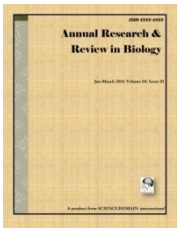

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Physiological Changes in Wister Rats Induced with Bacillus Species Used as Bio Control Agent

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Authors' contributions

This work was carried out in collaboration between all authors. Author FAA designed the study. Author FOO wrote the protocol, wrote the draft of the manuscript and performed the statistical analysis. Author BB managed the analysis and literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This work is aimed at toxicological effects of three Bacillus species employed as bio control agent against mosquito larvae in terrestrial environment.
Study Design: Using animal model where weight, hematology and histopathology were considered.
Place and Duration of Study: At Microbiology Department, Federal University of Technology, Akure, Nigeria, between December 2009 and February, 2010.
Methodology: Twenty wistar rats weighing 180±0.61g were used and fed with mice feed and water *ad libitum* for one week to be acclimatize before dividing into four groups of five. Group 1 was the control and was allowed to normal rat feed and water only, group II – IV were subjected to daily single subcutaneous injection with 200µl of 10^7cfu/ml cell dose of *Bacillus thuringiensis*, *Bacillus subtilis* and *Bacillus cereus* respectively for seven days. The rats were observed shortly after each dosing and thereafter twice daily for one week for general behavioral signs of toxicity and possibly mortality.
Results: The rats infected with *B. cereus* lost weight by 3.8% during the period of study while those in the control, the treated with *B. thuringiensis var. isrealensis* and *B. subtilis* gained weight by an average of 3% of the initial weight. In the hematological analysis of the rats, there was significant difference ($P<0.05$) in values when compared with the

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control group. Though decrease in differential counts was observed in the rats injected with *B. subtilis* and *B. thuringiensis,* significant difference ($P<0.05$) in values was not observed with the control group. Histopatological examinations of the liver and small intestine of the rats treated with *B. cereus* had observable pathological changes when compared with control, *B. thuringiensis* and *B. subtilis* treat groups.

Conclusion: There were no significant toxicological changes in the groups treated with *B. subtilis* and *B. thuringiensis* when compared with the control group. Based on these observations, it could be concluded that these bacterial species could be used as bio control agent in human environment without causing havoc to humans and animals.

Keywords: Toxicology; bio control; mosquito larvae; bacillus species.

1. INTRODUCTION

Biolarvicides of bacterial origin, particularly *Bacillus thuringiensis var. isrealensis* (*Bti*) have proved to be highly effective against mosquito larvae at very low dosage and safe to non-target organisms [1]. *Bti* is effective against several mosquito species [2,3]. According to Regis et al. [4], *Bti* offers many other advantages including high efficacy against target species, causing catastrophic larval mortality in 24 h. The productions of the characteristic insecticidal (Cry) proteins deposited in crystals in the mother cell have been shown to mainly start from the onset of sporulation [5,6]. It has also been shown that some *Bti* insecticidal proteins are produced and secreted into culture medium during vegetative growth [7,8].

Despite the larvicidal potency of *B. thuringiensis var. israelensis*, it is however observed that its insecticidal and larvicidal activity against *Anopheles* mosquitoes causing malarial menace in African countries have not been adequately tested, well documented and adopted in sub Saharan Africa. This particular bacterial species has also been observed to suffer many limitations, among which is the short persistence of action. According to Margalith and Ben-Dov [9], the application of *B. thuringiensis* for mosquito control is limited by short residential activity of current preparations under field conditions. Loss of persistence of *B. thuringiensis* activity might result from multiple environmental factors such as temperature, water, and sunlight. *B. thuringiensis* adapts poorly to surface exposure to sunlight, particularly ultraviolet light. It has been observed that one of the major problems affecting the efficacy and economy of *B*. *thuringiensis* is its inactivation by sunlight in the field [10].

Owing to the widespread suffering and death caused by malaria and the failure of the safest and most affordable antimalarials to treat the disease malaria [11,12], there is an urgent need for alternative methods for treatment, management, prevention and control of malaria. The biological means of control using microorganisms has proved to be a suitable and effective alternative. According to Weinzierl et al. [13], microbial insecticides are especially valuable because their toxicity to non target animals and humans is extremely low. Compared to other commonly used insecticides, they are safe for both the pesticide users and consumers of treated crops. Microbial insecticides also are known as biological pathogens, and biological control agents. So owing to the drawback associated with *B*. *thuringiensis var. isrealensis*, it becomes very imperative that alternative bacterial organisms that could be used to control mosquitoes be identified, with their methods and mechanisms of microbial control well understood and comparable. In previous work, *B*. *subtilis* and *B*. *cereus* have been found effective as larvicidal. Hence the use of these bacillus species are

going to be employed in human environment, their toxicity was carried out using animal model in order to predict their safe use in an environment.

2. MATERIAL AND METHODS

2.1 Toxicity Test

Potential toxic activity for mammals was determined according to Peng et al. [14]. Twenty Wistar rats were purchased from Veterinary research institute, Vom, Jos. The rats were bred in cages made of fabricated metals iron having a measurement of 100 x 11cm in the animal house of department of Microbiology, Federal University of Technology, Akure. The cage was made up of four compartments 20 x 11 cm each. The floors are composed of flat metal of 1-2 cm diameter with 1 cm spacing to facilitate the passage of faeces. The animal house temperature was 28±2ºC during the experiment. The rats weighing 180±0.61g were fed with mice feed and water *ad libitum* for one week to acclimatize and were starved for 24 hrs prior treatments. They were divided into four groups of five each: Group 1 served as the control and was allowed to normal rat feed and water only, group II – IV were subjected to daily single subcutaneous injection with 200µl of 10^7cfu/ml cell dose of *B. thuringiensis*, *B. subtilis* and *B. cereus* respectively for seven days. The rats were observed shortly after each dosing and thereafter were observed twice daily for the period of one week infection for a general behavioral signs of toxicity and possibly mortality. The behavioral signs of toxicity observed included changes in the skin and fur, rate of food consumption, agility, eyes and mucous membrane and change in body weights. The tested rats were sacrificed by anaestizing with fume of chloroform at the end of experiment. The animals were conducted in compliance with NIH Guide for Care and Use of Laboratory Animals.

2.2 Collection of Blood

Blood was collected from the rats by cardiac puncture with the aid of disposable sterile syringe and needle into heparinised vials. Sterile heparinised capillary tubes were used to collected blood directly for packed cells volume determination. The heparinised vials containing blood samples were taken to the laboratory for immediate haematological analysis.

2.3 Hematological Parameters

The hematological parameter including, Hemoglobin, total red blood cells count (RBC), white blood cells count (WBC), differential count of leucocytes such as neutrophil (%), lymphocyte (%), monocyte (%) and packed cell volume (PVC) were enumerated.

2.3.1 Packed cell volume (PCV)

This was estimated by spinning 75% of each blood sample in a haematocrit micro centrifuge at 1200 rpm for five minutes and the value was read on the haematocrit reader as a percentage of the total blood volume using the equation:

$$\text{PCV} = \frac{\text{Height of packed cell column}}{\text{Height of whole blood column}} \times 100$$

2.3.2 White blood cell

Ethylenediamine tetra acetic acid (EDTA)-treated whole blood at 1:20 ratio with blood cell diluting fluid (made up of 3.8g sodium citrate, 0.21g neutral formalin and 0.5g brilliant cresol blue and 100ml distilled water). The diluted sample was then mixed and loaded into counting chamber. The WBC in the chamber was counted leaving out the edges of the chamber. Total WBC was determined using the equation.

$$WBC = \frac{N \times DF \times 10^6}{A \times D}$$

Where A = the area counted, N = number of cell. DF = Dilution factor, D = depth of chamber.

2.3.3 Red blood cell

This was determined using the Neabeauer counting chamber, dilution pipette, dilution fluid (formol-citrate prepared by mixing 10ml of formalin with 1 litre of trisodium citrate solution). The Neabeauer counter was mounted under compound microscope and cells were counted with a hand tally. The equation below was used to express total RBC.

$$\text{Red cell count} = \frac{N \times DF \times 10^6}{A \times D}$$

Where A = the area of chamber counted, N = number of cell, DF = Dilution factor, convert to cell per litre D = depth of chamber.

2.3.4 Hemoglobin

Blood samples (0.02 ml) were mixed gently for one minute and drawn using a 0.02ml micro-pipette and expelled into a tube containing 4ml of Drakins solution. The tube was stopped, mixed and left to stand for 5 minutes to allow full colour development. A standard was prepared using a blood sample of known hemoglobin concentration. Using plain Drakin's solution both the sample and standard blood dilution were then read on the colorimeter (Corning colorimeter, 253) at 550nm. The hemoglobin concentration in the blood samples were calculated using the formula:

$$\text{Sample Hb concentration} = \frac{\text{Reading of text}}{\text{Reading of standard}} \times \text{Standard Hb concentration}$$

2.4 Histopathological Analysis

The internal organs namely liver and intestine were preserved in 10% formalin solution. After which they were processed for histopathological studies [15,16]. The tissues were cut into small sizes of about 3cm and were dehydrated in different grades of alcohol from 50% through 100%. Thereafter, the tissues were cleared in xyline for 2 hours and impregnated in molten wax. The impregnated tissues were embedded in paraffin wax, allowed to solidify, marked out with sharp knife and mounted on wooden block for sectioning. The tissues were sectioned with microtome at micron 5. The sectioned tissues were spread out in a water bath regulated at 45^{o}C and picked with slides previously rubbed with egg albumin. The sectioned tissues were de-waxed and hydrated in alcohol grades from 100–50%, stained with haematoxylin and eosin, excess stain was washed with 70% ethanol, clear in xyline and

mount in Canada balsam. Liver sections were graded numerically to assess the degree of histological changes in the acute hepatic injury. The scoring of liver damage was done [17,18] as follows: portal fibrosis (0-6), lobular infiltration and necrosis (0-3), Mallory bodies (0-3), hepatocyte ballooning (0-3) and fatty changes (0-3). The parameters were graded from score 0 to 6, with 0 indicating no abnormality, 1 to 2 mild injury, 3 to 4 moderate injury and 5 to 6 with severe liver injury.

2.5 Statistical Analysis

The results were expressed as mean ± standard deviation (SD) and were subjected to one way analysis of variance (ANOVA), using statistical package for social sciences (SPSS-15) at 95% level of confidence. The least significant difference (LSD) was performed for the pair wise mean comparisons, to determine the significant treatment dose at 95% level of confidence. Values were considered statistically significant at (P<0.05) and denoted by different alphabets.

2.6 Bacterial Organisms

B. cereus is closely related to *B*. *thuringiensis* but can be distinguished from *B*. *thuringiensis* as it cannot produce parasporal bodies [19]. *B. cereus* spore coats contain a 13,000-dalton monomer and a 26,000-dalton dimmer [20].

Among the Bacillus genus, *B. subtilis* produces a broad spectrum of bioactive lipopeptides having a great potential for biotechnological and biopharmaceutical applications such as their use as antiviral, antibacterial and antitumor agents and immunomodulators. Unlike other bacillus species *B*. *subtilis* is spore forming pupicidal bacteria whose metabolite can kill both the larval and pupal stages of mosquito [21,22], observed that the crude mosquitocidal toxin (CMT) produced by *B*. *subtilis* could be more toxic to the pupae of mosquito than to the larvae.

B. thuringiensis var isrealensis is a natural pathogen of some pests, and the insecticidal protein produced by *Bti* are extremely toxic to certain pests, but cause little or no harm to people, wildlife and most beneficial insects [23]. The enthomopathogenic activity of *Bti* is caused primarily by the sequential action of Cry toxin ingested by susceptible insect larvae that include solubilisation and activation via enzymatic processing, interactions with insect midgut epithelium, and disruption of the structural and functional integrity of the epithelium, leading to complete tissue destruction and death of the insect [24].

3. RESULTS

Table 1 shows the hematological parameters of Wister rats treated with *Bacillus cereus*, *Bacillus subtilis* and *Bacillus thuringiensis var isrealensis* HD522. There was a slight decrease in the PCV, RBC and WBC counts of Wister rats treated with the Bacillus species except with *B*. *cereus* where much decrease was noticed when compared with the control group. *B*. *cereus* PCV was the least among the Bacillus species treatments where it was 32.00±2.00%, *B*. *subtilis* (42.30±1.52%), *B*. *thuringiensis* (39.30±0.57%) as compared with the control group value of 41.00±1.73%). The RBC of the control group was 9.28±0.06m/cu.mm as against 8.19±0.23, 7.90±0.40 and 7.55±0.43m/cu.mm for *B*. *thuringiensis*, *B*. *subtilis* and *B*. *cereus* treatments respectively. WBC values in that order was 168±1.15, 174±4.5, 172±2.00 and 265±1.15 t/cu.mm. Because of the low RBC counts in

the *B. cereus* treated group of rats, hemoglobin became higher in value (14.00±1.05) than *B. subtilis* (12.5±0.48%), *B. thuringiensis* (12.7±0.12%) and control group (11.1±0.22%). The differential counts of lymphocytes, eosinophil, neutrophil and basophil were also more affected in the *B. cereus* treated rats than observed in *B. thuringiensis* and *B. subtilis* treatments Table 2. Mean weight of between 180.23-180.40g increased to between 184.23-184.82g in *B. thuringiensis* treated group of rats. Also, in *B. subtilis* treated group rats, weights from between 178.85-180.10g increased to between 183.04-183.8g. The control experiment weights of between 178.32-180.68g increased to 185.04-185.88g at end of experiment. Meanwhile, decrease in weights was only observed in *B. cereus* treatment Table 2.

Pathological observations of some tissues namely, liver and intestine of wistar rats injected with *Bacillus cereus*, *B. subtilis, B. thuringiensis var israelensis* HD522 and the control group are presented in Figs. 1 – 8. The results of histological studies of wistar rat's liver injected with *B. cereus* are shown in Fig. 1. However, there were observable pathological changes in the liver Fig. 1 and intestine Fig. 7 of wistar rats injected with *B. cereus.* In the *B. cereus* treated mice, liver and intestine had signs of hemorrhagic lesion, gangrenous edges and shrinkage thereby weakening and reducing the ventricle size of the organ for its appropriate body functions. The intestines were inflamed with enlargement of the epithelium of villi in the *B. cereus* treated rats Fig. 7 which may result to poor absorption rate and whose effects was seen in the weight loss and hematological parameters studied. No significant damages were recorded in the intestine of Wistar rats treated with *B. thuringiensis var israelensis* HD522 Fig. 5 and *B. subtilis* Fig. 8 when compared with the uninfected intestine Fig. 6.

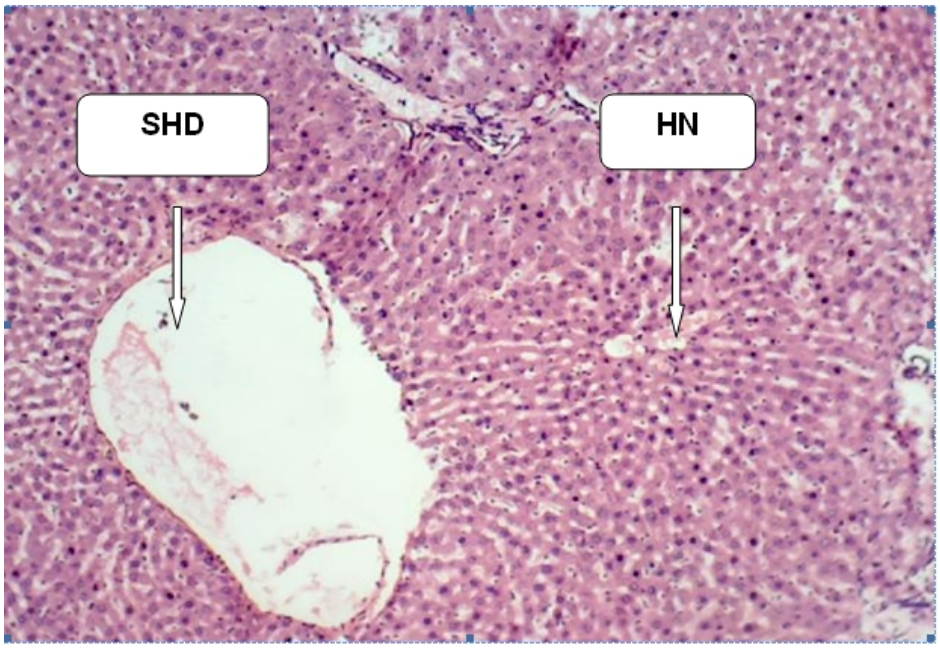

Fig. 1. Histopathological section of rat liver treated with *B. cereus* showing severe hepatic degradation (SHD) and Hepatocellular necrosis (HN)

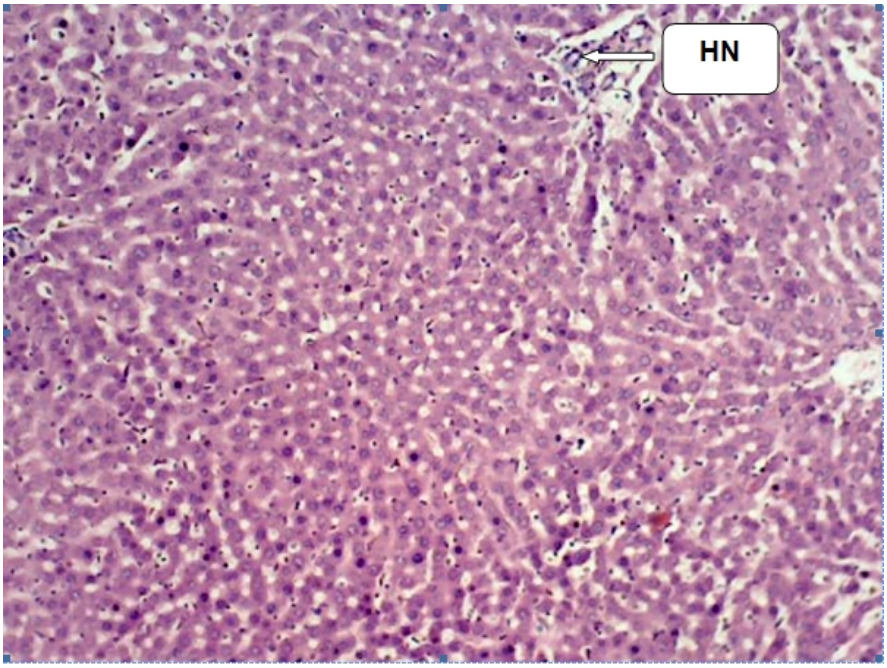

Fig. 2. Histopathological section of rat liver treated with *B. subtilis* showing hepatocellular necrosis (HN)

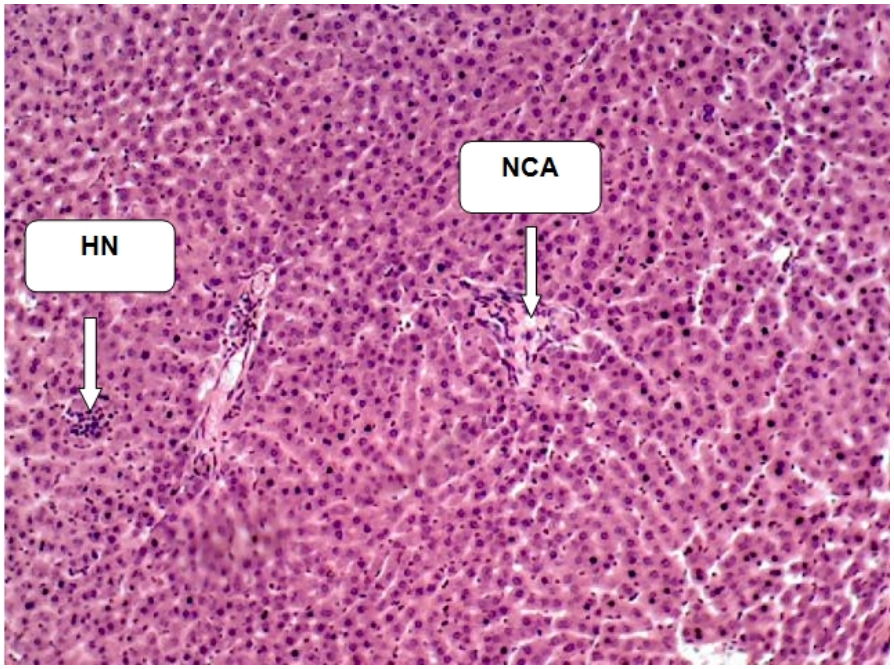

Fig. 3. Histopathological section of rat liver treated with *B. thuringiensis* showing Normal cellular architecture NCA) hepatocellular necrosis (HN)

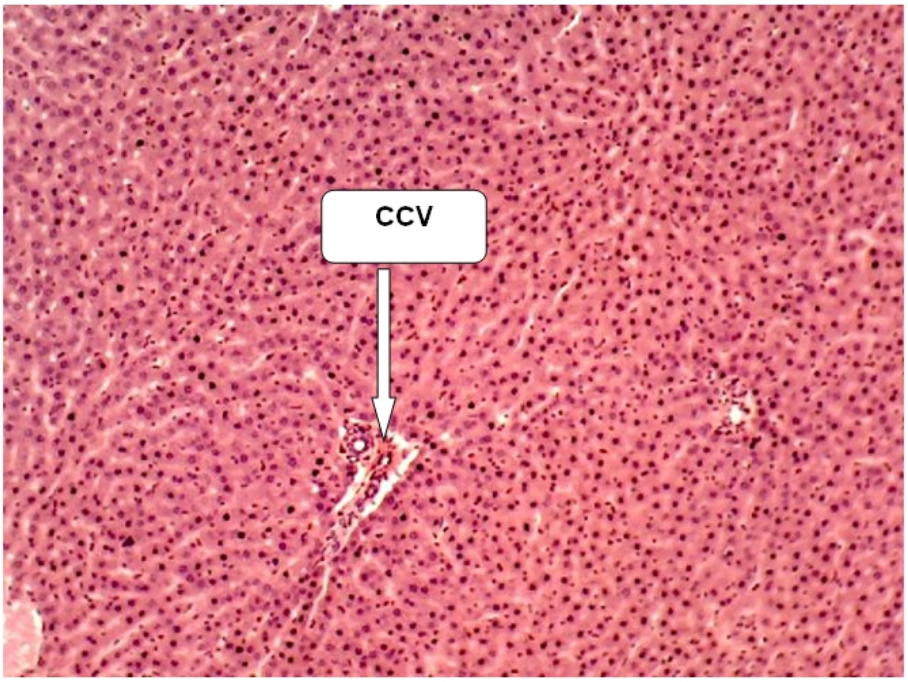

Fig. 4. Histopathological section of control rat liver showing Central cellular vein (CCV)

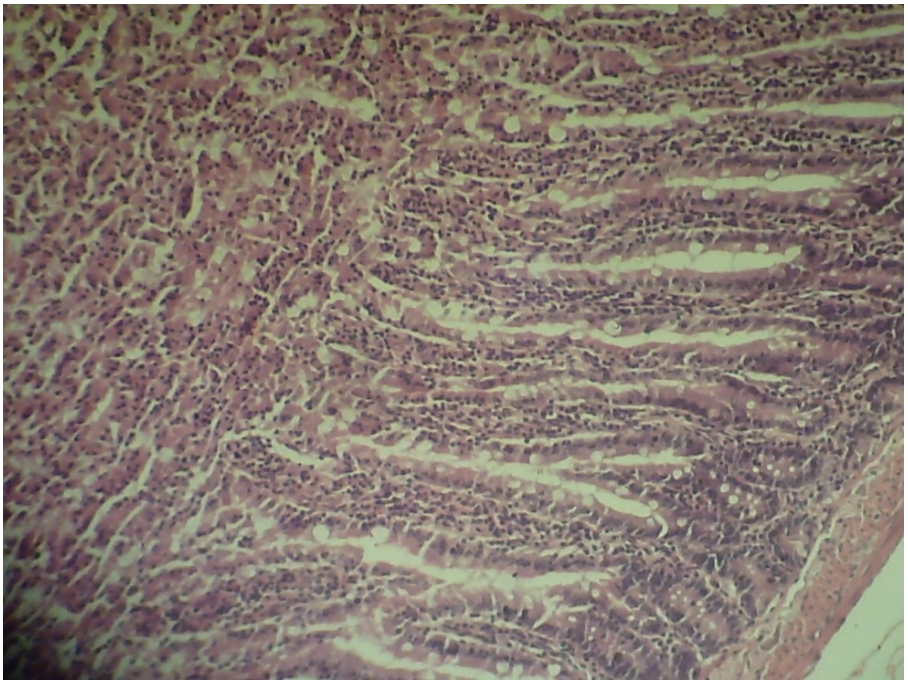

Fig. 5. Photomicrograph of histopathological section of rat's intestine infected with *Bacillus thuringiensis*

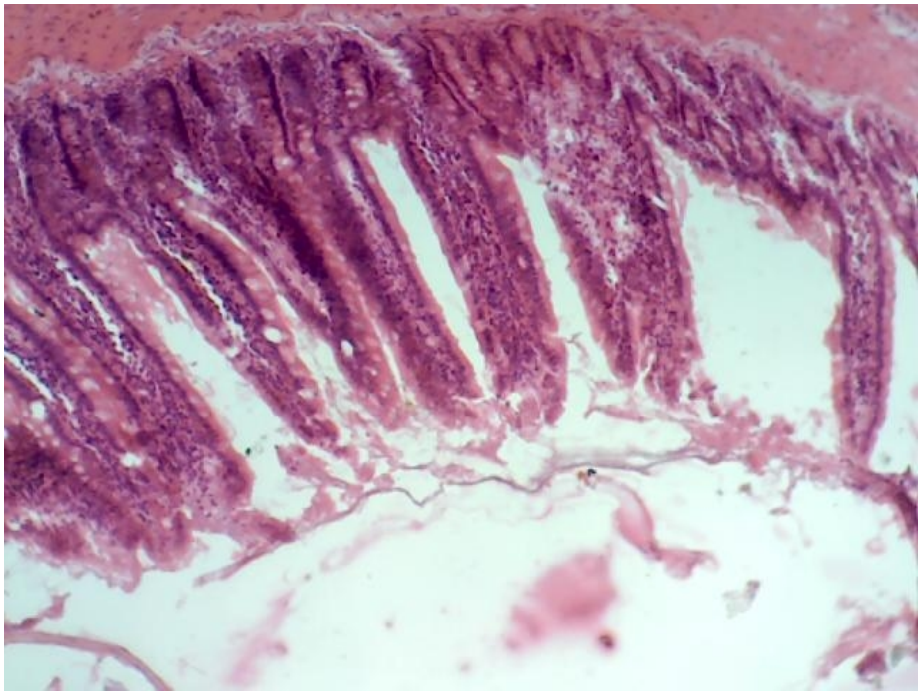

Fig. 6. Photomicrograph of Histopathological section of uninfected rat's intestine

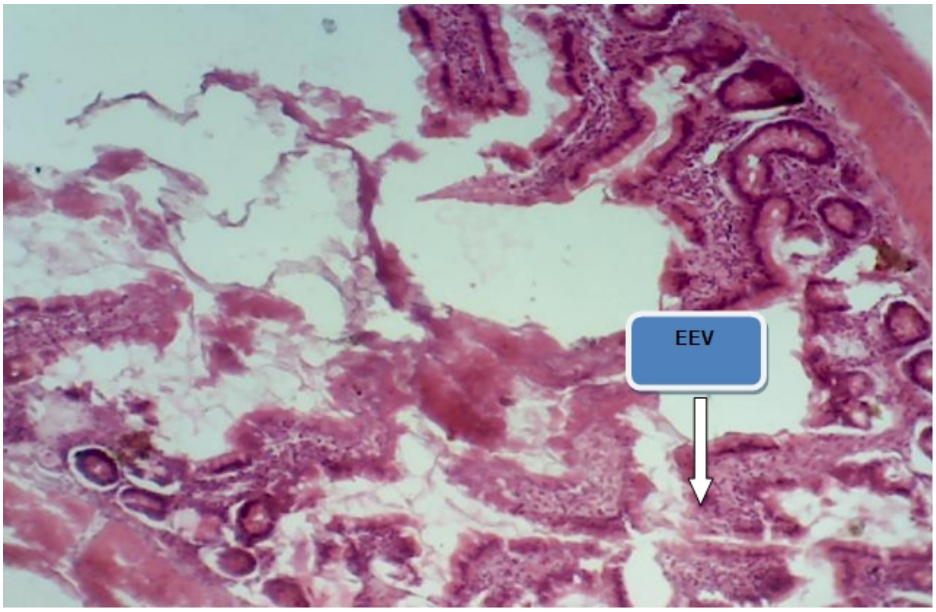

Fig. 7. Photomicrograph of histopathological section of the intestine of rat infected with *B. cereus* showing enlargement of the epithelium of villi (EEV)

Table 1. Hematological parameters of wistar rats treated with Bacillus species

Test organisms	PCV (%)	RBC x 10 (m/cu.mm)	WBC (t/cu.mm)	Hemoglobin (%)	Lymphocyte (%)	Eosinophil (%)	Neutrophil (%)	Monocyte (%)	Basophil (%)
Bacillus cereus	32.0±2.00[b]	7.55±0.43[b]	205±1.15[a]	14.0±1.0[a]	59.0±1.0[b]	1.9±0.[11a]	35.33±0.6[a]	2.26±0.64[b]	0.6±0.57[b]
Bacillus subtilis	40.30±1.6[a]	7.90±0.40[b]	172±2.00[b]	12.5±0.48[b]	66.3±1.15a	1.96±0.06[a]	29.0±1.00[b]	1.97±0.57[c]	1.0±0.00[a]
Bacillus thuringiensis	39.3±0.57[b]	8.19±0.23[b]	174±4.5[b]	12.7±0.[12b]	66.0±1.0[a]	1.97±0.[50a]	28.0±1.00[b]	4.96±0.06[a]	0.67±0.57[b]
Control	41.0±1.73[a]	9.28±0.06[a]	168±1.15[b]	11.1±0.[22c]	66.0±1.00[a]	2.0±0.0[a]	38.3±0.64[a]	4.9±0.01[a]	1.0±0.00[a]

Each value represents the mean ± Standard deviation and values in the same column with different superscripts are significantly different from each other (P<0.05)

Table 2. Effect of Bacillus species on wistar rats body weight

Tested organisms	Initial rats weight (g) Mean ± standard error	Final rats weight (g) Mean ± standard error
Bacillus thuringiensis	180.23±0.15[a] (180.23-180.43)	184.15±0.58[c] (184.23-184.82)
Bacillus subtilis	179.03±0.39[a] (178.85-180.10)	183.94±0.61[b] (183.04-183.87)
Bacillus cereus	180.13±0.22[a] (179.73-180.32)	173.20±0.26[a] (173.41-173.85)
control	180.30±0.22[a] (178.32-180.68)	185.00±0.41[d] (185.04-185.88)

Values in parenthesis = range, sample population for each mean value = 4; Values in the same column with different superscripts are significantly different from each other (P<0.05)

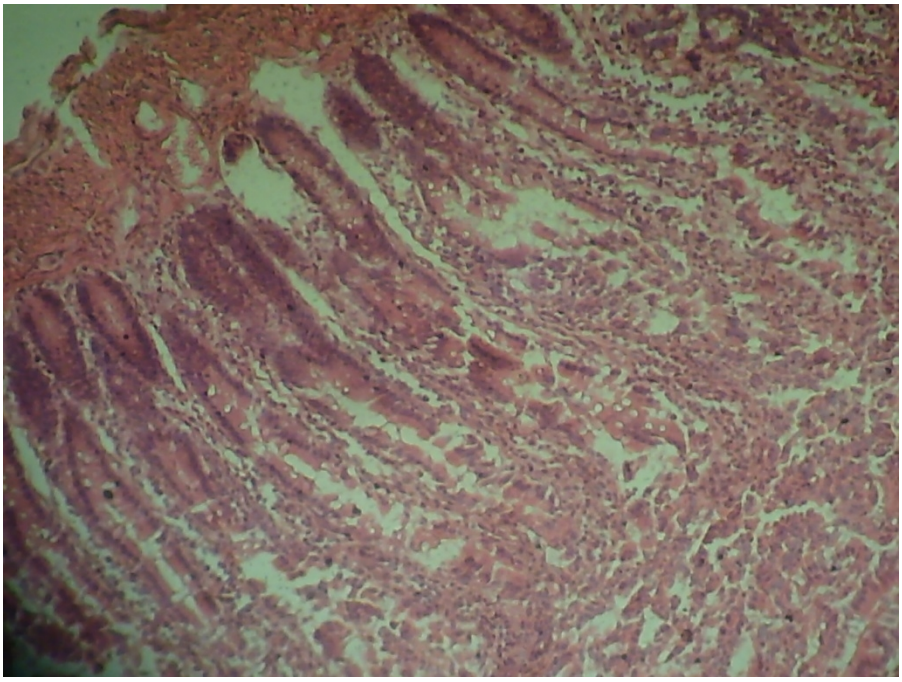

Fig. 8. Photomicrograph of histopathological section of rat's intestine infected with *B. subtilis* showing normal architecture of villi (NAV)

4. DISCUSSION

In many larvicidal bioassay, designs have revealed that numerous intrinsic and extrinsic factors may influence the toxicological performances of a given insecticide against a given target mosquito larvae. These factors need in-depth consideration for real interest of any larvicidal strategy and most importantly its safety use in human environment. Emphasis is laid on the toxicology of Bacillus species (*B*. *thuringiensis*, *B*. *subtilis* and *B*. *cereus*) because they have been found to be of potential use in killing mosquito larvae of *Anopheles* mosquito species which are vectors of *Plasmodium falciparium* in Nigeria and other African countries. If their adequate use in environment must be fully established, toxicity effect on man and his environment is necessary which have revealed that *B*. *subtilis* and *B*. *thuringiensis* are of adequacy in environmental use for mosquito larvae elimination or reduction than *B*. *cereus*. The extensive and widespread use of synthetic insecticides has caused some concerns regarding the toxicological, economical and environmental impacts of xenobiotics [25;26]. Hence practical and environmental difficulties results in the use of the available larvicidal agents, devised means of mosquito larvae eradication with natural influences that will be efficient and environmentally safe and target-specific is a point of interest. *B*. *thuringiensis* and *B*. *subtilis* are spore forming bacterial species which are able to resist adverse effects in water column to make their bioavailability for mosquito larvae ingestion in water column. The toxicological performance of the Bacillus species with terrestrial animal is of concern at the operating level on environment and human safety hence use of the Bacillus species is in human environment conducive for mosquito larvae breeding. The safety assessment of the Bacillus species on terrestrial animal using Wistar rats revealed that there were slight changes in food consumption rate of rats treated with *B. cereus.* The significant changes most especially in reduction of activities by the group of

experimental animals treated with *B. cereus* was as a result of its toxicological effect which caused minor alteration in the haematological parameters and histopathological changes in the tissue, resulting to malfunction. Weight was monitored as an indicator of the general well-being of animals. The results obtained when the animal's body weights were determined agreed with the changes that were recorded in food consumption rates of the rats. Wistar rats treated with *B. cereus* showed reduction in their weight ($P<0.05$) when compared with control group. The slight weight gained by those groups treated with *B. subtilis and B. thuringiensis* was not significant when compared with the control group. The haematological studies of Wistar rats treated with the *Bacillus* species encountered general reduction in values of Packed Cell Volume (PCV), White Blood Cell (WBC) and hemoglobin (Hb) when compared with the values of control rats. However, the WBC, PCV and Hb of rats treated with *B. cereus* were lower than that of the control and all other groups. The Decrease in WCB count normally reflects a decline in the production of defensive mechanisms to combat infections. Such decrease would naturally make the rats more susceptible to various psychological stresses resulting in disease, poor growth and greater mortality [27]. Though neither infection nor mortality was observed from the study, the disruption in the metabolism of the various cell types involved in the defense mechanism would predispose the animals to infection if there was any attack. On the other hand, leucocytosis may occur as an indication of immunological response against foreign bodies. The basophils, neutrophils, eosinophils and lymphocytes in rats treated with *B. cereus* were significantly different from that of the control.

The histopathological changes in the liver and intestine of wistar rats were observed in *B. cereus, B. subtilis* and *B. thuringiensis var isrealensis* treatments. The intestines were inflamed with enlargement of the epithelium of villi in *B. cereus* treated rats. In liver, there were signs of hemorrhagic lesion gangrenous edges and shrinkage thereby weakening and reducing the ventricle size of the organs for their appropriate body functions whereas no significant changes were observed in the rats infected with *B. subtilis* and *B. thuringiensis var isrealensis* when compared with the control group. Injection of *B. cereus* exotoxin into the skin of rabbits has caused increased vascular permeability and necrosis; production of this toxin seems to correlate with the severity of clinical infection [28]. Liver being the detoxification organ of animals and kidney the most important excretory organ is very susceptible to toxins. From this study, *B. cereus* could cause damage to liver. The damages observed in these vital organs might have led to reduction in the rate of consumption of food by rats in this group which eventually led to weight loss.

Haematocrit (packed cell volume) is the measurement of the percentage of red blood cells in whole blood. Anemia may arise from reduced production of red blood cells, which may result from deficiency in nutrients or hormones, or from disease or other conditions and excessive destruction of red blood cells [29]. Reductions which were significant ($P<0.05$) in the haematocrit in this group may imply anemia. This report also agrees with the findings observed on studies in the effect of electroplating effluent on hematological parameters of *Oreochromis mossambicus* [30,31].

Hemoglobin is a protein in the red blood cells and it is the most prevalent of the special blood pigments that transport oxygen from the lungs to the body cells, where it picks up carbon dioxide for transport back to the lungs to be expired. Distinct decrease in the level of hemoglobin observed suggests a haemodilution mechanism being operational. Haemodilution has been interpreted as a mechanism that reduces the concentration of an irritating factor in the circulatory system [30].

Red blood count determines the number of red blood cells in one cubic meter of blood. Red blood cells are the means by which oxygen is carried to the various parts of the body. Thus shortage in red blood cells would therefore lead to less oxygen supply by the red cells. Hypoxia is a state of acute oxygen deficiency. It produces a variety of reactions in the body which includes mild intoxication and stimulation of the nervous system, followed by progressive loss of attention and judgment until unconsciousness occurs. Respiration and pulse rate increase, and the systemic oxygen content is reduced. Prolonged lack of oxygen may cause damage to the brain [29].

This study investigation revealed that *B. cereus* has pronounced hepatic histopathology evidenced by histological alternations in liver which include focal necrosis, dilation of central vein and sinusoids. These findings are in support with [32].

B. cereus hepatotoxicity could lead to vacuolization of the cells, polymorphism of the nuclei and a decrease in glycogen content of the hepatocytes as observed.

This study's results showed that the epithelium villi damages were severe in the intestine of rats treated with *B. cereus* than the other Bacillus species in comparison to the control group of mice. This could be due to the fact that villi are the sites of absorption and connected to the active transport leading to higher concentration of *B. cereus* toxins in the epithelial lining of the villi. The intestinal vacuolization and necrosis could be due to the released toxins which the rats immune system could not overcome thus the observed elaborate vacuolization of epithelium of the villi.

5. CONCLUSION

There were no significant toxicological changes in the groups treated with *B. subtilis* and *B. thuringiensis* when compared with the control group. Based on these observations, it could be concluded that no adverse effect was recorded against these organisms. Hence *B. subtilis* has been described as an environmental friendly organism, thereby documenting its bio safety for non-target terrestrial animals. Hence *B. subtilis* did not change the normal physiological characteristics and blood parameters of the animals, it could be considered as a safe bio control agent.

CONSENT

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Walton WE, Mulla, MS. Impact and fates of microbial pestcontrol agents in the aquatic environment. In: Rosenfield, A., Mann, R. (Eds). Dispersal of living organisms into aquatic ecosystems. Maryland Sea Grant College, University of Maryland, College Park, MD. 1992;205-237.
2. Ignoffo C M, Garcia C, Kroha MJ, Fukuda T, Couch TL. Laboratory tests to elevate the potential efficacy of *Bacillus thuringiensis* serovar israelensis for use against mosquitoes. Mosquito News. 1981;41:85-93.
3. De Barjac H. Characterization and prospective view of *Bacillus thuringiensis* serovar israelensis. In: Bacterial control of mosquito and Blackflies: Biochemistry genetics and application of *Bacillus thuringiensis* israelensis and *Bacillus sphaericus*. De Barjac. H and Sutherland, D. J. (eds). Rutgers University press, New Brunswick, New Jessey. 1990;10-15.
4. Regis L, Da Silva SB, Alice M, Santos V. The use of bacterial larvicides in mosquito and black fly control programme in Brazil, Mem. Inst Oswaldo Cruz Rio de Janeiro. 2000;95:207-210.
5. Guidelli-Thuler AM, De Abreu IL, Lemos MVF. Expression of the sigma35 and *cry2ab* genes involved in *Bacillus thuringiensis* virulence. Sci Agric (Piracicaba, Braz) 2009;66(3):403–409.
6. Pérez-García G, Basurto-Ríos R, Ibarra JE. Potential effect of a putative σH-driven promoter on the over expression of the Cry1Ac toxin of *Bacillus thuringiensis*. J Invertebr Pathol. 2010;104(2):140–146
7. Singh G, Sachdev B, Sharma N, Seth R, Bhatnagar RK. Interaction of *Bacillus thuringiensis* vegetative insecticidal protein with ribosomal S2 protein triggers larvicidal activity in *Spodoptera frugiperda*. Appl Environ Microbiol.2010;76(21):7202–7209.
8. Abdelkefi-Mesrati L, Boukedi H, Dammak-Karray M, Sellami-Boudawara T, Jaoua S, Tounsi S. Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of Spodoptera littoralis. J Invertebr Pathol. 2010;106(2):250–254.
9. Margalith Y, Ben-Dov E. Biological control by *Bacillus thuringiensis* subsp Israelensis. In: Insect pest management: techniques for environmental protection. Rechcigl JE, Rechcigl NA, (eds). Lewis Publishers, Boca Raton, Florida. 2000;243-301.
10. Myasnik M, Manasherob R, Ben-Dov E, Zaritsky A, Margalith Y, Barak Z. Comparative sensitivity to UV-B radiation of two *Bacillus thuringiensis subspecies* and other Bacillus sp. Current Microbiology. 2001;43:140-143.
11. Kilama WL. Ethical perspective on malaria research for Africa, Acta Tropica. 2005;95:276-284.
12. Waako PJ, Gumede B, Smith P, Folb PI. The in vitro and *In vivo* antimalarial activity of *Cardiospermum halicacabum* L. and *Momordica foetida* Schumch. Et Thonn Journ Ethnopharmacology. 2005;99:137-143.
13. Weinzierl R, Henn T, Koehler PG, Tucher CL. Microbial insecticides. Entomology Nematology Series, ENY-275 (IN081), Florida Cooperative Extention Services, Institute of Food and Agricultural Sciences, University of Florida. 2005. Available: http://edis.ifas.ufl.edu.
14. Peng D, Shouwen C, Lifang R, Lin L, Ziniu Y. Safety assessment of transgenic *Bacillus thuringiensis* with VIP. Insecticidal protein gene by feeding studies. Food and Chemical Toxicology. 2007;45:1179-1185.
15. Hawk PK. Urine qualitative analysis. In Hawk's physiological chemistry, edited by Oser, BL. 14th edition. Tata McGraw Hill Books Company Ltd, New Delhi, India. 1965;1032-1034.

16. Varly H, Gowenlock AH, Bell M, Prophyrins R. Haemoglobin and related compounds. In: Gowenlock AH, (editor). Practical Clinical Biochemistry, 5th edition. Williams Heinemann Medical Books Ltd., London. 1980;451.
17. Brunt EM. Grading and staging the histopathological lesions of chronic hepatitis: The Knodell histology activity index and beyond. Hepatology. 2000;31(1):241-6.
18. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW. Non-alcoholic steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for non-alcoholic fatty liver disease. Hepatology. 2005;41(6):1313-21.
19. Yuang Z, Hansen BM, Andrup L, Eilenberg J. Detection of enterotoxin gene in Mosquito-larvicidal bacillus species. Current Microbiology. 2002;45:221-225.
20. Aronson I, Beckman W, Dunn P, *Bacillus thuringiensis* and related insect pathogens. Microbiology Review. 1986;50:1-24.
21. Geetha I, Prabakaran G, Paily KP, Manonmani AM, Balaraman K. Characterisation of three mosquitocidal Bacillus strains isolated from generation of protein database search programs. Nucleic Acids Research. 2007;32:45-52.
22. Geetha I, Manonmani AM. Mosquito pupicidal toxin production by Bacillus subtilis subsp. Subtilis. Biological Control. 2008;44:242-247.
23. Tabashnik BE, Liu YB, Malvar T, Heckel DH, Masson L, Ferric J. Insect resistance to B. thuringiensis: uniform or diverse? Philosophical Transactions of the Royal Society B: Biological Sciences. 1998;353:1751-1756.
24. Candas M, Loseva O, Oppert B, Kosaraju P, Bulla LA Jr. Insect resistances to B. thuringiensis alterations in the Indian meal moth larval gut proteome. Molecular and Cellular Proteomics. 2003;2:19-28.
25. Casida JE, Quistad, GB. Golden age of insecticide research: past, present, or future? Annu Rev Entomol. 1998;43:1-16.
26. Rey D, Jean-Philip D, Jean-Claude M. Factors influencing the toxicity of xanobiotics against larval mosquitoes. Comptes Rendus Biologies. 2003;326:317-327.
27. Agrawal NK, Mahajan CI. Heamatological changes due to vitamin C deficiency Channa punctaus block. Journ Nutri. 1980;110:2172-2182.
28. Baron DN, Whicher JT, Lee KE. A new short textbook of chemistry pathology, ELBS, 5th edition. Cambridge University Press, Cambridge, England. 1994;155-156.
29. Encyclopaedia Wikipedia. Wikipedia, the free encyclopaedia, 11th ed. Encyclopaedia Britannica Inc., USA; 2001.
30. Navaraj PS, Kumaraguru, AK. Effects of electroplating effluent on haematological parameters of *Oreochromis mossambicus*. Jour Physiol. 2003;107:935-939.
31. Smith GL, Hatting J, Burger AP. Haematological assessment of the effects of the anaesthetic MS222 in natural and neutralized form in three fresh water fish species: interspecies differences. Jour Fish Biol. 1979;!5:633-643.
32. Shalan MG, Mostafa MS, Hassouna MM, Hassab SE, El-Nabi a Elrafaie. Amelioration of lead toxicity on rat liver with vitamin C and silymarin supplements. Toxicology. 2005;206:1–15.

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