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Assessment of *B. subtilis* Toxicity in Wistar Rats through the Intraperitoneal Route

Babatunde Kelly[a*], Tolulope Oyedeji[b], Funmi Olatujoye[c], Olorunjuwon Bello[d]

[a]*Adekunle Ajasin University, Akungba-Akoko Ondo State, 342111, Nigeria*
[b,d]*University of Medical Sciences, Ondo State, 351104, Nigeria*
[c]*Wesley University Ondo, 351110, Ondo State, Nigeria*
[a]*Email:babatunde.kelly@aaua.edu.ng,,*[b]*Email:cutestoyosi@gmail.com*
[c]*Email:funmi.olatujoye@wesleyuni.edu.ng,*[d]*Email:obello@unimed.edu.ng*

Abstract

The effect of exposing wistar rats to insecticidal strains of *B. subtilis* was evaluated in this study to determine the safety of the strain as a biocontrol agent. *Z. variegatus* were collected from the university agricultural fields with the aid of an insect sweep net, brought to the lab, allowed to acclimatize and watched for the onset of diseased symptoms. The entomopathogenic bacteria was isolated from diseased *Zonocerus variegatus* and entomopathogenicity was confirmed through reinfection of healthy batch. Diseased *Zonocerus variegatus* were removed for microbial analysis and B. *subtilis* was fingered as the main entomopathogen. The microbe was cultured in liquid media, allowed to sporulate, centrifuged and washed with saline buffer. The optimal dose of the bacteria was prepared and introduced into the experimental animals through the intraperitoneal route. Animals were watched for five days before the tissues and organs were taken for hematological and histopathological analysis. Histopathological analysis showed benign effect of the microbe on the experimental animals – a further testimony to the assertion that most entomopathogenic microbes are non-lethal to other non-target animals within the environment thus making *B. subtilis* a potential candidate for the preparation of microbial pesticides.

Keywords: Entomopathogens; Zonocerus variegatus; Bacillus subtilis.

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* Corresponding author.

1. Introduction

An entomopathogen refers to a microbe which can be typically a bacterium, virus, fungus, protozoan or nematode that has the tendency of causing diseases in insects and sometimes arachnids. These set of microbes have evolved mechanisms to exploit and infect their insect hosts and more often than not cause disease conditions thus leading to high mortality rates within the affected insect populations. Entomopathogens are important in natural ecosystems because it is nature's way of checking and regulating insect populations and balancing ecosystem dynamics [1].

Zonocerus variegatus is a common grasshopper in parts of west and equatorial Africa. The insect feeds on a wide range of foliage belonging to crops such as coffee, cassava and cocoa. Apart from crops, some weeds also suffer from the menace of *Z. variegatus*. The insect is a polyphagous species capable of consuming many plants [2]. It also destroys the stem bark of food crops at the end of the dry season sometimes resulting to about 90 and 30 percent yield losses. This is of great economic consequence to the African farmer [3].

Several attempts have been made to control them using chemical insecticides and these attempts have not been without certain peculiar challenges which include the contamination of the environment, disruption of the ecosystem, pests' resistance, health risk to users as well as harming the beneficial species [4]. As a result, using microbial-based biopesticides is gradually being embraced as it is free of the usual encumbrances being witnessed with the usage of synthetic pesticides. The microbes used in the formulation of these biopesticides must however be continually researched in order to ascertain their safety on non-targets within the environment thus necessitating their toxic evaluation on experimental animals.

2. Materials and Methods

2.1 Collection and observation of the insect population for diseased individuals

Grasshoppers were sourced from the University's cassava farm using sweep nets. These insects were then taken to the laboratory. They were provided with a daily supply of fresh cassava leaves and sterile water [5,6]. The grasshoppers were allowed to acclimatize and incidence of disease symptoms were watched out for. The population was closely watched for 3 weeks after which individuals showing morbid and mortal symptoms probably induced by the combine effect of environmental stress and pathogenic microbes acquired while still in the fields. Symptoms shown were in the form of lethargy, reduced activities, colour change, reduced feeding rate and subsequent death.

2.2 Isolation of bacteria from diseased grasshoppers

Isolation of bacteria from grasshopper was done by plating the appropriate aliquot dilutions from the macerate obtained from the cadavers. The cadavers were surface-sterilized by washing in saline buffer followed by a quick immersion in 70 percent ethanol. This was followed by macerating the insect cadavers inside a sterile mortar and pestle which was sterilized using 75 percent ethanol until they became powdery. This was emptied

into a sterile test tube followed by the addition of sterile distilled water to dislodge the bacterial cells from the macerate. The aliquots were then serially diluted to obtain the appropriate diluents after which they were pour plated using the various media already prepared. After solidification of the media, the plates were incubated at a temperature of 37 °C for 24 hours [3].

2.3 Purification and identification of isolated bacteria cells

The mixed visible colonies obtained from the initial isolation were sub-cultured by re-streaking each colonies on fresh plates. Identification of cultures was performed using different biochemical means and cultural attributes most especially from the growth patterns exhibited on the specialized media. *Bacillus* sp. was further confirmed by allowing the culture to sporulate and heating the suspension spores to 80°C for 10 minutes which ultimately kill the non-sporulating bacteria. Spore suspension is plated on LB agar and colonies checked. The identified cultures were then streaked on double strength nutrient agar slants and kept for further analysis to be carried out on them [5].

2.4 Preparation of bacterial suspension for infection of insects

Bacterial suspensions were prepared from each of the isolated organisms by inoculating a loopful of the colony into 50 Ml of sterilized broth. This was incubated at 37^{o}C for 24 hours. The resulting broth cultures were centrifuged to separate and obtain their cells from the broth. The cells were further washed by reconstituting with sterile saline and centrifuging again. The cells and spores obtained were reconstituted by adding 50 Ml of water and dispensed into special containers capable of aerosolizing the microbial suspensions. Each reconstituted suspension was used in spraying different groups of *Z. variegatus* to know the suspension capable of causing infection [7;3].

2.5 Collection and rearing of wistar rat for animal studies

Adult male wistar rats were used in this experiment. The rats were reared and purchased from the animal house of the Department of Physiology, University of Ibadan, Nigeria. The rats were housed in clean and spacious cages which freely permit the flow of air. Wood shavings were used as beddings. Animals were kept under standard conditions in a well-ventilated room at temperature of 26.0 ± 2.0^{o}C. Rats experienced conditions of 12 hours light/dark cycle for 5weeks to acclimatize to their new environment and fed with a standard rodent pellet and water. Pellets were purchased from certified Top feed mills outlet in Ondo City. Tissues were refrigerated to preserve the integrity of cells and prevent enzyme denaturation [8].

2.6 Preparation of bacterial suspension for the infection of wistar rats

The bacteria was inoculated into a sterile broth and incubated for 24 hours at 27 ^{o}C. Culture was centrifuged at 2500 rpm for 15 minutes in order to separate the cells and the cells were washed with buffer saline twice. After separation, serial dilutions were carried out to and the suspension concentration was adjusted to 10^{6} cfu/ml with the aid of a spectrophotometer for subsequent inoculation into the experimental animal [3].

2.7 Inoculation of entomopathogenic microorganisms into albino rat

Laboratory animals were inoculated with 1ml of the prepared microbial suspension. The bacterial cells were injected into the first batch of each rat intraperitoneally using the 1ml syringe. The second batches were inoculated intradermally for the organism effect on the skin. The third batch were inoculated with sterile saline buffer used in washing and reconstituting the cells. Rats were watched for seven days before being sacrificed and their organs and blood harvested for haematological and histopathological tests [9].

2.8 Physical examinations of experimental rats after inoculation

After the test rats were inoculated, they were observed for some new and unusual traits and signs after seven days. These signs were compared to how they initially behave before they were inoculated with the test organisms. They were also compared to the behaviour of the ones in the control section of the cage. All of the parameters observed were based on the general behaviour, signs of toxicity, possible lesions, abnormal outgrowths and mortality. The examinations were carried out twice every day. The observations made include changes in the skin, and fur appearance, the look of their eyes, their agility, the rate at which their foods were consumed and also the changes in their body weight [10].

2.9 Collection of blood from infected wistar rat

The albino rats were not fed the night before. The following morning, they were put into air-tight jar containing diethyl ether for a slight anaesthetization. Blood samples were collected under anaesthesia thorugh tail and ocular vein puncture using a one ml blood sample collection EDTA tubes placed in a slant form for haematology tests [10].

2.10 Haematological tests on blood

An automated full blood count was carried out on each blood sample to determine parameters such as the total red blood cells, the hemoglobin, packed cell volume (PCV) used in investigating anemia, the total white blood cells (neutrophils, eosinophils basophils, monocytes and lymphocytes) [10].

2.11 Histopathological examinations

The animals in the test and control groups were sacrificed and their internal organs were harvested. Organs such as liver, spleen, heart, kidney and intestine (ileum, duodenum, jejunum) were harvested by surgical blade and cleansed from blood stains. They were transferred inside universal bottles which contained 10% formalin solution and were taken for histopathological test.

The tissues of organs excised were diced into smallers sizes of around 3mm length. Tissues were treated with different grade alcohol of concentration ranging from 50 percent to 100 percent for dehydration. Thereafter, tissues were cleared using xylene for a period of 2 hours and impregnated in molten wax. They were further

embedded in paraffin wax and left to solidify, marked out with a sharp surgical knife and then hung on a wooden block for sectioning. Sectioning as carried out with a microtone at 5microns and was stained with haematoxylin-eosin. The excess stained removed using tap water. It was further cleared in xylene and subsequently mounted in Canada balsam. The sectioned tissues were spread out in a water bath regulated at temperature of 45^{o}C. They were then collected with slides rubbed with egg albumin and allowed to dry in the oven with temperature set at 40^{o}C. They were then examined under the microscope slide using the low and high power objectives [11].

2.12 Statistical analysis

All data were expressed as mean ± SEM. Analysis of variance (ANOVA) was performed on the data and the means compared by Duncan Multiple Range Test using SPSS version 15. Differences were judged to be statistically significant at $p < 0.05$.

3. Results

Bacteria isolated from diseased *Z. variegatus* include *Salmonella typhii, Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermis, Klebsiella pneumoniae, Bacillus subtilis, Bacillus sphaericus,* and *Streptococcus feacalis.* As shown in Figure 1, all eight bacteria isolated from *Z. variegatus* were used in the infection for the determination of possible entomopathogens out of which *Bacillus subtilis* is the only microbe with insecticidal potentials. The microbe was also recovered in large quantities from the insect cadaver.As shown in tables 1a and 1b, haematological results showed that the Packed Cell Volume, Red blood cells and haemoglobin count of the infected rats are slightly lower when compared to that of the rats in the control group. However, total white blood cell count of the rats within the experimental group is relatively higher than that of the rats within the control group.

The histopathological tests of the organs removed from rats within the control group generally showed no visible lesions or anomaly while those harvested from the rats within the test group showed occasional mild symptoms. Figure 2 shows Liver harvested from the rats within the control group which showed a radially arranged hepatic plates with uniform thickness as well as closely-packed hepatic plates with no visible lesion. The hepatic plates are also devoid of signs of fibrosis, inflammation, necrosis, or other pathological changes. Figure 3 also shows histopathological test on the liver of the infected rats showed widespread moderate vacuolar change of hepatocytes. Figure 4 shows Kidney of rats within the control group. The kidney histopathology showed normal glomeruli, tubules and renal interstitium while that of the infected group (Figure 5) showed a moderate sloughing off of tubular epithelium. The heart obtained from rats within the control and test group both showed no visible lesion on the cardiomyocytes (Figures 6 and 7). Spleen harvested from rat within the control group shows no visible lesion with no evidence of pathological alterations in splenic architecture or cellular composition (Figure 8). Spleen removed from rats within the control group however shows moderate amounts of widely-spaced discrete PALSs (Figure 9).

Histopathological test results on skin of both test and control rat group showed no visible signs of lesions at the

site of inoculation. There is no hair loss and no signs of epithelial degeneration or necrosis (Figures 10 and 11). The histopathology test on intestine of rats from both control and experimental group showed no visible lesion with normal crypts and villi (Figures 12 and 13).

Table 1: Haematological parameters of infected and controlled rats

Organisms	PCV	Hb	RBC	WBC
B. subtilis	36.33 ± 1.15^{c}	12.37 ± 0.21^{cd}	6.65 ± 0.10^{bcd}	4033.33 ± 76.38^{bc}
Control/Saline buffer	37.33 ± 0.56^{c}	13.27 ± 0.15^{e}	6.93 ± 0.04^{e}	3816.67 ± 28.87^{a}

Mean±SD in the same column with homogenous superscript are not significantly different (p>0.05)

Key: Pcv - Packed Cell Volume, Hb – Haemoglobin, Rbc - Red Blood Cells, Wbc - White Blood Cells, Lym – Lymphocytes, Ne – Neutrophils, M – Monocytes, E – Eosinophils

Table 2: Haematological parameters of infected and controlled rats

Organisms	Platelets	Lymphocytes	Neutrophils	Monocytes	Eosinophils
B. subtilis	129333.33 ± 1527.53^{c}	56.00 ± 2.00^{ab}	33.33 ± 1.53^{ab}	0.67 ± 0.58^{ab}	1.33 ± 0.58^{ab}
Control/Saline buffer	116666.67 ± 1527.53^{a}	56.33 ± 2.52^{ab}	31.67 ± 1.16^{a}	0.33 ± 0.58^{a}	0.67 ± 0.58^{a}

Mean±SD in the same row with homogenous superscript are not significantly different (p>0.05)

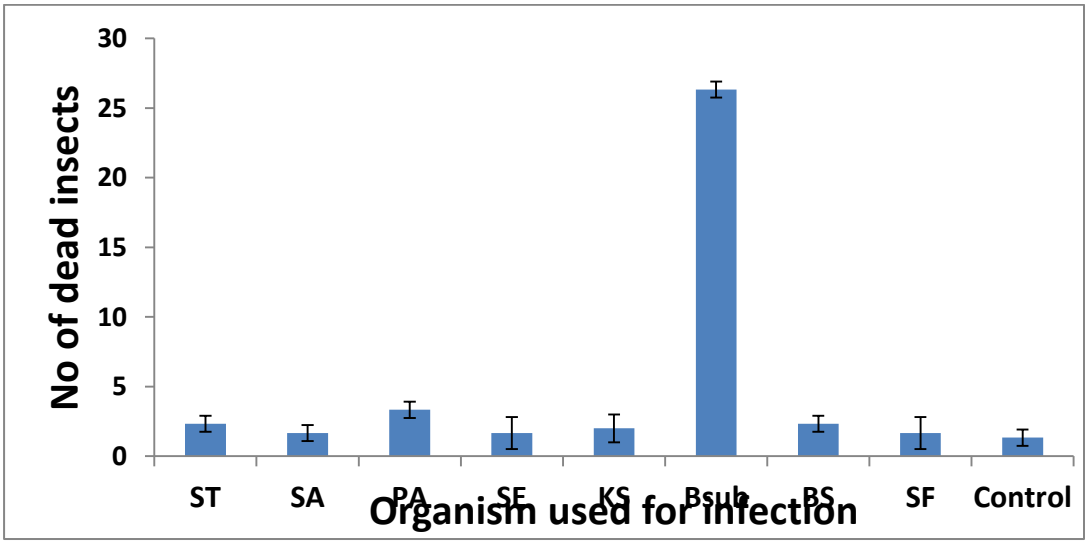


Figure 1: Lethal effects of selected bacteria on *Z. variegatus*

Key:

ST – *Salmonella typhi*, SA – *Staphylococcus aureus*, PA – *Pseudomonas aeruginosa*, SE – *Staphylococcus epidermidis*, BSub – *Bacillus subtilis*, KS – *Klebsiella pneumoniae*, BS – *Bacillus sphaericus*, SF – *Streptococcus feacalis*

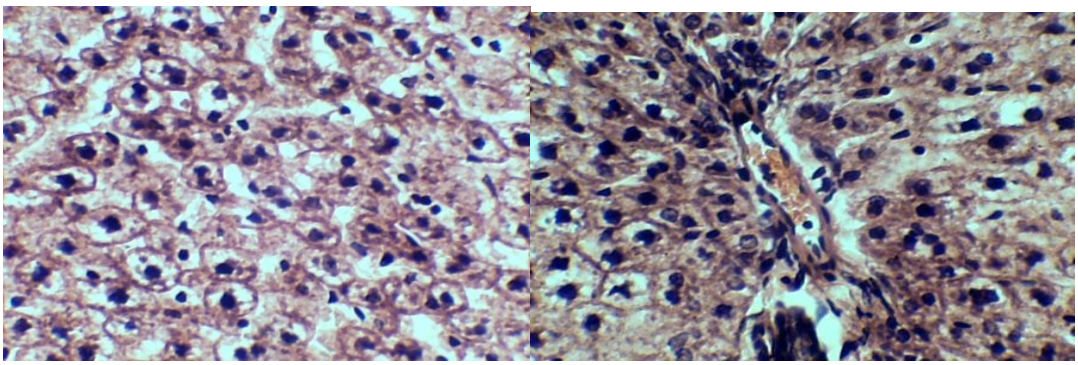
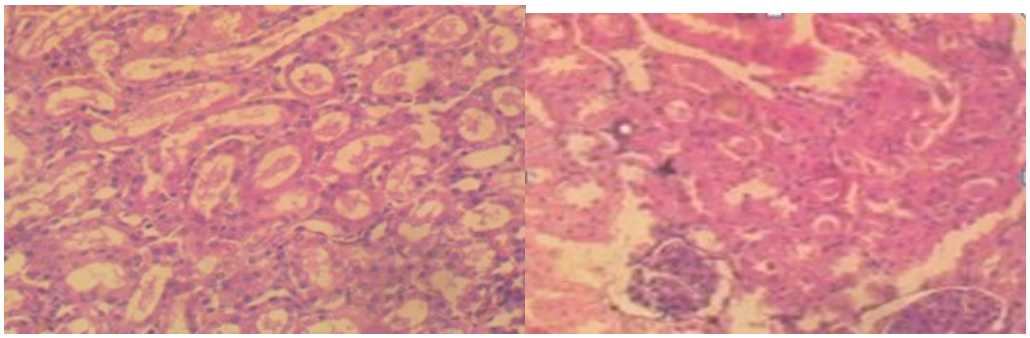


Figure 2



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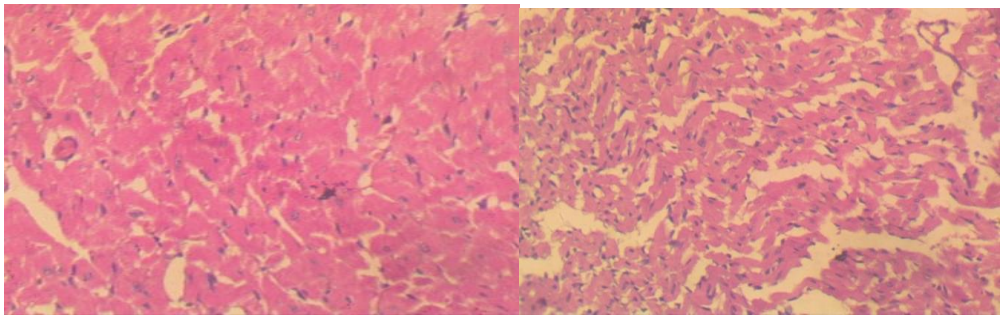

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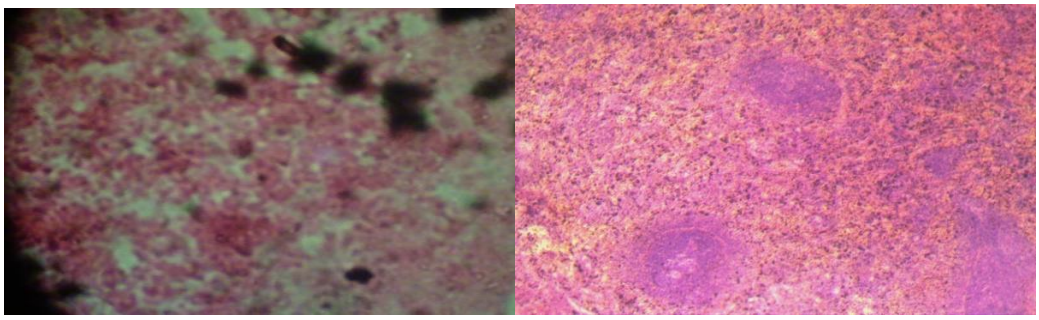

Figure 5

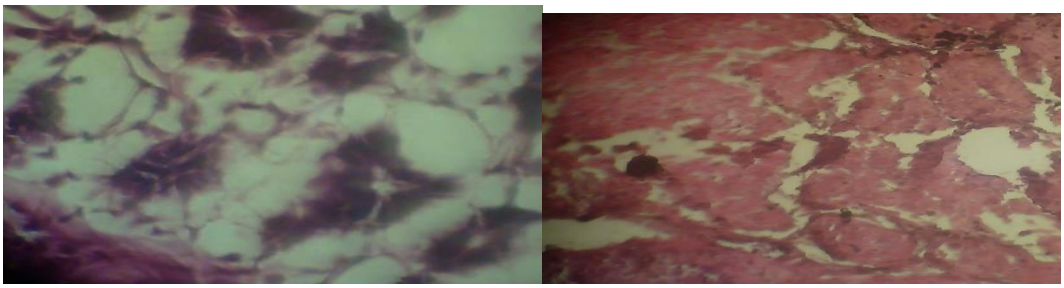

Figure 6

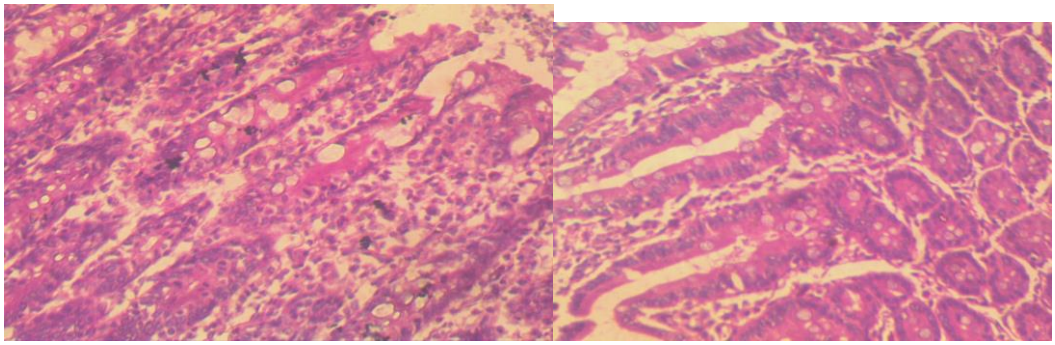

Figure 7

Key: Plate 1, Liver of uninfected rats, Plate 2, Liver of rat infected with *B. subtilis*, Plate 3, Kidney of uninfected rats, Plate 4, Kidney of rat infected with *B. subtilis*, Plate 5, Heart of uninfected rats, Plate 6, Heart of rat infected with *B. subtilis*, Plate 7, Spleen of uninfected rats, Plate 8, Spleen of rat infected with *B. subtilis*, Plate 9, Skin of uninfected rats, Plate 10, Skin of rat infected with *B. subtilis*, Plate 11, Intestine of uninfected rats, Plate 12, Intestine of rat infected with *B. subtilis*,

4. Discussion

Occurrence of bacterial entomopathogens as seen in diseased *Zonocerus variegatus* in this study has been described in literatures and research findings. Some entomopathogens are known to colonize plant surfaces, where they can infect insects that come into contact with them during the process of feeding. Author [12]

describes the presence of *Bacillus thuringiensis* known to produce insecticidal toxins that are commonly used in biopesticides on certain plants. These toxins are ingested by insects feeding on such plants and this may account for the reason why the *Z. variegatus* in this study came in contact with the microbe since it is known to feed on leaves of plants. Author [13] also described the abundant existence and interaction of entomopathogens in natural environments.

The Haematological parameters obtained inform the analyses of the infected rat blood sample gives an indication of infection when compared with that of the control rats. In the analysis carried out for the enumeration of the red blood cells, there was a slight general reduction in total numbers obtained for the infected rats in contrast to what was obtainable in the uninfected rats.

This points to the fact that during the course of an infection, haemolysis can occur and most times, this leads to a reduction in the total number of the red blood cells available [14]. The same is also applicable to the reduction in the packed cell volume and haemoglobin.

Other haematological analyses carried out showed an increase in the total white blood cells which may be due to the increase in the production of antibodies against the entry of the insect. An increased amount of white blood cells in the blood suggests that such individual or animal is either reacting to an allergen or building immunity against an antigen, pathogen and foreign particles into the body.

Previous works on the biosafety of *M. anisopliae* which was carried out by Authors [15;16] showed that the crude allergenic extract inoculations obtained from a *M. anisopliae* entomopathogenic strain and injected into mice contained components that induced immunological, inflammatory and histopathological responses which are characteristics of allergy. Thus, this may also worsen allergy in susceptible individuals [17]. Such is the case of the first reported human case of possible disseminated infection with *M. anisopliae var. anisopliae*. A 9-year-old, immunosuppressed boy with a 5-year history of acute leukaemia was under chemotherapy and during this period exposed to *M. anisopliae var. anisopliae*. This eventually led to his death according to research by Author [18]

Histopathological examinations carried out on the heart, intestine, spleen and skin of rats challenged with *B. subtilis* showed no signs of major histopathological anomalies. Similar work by Author [19] in other animal models and in-vitro systems evaluating the acute and chronic toxicity, genotoxicity as well as other safety parameter of *B. subtilis* also generally showed that *Bacillus subtilis* is safe within certain dosage ranges. Author [20] even reported that *B. subtilis* can confer health benefits by supporting digestion and modulating the immune system when used as a probiotic in rats, including Wistar rats, without significant toxicity.

Although liver of infected mice showed widespread moderate vacuolar change of hepatocytes with *M. anisopliae* moderately showed no vacuolar change of hepatocytes. Similar experiments on mice also revealed that some insecticidal strains were not toxic to certain organs of experimental animals even though they may result in certain mild changes. The spores and conidia of such strains usually remain viable for at least one month in the body tissue after which they are continuously eliminated in the subsequent days Author [21].

Viable spores and conidia have been recovered from the spleens of rats for as long as 18 days after intra peritoneal injection. However, such infection is self-limiting and usually disappeared from the lungs after 14 days.

5. Limitations of the Study

While this study provides valuable insights into the safety of entomopathogens on nontargeted animals, certain limitations should be acknowledged. The lethality of *B. subtilis* was evaluated only on adult *Z. variegatus* with no consideration for their sex. Although, it seems unlikely but there might be a possibility that nymphs might have a slightly different reaction to the entomopathogens. Secondly, only male rats are used for the animal studies. Due to hormonal variations, females might have a different histopathological outcome. Thirdly, financial and time constraints as well as limited access to the required equipment prevented the identity confirmation of the entomopathogen using molecular method through Nucleic acid amplification and 16S rRNA Gene Sequencing. Future research in this area can therefore build on this research outcome and extend our understanding beyond its peculiar limitations.

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