

Research Article

Comprehensive biosafety assessment of plant growth-promoting endophyte *Burkholderia contaminans* NZ

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ABSTRACT

Burkholderia contaminans NZ is a potent candidate for sustainable agriculture because of its ability to promote plant growth and exhibit antifungal activity. Being a member of the *Burkholderia cepacia* complex (BCC), however, this bacterium needs thorough biosafety assessment before its application in the environment. In the present study, a comprehensive panel of assays was employed as an integrated approach to evaluate its potential risks to human health and the environment. The dual microbial culture of NZ with *Escherichia coli* DH5α and *Bacillus subtilis* 168 showed no antagonism and a cross-streak assay resulted no inhibitory activity against different jute endophytes e.g., *Staphylococcus arlettae*, *Staphylococcus pasteurii* and *Micrococcus* sp. Exposure of soil nematodes and earthworms to NZ had no significant effect on their survival. Pathogenicity assessments in mice also showed no significant adverse effects on phenotypic behavior or histological characteristics. Overall, our findings demonstrate that *B. contaminans* NZ does not exhibit adverse effects on the tested non-target model organisms, supporting its potential safety as a bioinoculant for sustainable agricultural practices.

Introduction

Food security for a growing population is now a global concern, and this has given rise to the need to explore alternative ways to enhance crop production. From 1961 to 2020, the world meet rising food demand through an extraordinary technological feat known as conventional agriculture (Abdo et al., 2025; Sumberg and Giller, 2022). Conventional agriculture typically includes the use of synthetic fertilizers, pesticides, and other modern inputs, which have hazardous effects on people, animal and the environment (Kumar et al., 2012; Mahmood et al., 2016). However, scientific studies have shifted from chemical inputs to

sustainable agriculture, using agriculturally important microbes (AIMs) as an eco-friendly alternative to synthetic counterparts. These beneficial microbes have been used since the 1970s (Rovira et al., 1974; Bisen et al., 2015; Prakash et al., 2015; Manzanera et al., 2015) for their potential plant growth-promoting effects in various ways. These microorganisms are termed as plant growth-promoting bacteria (PGPB), widely recognized for their ability to enhance crop productivity through secretion of growth-promoting hormones, volatile organic compounds, secondary metabolites, exhibit immense antipathogenic

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activities against a broad range of pathogens, they can overcome the detrimental effects of various biotic and abiotic stresses, making them key components for sustainable agriculture. These PGPBs live in close association with plant roots, shoots, leaves, fruits, or seeds, either extracellularly or intracellularly (Laca et al., 2006; Figueiredo et al., 2008). PGPBs that live within internal plant tissues and spend at least part of their lifecycle without affecting their hosts (Hardoin et al., 2015), have been intensely studied for years for their diversity, plant growth promotion, biocontrol, bioremediation, etc. (Kaul et al., 2016). To promote agronomic sustainability, researchers have conducted extensive global research and found that biofertilizers are the most promising tool for sustainably increasing agricultural productivity (Kumar et al., 2022). Biofertilizers are those substrates containing living microbes that can be used for plant growth promotion, nutrient acquisition, and disease control in an eco-friendly manner (Aamir et al., 2020). These bioproducts are essential for provide an appropriate environment for bioinoculants and protecting them for an extended period.

As global awareness of sustainable farming and environmental protection grows, research on beneficial microbes has expanded significantly, enhancing the use of PGPBs as biofertilizers. This growing interest has led to extensive screening of eco-friendly bacteria that can act as potential biofertilizers. These efforts have resulted in the successful isolation of promising bacterial strains for use as biofertilizers. These microbial-based bioformulations, while supporting sustainable agricultural techniques, may pose unintentional risk to human health if they contain opportunistic pathogens. Recently, it has been recognized that some of these microorganisms act as opportunistic pathogens and belong to the genera *Stenotrophomonas*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Burkholderia*, and the *Bacillus cereus* group (Keswani et al., 2019). Despite their risk assessment, these bacteria are still used in the laboratory because of their growth-promoting potential.

Jute (*Corchorusolitorius* var. O-4) is an important natural fiber-producing cash crop in Southeast Asia (Babu et al., 2007), and *Macrophomina phaseolina* is a necrotrophic fungal pathogen that causes charcoal rot disease in it. While screening for jute endophytes for potential inhibitory effects against *M. phaseolina*, the Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka isolated the endophytic bacterium *Burkholderia contaminans* NZ from jute seed (Zaman et al., 2020). Zaman and co-authors (Zaman et al., 2021) stated that this bacterium has siderophore production, ACC deaminase activity, Indole Acetic Acid (IAA) production, and nitrogen fixation, making it a suitable candidate for a bioinoculant. However, *Burkholderia contaminans* NZ is a member of *Burkholderia cepacia* complex (BCC) (Degrossi et al., 2019), which include several closely related *Burkholderia* spp. Because of the difficulty of distinguishing beneficial from pathogenic strains, the release of *Burkholderia* spp. as a bioinoculant remains debated (Ellis and Cooper, 2010). Therefore, establishing standardized biosafety assessments for the PGPB strains is a critical step before field applications. In response to these efforts, this study aimed to determine the safety of *Burkholderia contaminans* NZ to protect personnel, consumers, and natural resources from potential hazards.

Materials and Methods

Biosafety assessment of *Burkholderia contaminans* NZ

To ensure the safety to humans, animals, and the environment, NZ underwent a biosafety test following the “One Health” approach. This step includes proper screening tests, e.g., toxicity and pathogenicity testing.

Microbial antagonism assay

To investigate NZ’s impact on the soil microbiome, we conducted an *in vitro* antagonistic assay using *Escherichia coli* DH5α and *Bacillus subtilis* 168 as non-pathogenic indicator strains.

We assessed NZ’s impact on *Escherichia coli* DH5α by co-culturing them in both Tryptic Soy Broth (TSB) and

Luria-Bertani (LB) broth, as *E. coli* grows well in LB and NZ grows well in TSB. The single cultures (NZ in TSB, *E. coli* in LB) and co-cultures (NZ + *E. coli* in TSB and NZ + *E. coli* in LB) were incubated at 37 °C with continuous shaking at 180 rpm. Samples were collected at 0, 3, 6, 9, 12, and 24 hours after inoculation and plated to estimate CFU/mL. Viable counts of NZ and *E. coli* from single and co-cultures were determined by serial dilution and spread-plating on Tryptic Soy Agar (TSA), Luria-Bertani Agar (LBA), and MacConkey agar, then incubation at 37 °C for 24 hours. Here, MacConkey agar was a selective medium for *E. coli* DH5 α .

To assess the impact of NZ on *Bacillus subtilis* 168, we used both single cultures (NZ, *B. subtilis*) and co-cultures (NZ + *B. subtilis*) which were incubated at 37 °C with continuous shaking at 180 rpm. Samples were collected as before at 0, 3, 6, 9, 12, and 24 hours after inoculation and plated to estimate CFU/mL. We determined the viable counts of NZ and *B. subtilis* 168 from single and co-cultures using the same procedure: serial dilution and the spread plate technique on Tryptic Soy Agar (TSA) medium, followed by incubation at 37 °C for 24 hours. Colonies from the co-cultures can be easily distinguished by their colony morphology. The antagonistic activity of NZ against selected jute endophytes was evaluated using a cross-streak assay. Briefly, NZ was streaked as a single line across the TSA plate and the test endophytes including *Staphylococcus arlettae*, *Staphylococcus pasteuri* and *Micrococcus* sp. Were streaked perpendicular to the NZ streak. The plate is incubated at 37 °C for 24 hours.

Survival and viability assay of soil nematodes (*Caenorhabditis* spp.) and earthworms (*Eisenia* spp.)

The easiest way to isolate nematodes from a sample is simply to place the sample around the bacterial lawn of a standard nematode (*Caenorhabditis* spp.) culture dish (Barrière and Félix, 2014). Soil nematodes were isolated using the nematode growth medium (NGM) (15g of Agar, 2.4g of NaCl, 2g of Tryptone, 2.72g of KH₂PO₄, 800 μ L of 1M CaCl₂, 1mL of 1M MgSO₄, 1mL of 5mg/mL Cholesterol in ethanol, 1mL of Ampicillin₁₀₀, 1mL of Nystatin₁₀, 1L dH₂O) with the pH 6 and harvested according to methods described by

Chaudhuri et al., (2010). Briefly, seed the NGM plate with 100 μ L of *E. coli* DH5 α , and allow the lawn to grow overnight at 37 °C. 1-2 grams of soil were spread around the bacterial lawn and moistened with 1mL of autoclaved M9 solution (3g of KH₂PO₄, 6g of Na₂HPO₄, 5g of NaCl, 1 mL of 1M MgSO₄, and 1L of H₂O) and incubated at room temperature overnight. Nematodes were crawled onto the *E. coli* DH5 α lawn, picked with a sterilized platinum wire, and transferred to two different NGM plates: one with *E. coli* DH5 α feed (control) and the other with NZ as feed (treatment). We transferred 5 nematodes to each plate, incubated them at room temperature, and observed and counted them at 10X magnification every 24 h for 7 days.

The earthworm (*Eisenia foetida*) survivability test was carried out with sixteen earthworms collected from the soil of Botanical Garden, Curzon Hall, University of Dhaka. Four containers were filled with 250 g of autoclaved soil each, and three contained three different concentrations of NZ (5 $\times$ 10⁸ CFU/mL, 10 $\times$ 10⁸ CFU/mL and 15 $\times$ 10⁸ CFU/mL) mixed with soil. Each container contained 4 earthworms, kept at 25 °C for 7 days in the dark with a regular supply of sterile distilled water to preserve soil moisture. We recorded individual length and weight daily. We repeated the experiment twice to ensure reproducibility.

Toxicity and virulence test in a mouse model

We tested the pathogenicity of NZ in Swiss albino mice (average weight 30–40 g), which were acclimated for two weeks before conducting the experiment. We housed the animals in rectangular polyacrylic cages and maintained them under constant laboratory conditions (25 °C) with a 12 h light/dark cycle. All mice were allowed free access to standard pellet diet and clean tap water ad libitum, and the food and water were changed daily. For the toxicity study, we handled the experimental animals in compliance with general methodology guidelines. We treated the mice with three concentrations of NZ suspensions (1 $\times$ 10⁷ CFU/mL, 2 $\times$ 10⁷ CFU/mL and 4 $\times$ 10⁷ CFU/mL) (Shah et al., 2023) via oral gavage once daily for 7 days. We recorded body weights and deaths daily. At the end of the experiment, on day 28th the mice in each group were anesthetized. Blood samples were collected through a cardiac puncture and placed in sample tubes

containing an anticoagulant, ethylene diamine tetra-acetic acid (EDTA), and used for determination of hematological parameters, e.g., red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), red cell distribution width (RDW), platelet count (PLT), white blood cell count (WBC), mean corpuscular volume (MCV), Mean corpuscular hemoglobin concentration (MCHC), and differential counts of each of the leucocytes were measured in an automatic analyzer. Similarly, serum alanine aminotransferase (ALT) was determined by standard techniques. For histopathological studies under light microscopy, tissue samples taken from the liver, lung, and kidney were immersion-fixed separately in Bouin's fluid at room temperature. According to Isaac et al. (2023), the tissues were processed for microscopic analysis and examined under a compound light microscope to evaluate histopathological changes relative to the controls. The study protocols and procedures described in this manuscript were approved by the Ethical Committee of the Faculty of Biological Sciences, University of Dhaka (ref. No. 315/Biol. Scs./2024-2025).

Statistical analysis

We collected, tabulated and statistically processed numerical hematology data from mice as mean±standard error of the mean (SEM) using SPSS 25.0. We applied one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. Differences were considered significant at $p < 0.05$.

Results

Effect of NZ on soil bacteria

The potential effect of NZ on microbial communities was assessed through microbial viability (sensitivity assay) analysis. The effect of NZ on *E. coli* DH5 α and *B. subtilis* 168 were studied in co-culture in comparison to single cultures in both TSB and LB broth. No significant changes in the growth of *E. coli* DH5 α and *B. subtilis* 168 were found from the CFU count in co-culture with NZ (Fig. 1A & 1B). Co-culture assay of NZ with jute endophytic bacteria *Staphylococcus pasteuri*, *Staphylococcus arlettae* and *Micrococcus* sp. on TSA plates also showed no visible growth inhibition at the intersection of streaks (Fig. 1C).

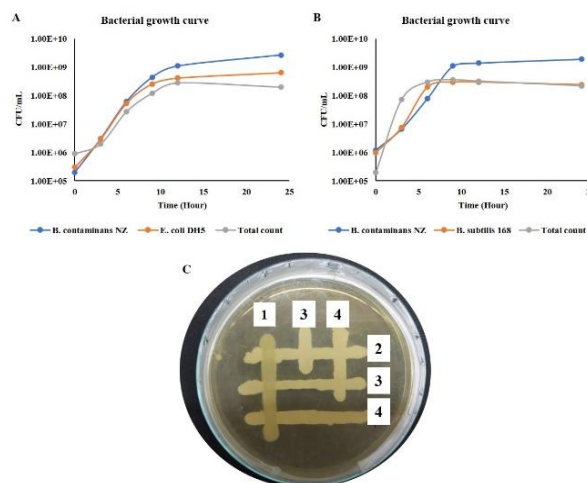


Fig. 1. Effect of NZ on *E. coli* DH5 α and *B. subtilis* 168 (A, B), cellular viability was assessed as CFU/mL at 0, 3, 6, 9, 12 and 24 hours, obtained through colony counts from both single and co-culture. (C) Cross-streak assay to evaluate the antagonistic activity of *Burkholderia contaminans* NZ (1) to other jute endophytes *Staphylococcus pasteuri* (2), *Staphylococcus arlettae* (3) and *Micrococcus* sp. (4). No inhibitory zones were observed at the intersection points indicating absence of antagonistic effect of NZ.

Survivability bioassay based on *Caenorhabditis elegans*

The survivability bioassay on soil nematodes was designed to evaluate the effect of NZ on nematodes numbers compared with nematodes on *E. coli* DH5 α , a well-established non-pathogenic strain. The number of transferred nematodes was similar in both *E. coli* DH5 α and NZ lawns. After 7 days of observation under a light microscope at 10X magnification, all nematodes were found alive in both bacterial lawns.

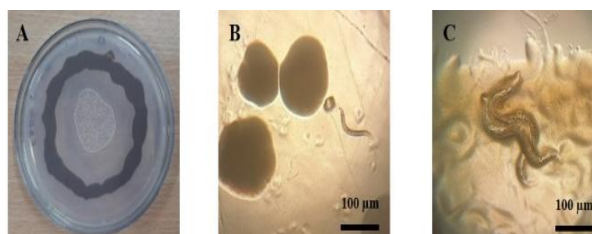


Fig. 2. Isolation of soil nematodes using Nematode Growth Medium (NGM) (A). Microscopic images of nematodes in the NGM on *E. coli* DH5 α lawn (B) and NZ lawn (C).

Survivability bioassay on *Eisenia foetida*

To evaluate the potential effect of NZ on earthworms, they were kept in NZ-treated soil at three different concentrations, and the experiment was performed twice. After 7 days of observation, all earthworms in each treatment were found alive, as was the non-treated control group. These results showed that NZ expose did not affect earthworms, even at concentrations higher than those normally used for plant inoculation.

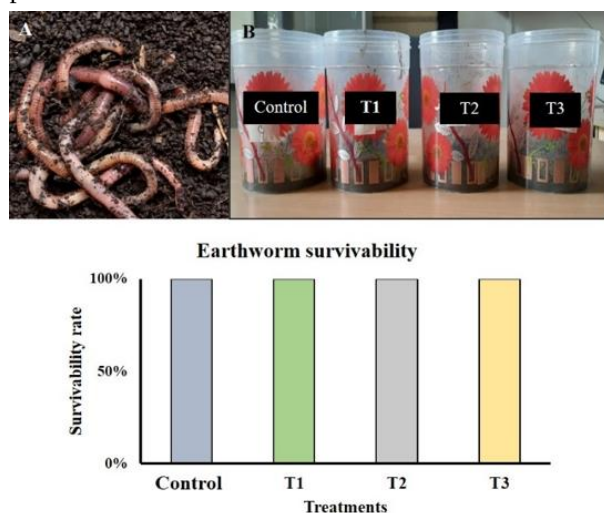


Fig. 3. Earthworm survivability assay. Earthworms collected from garden soil (A); Exposure of earthworms to one control pot (only in autoclaved soil) and the other three pots of autoclaved soil mixed with different concentrations of *B. contaminans* NZ (T1 = 5×10^8 CFU/mL, T2 = 10×10^8 CFU/mL, and T3 = 15×10^8 CFU/mL) (B) and monitored for seven days; Survivability rate of earthworms in different levels of NZ inoculum (C).

Pathogenicity test in laboratory mice

To ensure NZ safety, a toxicity test was conducted using Swiss albino mice. Mice were administered three different concentrations of NZ suspension (1×10^7 , 2×10^7 and 4×10^7 CFU/mL) through oral gavage for 7 days, and mortality and body weight changes were monitored for 28 days.

No mice showed any lethal effect of NZ. In addition, no significant changes in body weights were observed; in the control group, the mean body weight was 33.92 ± 3.73 , and in the treated groups it was 34.68 ± 3.43 , 34.03 ± 3.12 and 34.1 ± 2.7 , respectively. In the

complete blood count, the RBC value in the control group was 6.4 ± 1.5 , whereas the highest NZ-treated group showed 6.2 ± 2.1 . Furthermore, in the differential WBC count, the control mice had neutrophils 45.6 ± 27.9 , eosinophils 1.4 ± 1.02 , lymphocytes 49.2 ± 25.4 , and monocytes 3.8 ± 2.9 , whereas the highest-treated group had 26.8 ± 2.8 , 3 ± 2.3 , 54.8 ± 10.4 and 15.4 ± 10.8 respectively (Table 2). No significant tissue damage was also observed in the histopathological analysis of liver, kidney and lung tissues of the treated groups compared with the control group.

Table 1. Effect of NZ treatment on mice.

Groups	Number of mice used	Number of mice died in 28 days	% of mortality
Control	5	0	0
Treatment 1	5	0	0
Treatment 2	5	0	0
Treatment 3	5	0	0

Discussion

Bacteria that will be released into the environment as a biofertilizer or biopesticide should be safe for

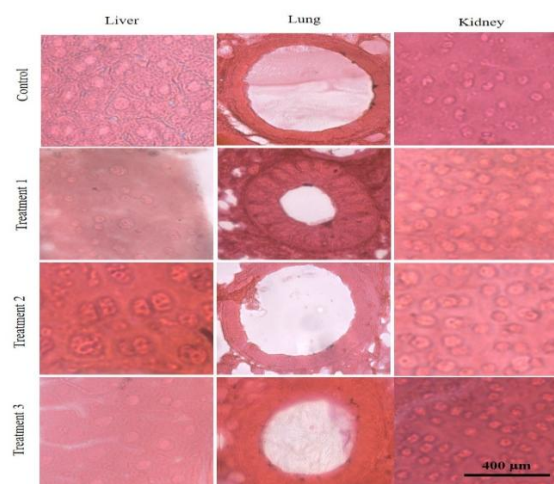


Fig. 4. Microscopic images of Eosin and Hematoxylin stained liver, lung and kidney sections of mice directly fed low, medium and high doses of NZ suspension 1×10^7 CFU/mL (Treatment 1), 2×10^7 CFU/mL (Treatment 2) and 4×10^7 CFU/mL (Treatment 3) through oral gavage for 7 days. Images showed no signs of abnormalities in the organs of mice treated with NZ.

Table 2. Effect of oral administration of NZ on some hematological parameters in mice.

Parameters	Control	Treatment 1	Treatment 2	Treatment 3
Hemoglobin (Hb%) (gm/dl)	11.1 ± 2.94	10.52 ± 5.3	11.24 ± 2.6	10.32 ± 3.7
Lymphocyte (%)	49.2 ± 25.44	56.6 ± 29.39	61.8 ± 12.24	54.8 ± 10.36
Monocyte (%)	3.8 ± 3	4.8 ± 4	15.6 ± 13	15.4 ± 10.82
Total WBC count (×10 ³ /μL)	2022 ± 414.2	1806 ± 1057.3	2974 ± 1649.4	2406 ± 1157.9
RBC count (m/ul)	6.4 ± 1.5	6.01 ± 2.96	6.54 ± 1.4	6.2 ± 2.1
HCT/PCV (%)	31.32 ± 7.5	29.8 ± 14.81	31.36 ± 6.8	30.24 ± 10.43
MCV (fL)	49.12 ± 1.15	39.68 ± 19.9	47.98 ± 0.76	48.92 ± 0.77
MCH (pg)	17.22 ± 0.54	14.04 ± 7.02	17.12 ± 0.43	16.56 ± 0.69
MCHC (g/dL)	35.1 ± 1.81	28.34 ± 14.22	35.68 ± 0.89	33.86 ± 1.00
RDW-CV (%)	17 ± 0.65	13.56 ± 6.79	16.36 ± 0.57	16.14 ± 0.53
PLT (×10 ³ /μL)	339.6 ± 90.95	187 ± 930.61	208.4 ± 767.08	193 ± 901.48
MPV (fL)	8.64 ± 2.29	9 ± 4.5	10.76 ± 1.00	10.3 ± 1.08
PDW (%)	8.12 ± 3.32	9.56 ± 4.8	12.26 ± 4.7	12.62 ± 5.3
(PCT (%))	0.29 ± 0.08	0.21 ± 0.1	0.23 ± 0.09	0.2 ± 0.1
S. ALT (U/dL)	112.79 ± 45.9	110.11 ± 40.16	194.65 ± 80.98	207.94 ± 63.37

humans, animals and the environment. The biosafety of NZ is a critical factor in its potential application as a bioformulation for plant growth promotion and biocontrol. Within the One Health approach, we performed a panel of tests to determine NZ safety by combining tests on microorganisms with pathogenicity assays in laboratory mice, aimed at reducing the need for vertebrate testing in experimental research. We studied the effect of NZ on *E. coli* DH5α, *B. subtilis* 168, *S. arlettae*, *S. pasteurii* and *Micrococcus* sp. and found no significant reduction or inhibitory zone when bacteria were exposed in co-culture and cross-streaked with NZ. This suggests that NZ does not inhibit common environmental and laboratory-associated bacteria or common jute endophytes, which is crucial for its potential agricultural application. Soil nematodes (*Caenorhabditis elegans*) serve as a reliable model for studying disease transmission and development in higher organisms, while the effects of chemicals

and metals on earthworms (*Eisenia foetida*) have been well documented. However, the impact of microorganisms on these organisms remains less explored (Vílchez et al., 2016). To address this, we examined the effects of NZ on *Caenorhabditis* sp. by assessing survivability and on *Eisenia* sp. by assessing weight and body length. Exposure of nematodes and earthworms to NZ at concentrations higher than those typically used for plant inoculation showed no adverse effect on their survival, indicating NZ does not pose a toxicity threat to these organisms. The laboratory mouse (Swiss albino) is a reference animal in experimental models for human safety and as a model for small mammals and other vertebrate organisms in ecosystems where agricultural products or biofertilizers may be used (Vílchez et al., 2016). We fed the mice different concentrations of NZ (1×10⁷, 2×10⁷ and 4×10⁷ CFU/mL) to verify NZ's safety for humans, animals, and the environment. The effect of 7 days of oral treatment with different doses of NZ on

hematological parameters in mice is shown in Table 2, and the results demonstrated that NZ did not induce any lethal effects in mice, even at the highest administered dose (4×10^7 CFU/mL). Additionally, no significant changes were observed in body weight between treated and control groups. In the present study, oral administration did not alter red blood cell indices (MCV, MCH and MCHC), total RBC count, HCT, and hemoglobin, indicating that it does not cause anemia. Besides, lymphocytes and monocytes showed greater increase in their percentage. In the control group, the lymphocyte percentage was 49.2%, whereas in treatment 2, it was highest (61.8%). Treatment 1 and treatment 3 groups also remained elevated compared with the control group indicating the activation of immune response. Several studies reported that *Lactobacillus acidophilus* administration increased lymphocyte numbers in mice and chickens (Markowiak and Śliżewska, 2017; Maragkoudakis et al., 2006; Gackowska et al., 2006; Sunada et al., 2008), suggesting it is a good probiotic bacterium for humans. In contrast, monocyte percentage increased gradually from the control group, indicating a dose-dependent elevation across the treatment groups. Meanwhile, recent studies suggest that an increased monocyte count is linked to some cardiovascular diseases (Gad and Zaghloul, 2013; Jeng and Swanson, 2006), though there were no statistically significant differences between the treatment groups and the control group, with p-values between each group and control were 0.998, 0.271 and 0.284, respectively. WBC count indicates systemic or physiological stress, suggesting the immune system is combating an infectious challenge. In this study, we found no significant alterations in WBC count across the treatment groups compared with the control group ($p=0.993$, 0.657 , and 0.965). Another parameter is ALT (Alanine aminotransferase) level, which is associated with liver infections. When liver cells are damaged, ALT is released into the bloodstream, causing symptoms like jaundice, fatigue, and loss of appetite (Shah et al., 2023). In this study, ALT levels

increased gradually in the treatment groups, indicating dose-dependence elevation, but the increase did not differ significantly from that of the control group. Furthermore, all the hematological parameters analyzed here, the alterations in the values of the treatment groups were statistically insignificant, indicating non-toxic nature of NZ. Moreover, histopathological analysis of liver, lung and kidney tissues showed no significant damage in the treatment groups compared to the control group, even at the high concentration, further confirming the non-toxic nature of NZ. Likewise, Yankey et al.(2022) found no abnormalities in mice inoculated with PGPB strain. Furthermore, Kaur et al.(2025) also found no negative influence of PGPB on the histopathological analysis of different organ tissues of mice. These results suggest that NZ could be safely used in agricultural applications without posing any risk to vertebrate animals.

Conclusion

This safety evaluation of *Burkholderia contaminans* NZ showed no antagonistic effect on microbial growth in co-culture. Exposure of nematodes and earthworms to different concentrations of NZ resulted no adverse effects confirming its non-toxic nature towards soil invertebrates. Oral administration of different concentrations of NZ also showed no serious adverse effects on body weights, hematological and histopathological parameters in mice. Nevertheless, the absence of morbidity and any significant signs of toxicity, even at high doses, supports the overall safety of NZ. Collectively, these findings confirm the safe release of NZ in the agricultural environment as a safe bioinoculant.

Authors contribution

Mohammad Riazul Islam, Fahmida Anika Khan, and Md. Abdul Karim: Conceptualization; Mohammad Riazul Islam, Fahmida Anika Khan and Adittya Sabhayasachi Khan: Experiment design; Fahmida Anika Khan, Haseena Khan, Md. Abdul Karim, Adittya Sabhayasachi Khan and Mahmud Hossain: Experiment conduction; Fahmida Anika Khan, Haseena Khan, Md. Abdul Karim, Mohammad Riazul Islam, Adittya Sabhayasachi Khan and Mahmud

Hossain: Result interpretation; Fahmida Anika Khan and Md. Abdul Karim: Writing draft; Fahmida Anika Khan, Mahmud Hossain, Md. Abdul Karim, Mohammad Riazul Islam and Haseena Khan: Review and Editing. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest regarding the publication of this article.

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