curative potential of purified protease against *S. enterica* infection (diarrhea pathogen) in *D. melanogaster*. The protease was isolated from the *B. clausii* UBBC07 strain, and the curative action of protease was determined based on reduced bacterial load, increased survivability, and reduced oxidative stress. Also, this study is a follow-up study concerning our previous study where the complete characterization of purified protease and its antimicrobial potential was successfully studied using *in vitro* assays against *Bacillus cereus* and *S. enterica*.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

All the reagents and chemicals used were of analytical grade. Phosphate buffer saline (PBS), sucrose solution, potassium phosphate buffer, reduced glutathione (GSH), 5,5′-dithiobis (2-nitro-benzoic acid) (DTNB), 1-chloro-2, 4- dinitrobenzene (CDNB), hydrogen peroxide (H_2O_2), catalase enzyme, Loperamide hydrochloride tablets I.P. (2 mg), pestle and mortar, Muller Hinton broth, Luria Bertani media.

Fly food composition included maize (35 g), sugar (30 g), yeast (12 g), agar (4 g), sodium benzoate (2 g), propionic acid (1 ml), 80% ethanol (8 ml), and distilled water (1 l).

2.2. Protease Isolation and Purification

The *B. clausii* strain UBBC07 an over-the-counter medicine under the name Bactolac was procured from a pharmacy in Jalandhar, India. A pure culture of *B. clausii* UBBC07 strain was grown in two-liter Muller Hinton broth for four days at 30ºC. Protein purification was performed as previously described by Liu *et al.* [16,17]. The bacterial culture was centrifuged and the supernatant was acid-precipitated with 1N hydrochloric acid. Partially purified protein was run on 15% Tricine SDS PAGE gel and the target protein band of 23 kDa was excised. Protein was identified using MALDI-TOFF mass spectrometry. Furthermore, the gel excised protein was manually purified using acetonitrile: ammonium bicarbonate (1:1) treatment. The concentration of purified protein was estimated using the Folin–Lowry assay. The protein was stored at 4ºC till further use.

2.3. *Drosophila melanogaster* Stock and Culture

The flies were cultured and maintained under standard environmental conditions, as described by Iorjiim *et al.* [18]. *Drosophila melanogaster* wild-type strain Oregon^R was received from Stock Centre, Animal Tissue Culture laboratory, Lovely Professional University, India. Flies were maintained on standard fly food unless specified for treatments, at 25ºC, with 60% humidity, 12:12 hour day: light cycle, throughout the experiment.

2.4. Bacterial Infection Dosage Size

Infection dosages were prepared as described previously by Harnish *et al.* [19]. The bacterial strain *S. enterica* (MTCC 1164) was obtained from the CSIR-Institute of Microbial Technology. Flies were fed the infection bacteria orally. Flies were first exposed to *S. enterica* infection using $O.D._{600\ nm} = 100$. The bacterial pellet was dissolved in a 5% sucrose solution. A sterile cotton was treated with the bacterial cell pellet and placed inside the test vials containing *Drosophila* food. Once the cotton had dried up, young flies were put inside the test vials. Control flies were given the cotton soaked in 5% sucrose only. The flies were observed for infection every 24-hour interval.

2.5. Experiment Design

2.5.1. Preliminary survival assay

To conclude the appropriate protease dosage for *Drosophila* treatment a preliminary study was performed [18], where bacterial load, bacterial shedding, and surviving flies were regularly recorded for 15 days during the treatment. For this, after infecting the flies with *S. enterica*, they were shifted to new vials (height 7 cm, diameter 2.5 cm) having fly food mixed with protease at variable concentrations, i.e., 2 mg/ 40 ml food, 4 mg/ 40 ml food, and 10 mg/ 40 ml food. Loperamide drug (4 mg/ 40 ml food) was used as a positive control, and healthy flies were used as a control. Positive control was used to confirm the oral drug consumption by flies. After determining the appropriate Dinb protease concentration for the effective treatment final assay was performed with a single protease dosage.

2.5.2 Final survival assay

After determining the appropriate protease dosage, a final assay was performed in two independent experiments followed by triplicates for all assays. The experiment set included the following groups:

Group I: Control flies with no infection or drug dosage.

Group II: Treatment flies with protease concentration of 10 mg/ 40 ml food after the infection.

2.6. Survival Assays

The five assays included bacterial load determination, bacterial shedding determination, survivorship curve, negative geotaxis, and reproduction [20]. 15 flies were distributed in each vial, flies were shifted to new respective food vials every 5-day interval.

2.6.1 Bacterial load determination

Bacterial load calculation was performed as described previously by Siva-Jothy *et al.* [21]. To confirm the oral infection, a live fly from the test vials (group of three for each control and infection) was removed and shifted to a 1.5 ml microcentrifuge tube. The fly was then surface sterilized by placing them in 70% ethanol solution for 30 seconds. To the microcentrifuge tube, 100 µl of 1X PBS and fly was homogenized. The homogenate was transferred to the top well of 96 well microtitre plate and dilutions up to 10^{-5} were made. 10 µl of each dilution was spread on Luria Bertani plates and colony-forming units were calculated using the Quebec colony counter (Thermo Scientific). The random bacterial colonies from the control plate were always subtracted from treatment plates and the experiment was performed with two independent experiments.

2.6.2. Bacterial shedding

Bacterial shedding was calculated as described by Siva Jothy *et al*. [21]. Flies from all test vials were transferred to a 1.5 ml microcentrifuge tube containing100 µl of 1X PBS, for 16 hours at 4ºC. The fly was removed and the tube was heavily vortexed. The solution of the tube was plated on an LB agar plate and colony-forming units were calculated.

2.6.3. Lifespan assay

The survivorship curve was plotted as previously described by Lopez *et al*. [22]. The dead flies from treatment and control vials were counted every day till day 30 of the treatment, and the mean survival rate was calculated.

2.6.4. Negative geotaxis

Negative geotaxis was performed in the same manner as described previously by Lopez *et al*. [22]. 15 treatment and 15 control flies were transferred without anesthesia to a clean, 15 cm tall glass vial, once every afternoon in light at room temperature. After 10 minutes of adaptation of flies, the glass vials were gently tapped three times, and fly climbing was observed for 30 seconds. The number of flies crossing a 10 cm mark was recorded until the movement of control and treatment flies became similar.

2.6.5. Fecundity rate

The reproductive abilities of the treatment flies were examined based on the protocol described by Iorjiim *et al*. [18]. A total number of 15 flies were placed in treatment and control vials in triplicates, where they laid eggs. The flies were shifted to a new vial every 48-hour interval and previous containers were monitored for 14 days for the appearance of new flies. The cumulative number was recorded and survivorship as an average mean was determined.

2.7. Biochemical Assays

2.7.1. Enzymatic assays

Enzymatic assays were performed as described by Iorjiim *et al*. [18] in triplicates. Sixty flies from Group I, and Group II were collected every 5 days, anesthetized using deep freeze treatment, and homogenized on ice in 1X PBS (0.1 M). The homogenate was tested for protein estimation using the Folin–Lowry method. The assays were performed for 25 days until all the treatment flies showed restored enzymatic activity similar to the control flies. Flies were shifted to fresh food vials every 5 days.

2.7.2. Determination of total thiol content

The total thiol content estimation was done using the Ellman method [18]. The reaction mixture consisted of 25 µl of homogenized protein sample, 30 µl of 5,5′-dithiobis DTNB (Ellman reagent) (10 mM), and 510 µl of 0.1M potassium phosphate buffer. The reaction mix was incubated for 30 minutes at room temperature and absorbance was calculated using a spectrophotometer at 412 nm. The standard curve of reduced GSH (100 mM GSH) was used to calculate the thiol content and expressed as µmol/mg of protein.

2.7.3. Determination of glutathione- S-transferase activity

Glutathione- S-transferase (EC 2.5.1.18) activity was determined by the method described by Adedara *et al*. [23]. Solution A was prepared by adding 20 ml potassium phosphate buffer, 10.5 ml of distilled water, 500 µl GSH 500 ml. The enzyme cocktail consisted of 270 µl of solution A, and 10 µl CDNB. To this mixture, a 20 µl test sample (1:5 dilution) was added. The increased absorbance was observed immediately at 340 nm for 2 minutes at 10-second intervals. The data were expressed as µmol/ml/minute of protein utilized in making conjugates. The molar extinction coefficient for CDNB conjugate was 9.6 $mM^{-1}cm^{-1}$.

2.7.4. Determination of catalase activity

Catalase (EC 1.11.1.6) activity was evaluated as described by Adedara *et al*. [23], with some changes in the protocol. Solution A was prepared by adding 1,800 µl of potassium phosphate buffer, pH 7.0 to 180 µl of 300 mM H_2O_2, 10 µl of the test sample and 990 µl of solution A were mixed well and absorbance was visualized at 240 nm for 2 minutes with a 10-second interval. Catalase enzyme was used as a standard. The results were expressed as µmol of H_2O_2 consumed/min/mg protein.

2.8. Statistical Analysis

Two independent experiments with triplicate for each assay were used. The statistical analysis of data was done by using Sigma plot 11.0 and the results were expressed as mean ± SE (standard error). One-way analysis of variance was done and all data with $p < 0.05$ was considered significant.

3. RESULTS

3.1. Preliminary Survival Assays

Flies fed on the protease concentration of 10 mg/40 meal, showed significant recovery, in the form of reduced internal bacterial load, reduced bacterial shedding, and increased survivability (Figs. 1–3). Flies fed on Loperamide tablets (4 mg/ 40 ml) also showed recovery, confirming the consumption of the drug from standard fly food. Data were collected by performing two independent sets of experiments for all biological samples, i.e., control, positive control, and treatment. Data were analyzed as Mean ± SEM (mean standard error) for all observations, the data were found to be highly significant for all three assays. For final assays, a protease concentration of 10 mg/ 40 ml meal was used to treat the infected flies.

3.2. Final Survival Assays

3.2.1. Bacterial load and bacterial shedding estimation

Flies were left to consume the bacterial infection until the colony-forming units reached approx. 1×10^9 CFU/ml. Day zero was taken as the day when the infection had reached the required amount and flies were shifted to the treatment vial (supplemented with protease).

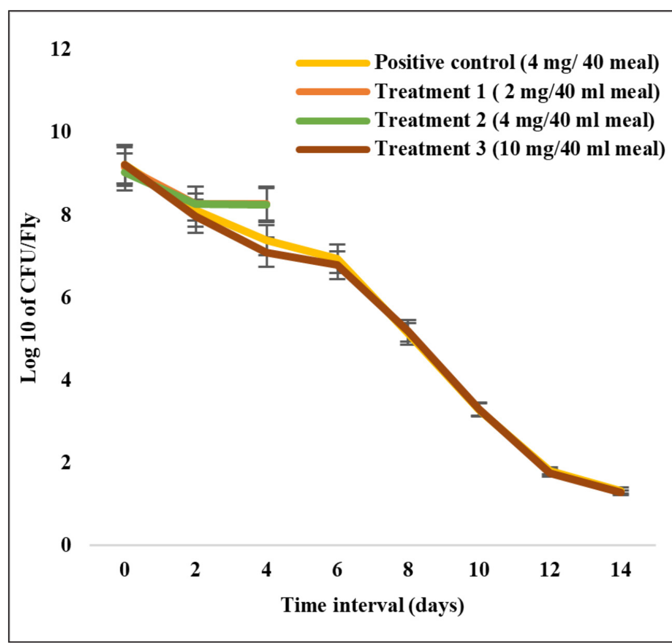

Figure 1. Preliminary analysis of protease effectiveness showing reduced bacterial shedding in treatment flies in a dose-dependent manner with the protease concentration of 10 mg/ 40 ml food. Data presented as Mean ±SEM of two independent experiments with duplicates, $p < 0.00$ versus control.

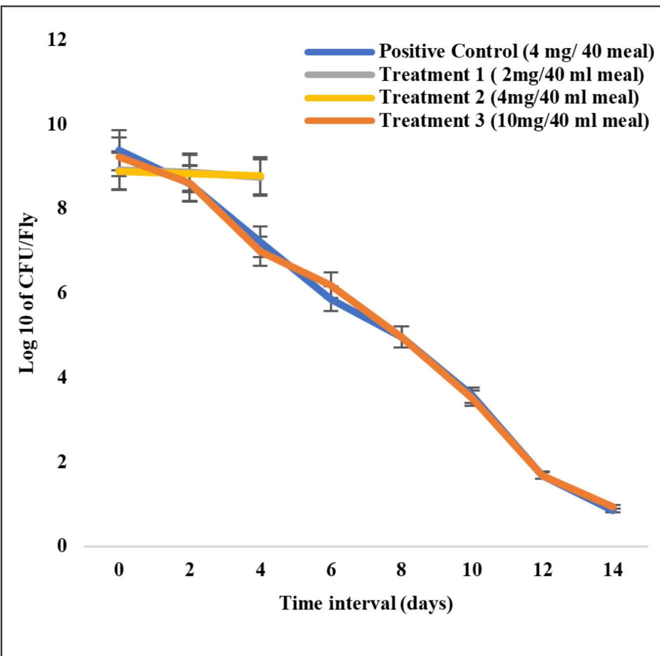

Figure 2. Preliminary analysis of protease effectiveness showing, reduced internal bacterial load in a dose-dependent manner with the protease concentration of 10 mg. Data presented as Mean ±SEM of two independent experiments with duplicates, $p < 0.00$ versus control.

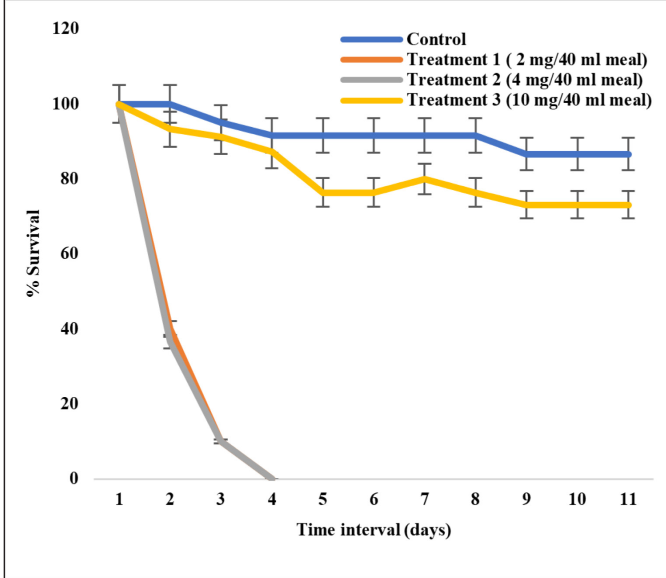

Figure 3. Improved survival rate in flies treated with 10 mg protease dosage. Data presented as Mean ±SEM of two independent experiments with duplicates, $p < 0.00$ versus control.

Flies were regularly analyzed for internal bacterial load with 48-hour intervals until no colony started to appear on LB media plates (Fig. 4).

3.1.2. Survivorship curve

The dead flies from control and treatment vials were counted every 48-hour interval for 30 days. The fly count initially decreased from 100% to 71% in treatment vials within 10 days during the treatment while the survival rate in control flies decreased to 93% (Fig. 5). After

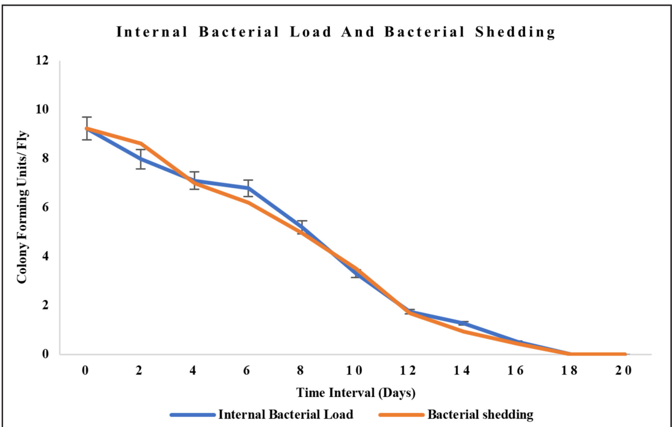

Figure 4. Bacterial load and bacterial shedding reduction in treatment fly were done in a dosage-dependent manner, using a single final Dinb protease concentration for 20 days. Random bacterial colony appearance in control samples was subtracted from treatment, and data from treatment flies was presented. Data were presented as Mean ± SEM of two independent experiments with duplicates, with $p < 0.0001$ for treatment and control.

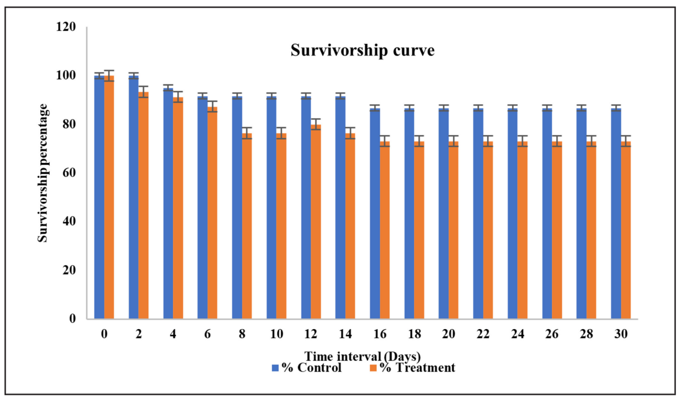

Figure 5. Dinb protease supplementation (10 mg/ 40 ml meal) rescued the flies from the *Salmonella enterica* induced bacterial infection in a dose-dependent manner, stabilizing the fly number between 15 and 30 days of treatment. Fly survival was calculated from day 1 of the treatment. Data were presented as Mean ± SEM of two independent experiments with duplicates each time, with a significance level of 0 for control and treatment.

15 days of treatment, the flies retained 69% of survival when observed for 30 days. Control flies retained an 87% survival rate till 30 days of observation. Data were significant with $p < 0.01$ for treatment and control.

3.1.3. Climbing assay

The climbing ability of treatment flies showed 80% recovery after 10 days of treatment, and the climbing ability of up to 96.6% was restored after 20 days of treatment which was similar to control flies. The climbing abilities of flies were stabilized after 15 days during the treatment with a significance of 0 for treatment versus $p < 0.01$ for control samples (Fig. 6).

3.1.4. Fecundity rate

During the first 10 days of the treatment, the fecundity rate in treatment flies increased from 38% to 67.5%. It reached 98% by the 20th day of treatment (Fig. 7). The reproduction rate in control flies rose from 100% to 125% in 20 days of observation with $p < 0.00071$ for treatment and $p < 0.001$ for control.

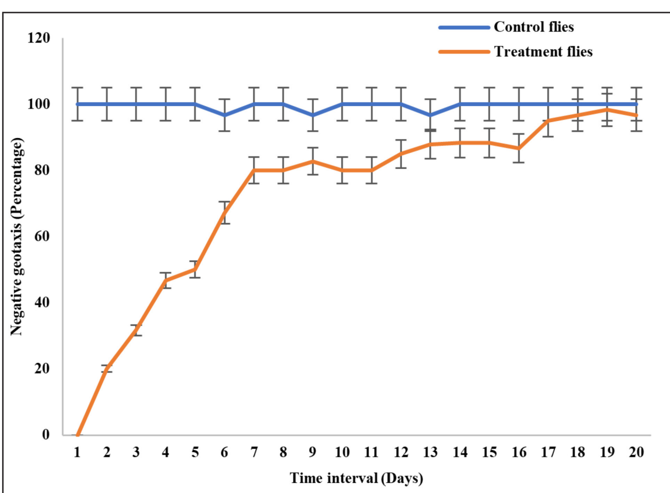

Figure 6. Dinb protease supplementation (10 mg/ 40 ml meal) significantly improved the climbing abilities in *S. enterica-infected* flies in a dose-dependent manner. In the 20-day treatment duration, the climbing ability of the treatment flies appeared similar to those of the control flies. Data were presented as Mean ± SEM of two independent experiments with duplicates each time, with significance 0 for treatment versus $p < 0.01$ for control samples.

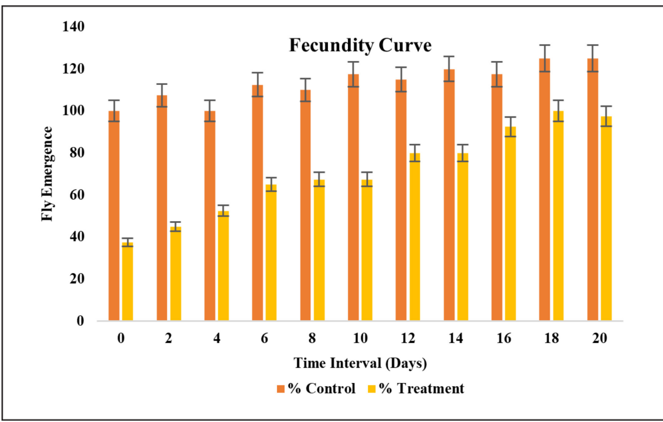

Figure 7. Dinb protease (10 mg/ 40 ml meal) treatment restored the reproduction abilities of infected flies, leading to an increased fly emergence during the 20-day duration of treatment. Data were presented as Mean ± SEM of two independent experiments with duplicates each time, $p < 0.00071$ for control and significance 0 for treatment were observed.

3.2. Biochemical Assays

3.2.1. Total thiol content

The protease supplementation (4 mg/ 40 ml food) significantly increased the thiol content from 9 µmol to 55 µmol when compared to control flies whose thiol content remained between 64 µmol to 62 µmol during 25 days of treatment (Fig. 8). Treatment data showed $p < 0.00002$ and control data showed $p < 0.1$.

3.2.2. Glutathione-S-transferase activity

Treatment groups that were exposed to protease (4 mg/ 40 ml) showed a significant increase in Glutathione-S-transferase activity from 0.74 U/ml to 3.67 U/ml within 25 days of the treatment, while the control flies showed the enzyme activity between 3.7 and 4.4 U/ml with $p < 0.02$ (Fig. 9).

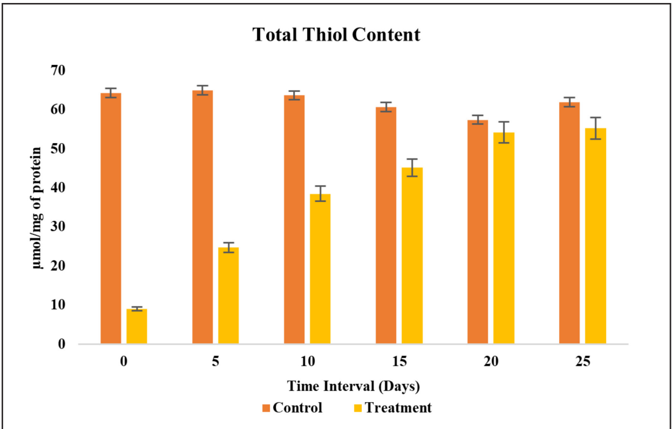

Figure 8. Total thiol content from Day 0 to Day 25 of the treatment duration. Thiol content increased in treatment samples in a dose (10 mg/ 40 ml meal) dependent manner from 14% to 86%, where for treatment $p < 0.00002$ obtained and for control data $p < 0.1$ obtained.

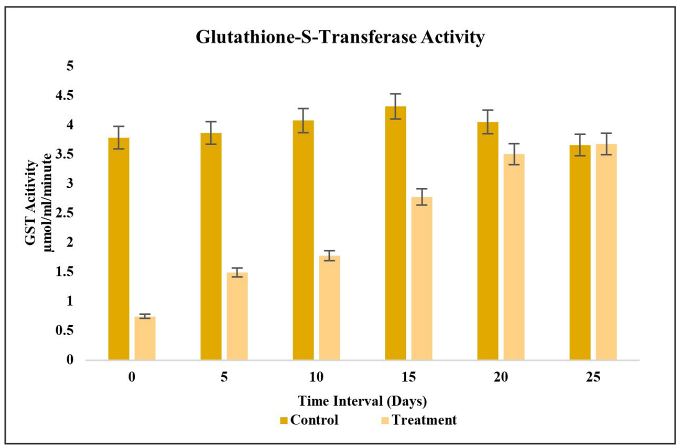

Figure 9. Glutathione-S-transferase levels were restored, with an increase from 19.7% to 97% when compared to the control flies. Data were presented as Mean ± SEM of two independent experiments with duplicates each time. The *p*-value for control was $p < 0.02$, and for treatment significance was 0.

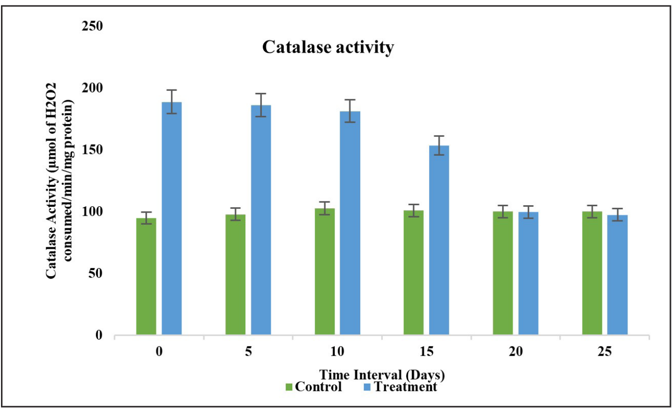

Figure 10. Dinb protease supplementation (10 mg/ 40 ml meal) restored the catalase activity to normal in infected flies, the catalase levels of fly homogenate from 20-day treatment showed a significant similarity to the control flies (healthy fly homogenate). Data were presented as Mean ± SEM of two independent experiments with duplicates each time, $p < 0.00001$ versus control $p < 0.001$.

3.2.3. Catalase activity

The enzyme activity significantly reduced ($p < 0.00001$) in the treatment flies from 188 U/ml to 97.5 U/ml, indicating the effect of protease supplementation in restoring normal cellular functions in flies. In control flies the catalase concentration remained majorly between 95 U/ml and 100 U/ml with $p < 0.001$, during the 25-days of treatment (Fig. 10).

4. DISCUSSION

Diarrhea is an enteric disease reported to cause the highest number of outbreaks in developing countries and is among the top four infections causing the maximum fatality worldwide [8,9]. Antibiotic-based treatment of diarrhea comes with problems of multidrug resistance, superbug formation, and other health issues. Researchers have reported the protective action of serine proteases isolated from *B. clausii* in Vero and Caco-2 cell lines against *B. cereus* and *Clostridium difficile* pathogens [7]. So far, no direct application of any protease in *Drosophila* concerning diarrhea treatment has been reported. However, the impact of many drugs and antibiotic administration on diarrhea infection in *Drosophila* has been reported [23,24]. Flies were infected with *S. enterica* and the infection reached 1×10^9 CFU/ml after 96 hours of incubation [8,25]. Infected flies showed increased fly death after 1×10^9 CFU/ml of bacterial load was attained. Harnish *et al.* [19] have reported fly death from *S. enterica* serovar *typhimurium (S. typhimurium)* after they had a bacterial load of 1×10^5 CFU/ml [10,19]. The purified protease concentration for the treatment was initially optimized under preliminary studies where protease was in 2 mg/40 ml fly food, 4 mg/40 ml fly food, and 10 mg/40 ml fly food. The initial treatments were exclusively observed for the reduced bacterial load and an increase in surviving flies. From the preliminary assays performed for 15 days, a final protease concentration of 10 mg was chosen for the final experiments.

From day 0 to the whole duration of treatment (30 days), protease treatment with 10 mg/ 40 ml standard fly food, in *Salmonella*-infected flies showed reduced bacterial load and bacterial shedding. Impaired climbing ability in infected flies was observed at the onset of infection. On the zeroth day of treatment, no flying in infected flies was observed, but the movement improved during the treatment, and by the end of the 20-day treatment climbing rates of treatment flies became similar to the control flies. The results were supported by Li *et al*. [26] where the bioactive compounds administration helped flies in their recovery. Treatment flies were rescued and mortality rates were stabilized after 15 days during the treatment, showing an increase in survival. The reproductive abilities of treatment flies also showed recovery and an increase after 12 days of the treatment.

Researchers have reported the association of oxidative stress with gut inflammation during diarrhea [27]. Xiu *et al*. [27] have reported the formation of ROS in the *Drosophila* intestine during Dextran Sulfate Sodium-Induced inflammatory bowel disease (IBD) [27]. The release of ROS is associated with the activation of signaling pathways in the host body and the release of antimicrobial peptides [28]. Various enzymes which are the biomarkers of oxidative stress were analyzed from zeroth day to day 25 of treatment. Initially, the total thiol content of treatment flies was 14% and it increased to 86% when compared to control flies during the treatment. Another marker enzyme of gut epithelial injury is Glutathione-S-transferase, which is involved in the function of cell protection, detoxification, and removal of ROS [29]. These enzymes are prevalent in the kidney, liver, and intestine. Analysis of Glutathione-S-transferase activity in infected flies showed a significant decrease in overall enzyme activity, but protease supplementation in infected flies showed an increase in the activity of Glutathione-S- transferase (EC 2.5.1.18) from 19% to 97% was observed when compared to the control flies. Another enzyme that is a part of an antioxidant defense system is catalase which negatively regulates ROS formation and reduces cytotoxicity [30,31]. Catalase dissolves the toxic peroxides in water and acts as an antioxidant [32]. The analysis of catalase (EC 1.11.1.6) in infected flies showed a 198% increase in catalase activity during peak infection which was restored to normal during 25 days of treatment when compared to the control cells.

5. CONCLUSION

The analysis concluded that the protease is effective in counteracting the *S. enterica* infection and has antimicrobial properties. The activity of different antioxidant enzymes proved the role of protease in the activation of the host immune system and signaling pathways. The study involved feeding the antibacterial protease to *Drosophila.* If the study had involved the direct injection of purified protein into the organism's gut, the treatment concentration and efficacy would have shown different results. Probably, a low concentration of protease would have been sufficient to induce antidiarrheal effects. Furthermore, the protease interaction with the host gut at the molecular level can provide insight into a better understanding of the protease's mechanism of action.

6. ACKNOWLEDGMENT

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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