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Research Article

Acute Toxicity Assessment of Cell-Free Supernatant Derived from *Lysinibacillus* sp. from Indonesian Peat Soil as a Potential Broad-Spectrum Antibiotic

¹Bayu Hari Mukti, ²Dede Mahdiyah, ³Dwi Sogi Sri Redjeki, ⁴Irfan Alias Kandek and ⁴Resha Surya Putri

¹Department of Industrial Engineering, Faculty of Sciences and Technology, Universitas Sari Mulia, Indonesia

²Department of Pharmacy, Faculty of Health, Universitas Sari Mulia, Indonesia

³Department of English Education, Faculty of English Education, Universitas Sari Mulia, Indonesia

⁴Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Sari Mulia, Indonesia

Abstract

Background and Objective: Antimicrobial resistance (AMR) drives an urgent need for new antibiotics. A *Lysinibacillus* sp. isolate from Indonesian peat soil has shown promising broad-spectrum antibacterial activity *in vitro*. This study aims to evaluate the acute toxicity profile of its cell-free supernatant (CFS) as an initial *in vivo* safety assessment. **Materials and Methods:** A single oral dose acute toxicity test was conducted on female mice (n = 3 per group). Treatment groups received CFS at volumes of 0.2 mL, 0.5 mL and 1.0 mL. Control groups received 0.5% Na carboxy methyl cellulose (CMC) and nutrient broth (NB). All animals were observed for 14 days for mortality, behavior and body weight changes, followed by necropsy on day 15. Statistical analysis was conducted using SPSS version 26, applying GLM repeated measures with Shapiro-Wilk normality, Mauchly's sphericity and Bonferroni *post-hoc* tests at a significance level of $p < 0.05$, with results expressed as Mean $\pm$ SD. **Results:** There was no mortality or significant behavioral changes. However, macroscopic necropsy revealed dose-dependent toxicity. At a dose of 0.2 mL, heart swelling and tracheal hemorrhage were found. A dose of 0.5 mL caused lung hemorrhage and a pale trachea. At the highest dose of 1.0 mL, all animals showed kidney swelling, lung hemorrhage and pale digestive tract. The Na CMC control group was normal, while the NB control showed mild abnormalities. **Conclusion:** The CFS of *Lysinibacillus* sp. did not show LD₅₀ toxicity or behavioral changes in mice. Some high doses showed organ-specific toxicity.

Key words: Acute toxicity, *Lysinibacillus*, peat soil, antibiotic, cell-free supernatant, mice

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Corresponding Author: Bayu Hari Mukti, Department of Industrial Engineering, Universitas Sari Mulia, Indonesia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The antimicrobial resistance (AMR) crisis has emerged as one of the most serious threats to global health in the 21st century. Between 2000 and 2023, there was a 65% global increase in resistant infections, with associated deaths reaching 4.95 million per year¹. The rising prevalence of bacteria resistant to various available classes of antibiotics has diminished the effectiveness of commonly used infection therapies. This has consequently led to increased morbidity and mortality rates, alongside a significant economic burden². Pathogenic bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* are prime examples of this challenge, causing difficult-to-treat infections that often require last-resort therapies which are more toxic and expensive³. The rate of discovery for new antibiotics has reportedly declined significantly in recent decades, while the dynamics of bacterial evolution continue to progress rapidly and relentlessly⁴. This situation confirms that the exploration of novel sources for innovative and effective antibacterial agents is no longer optional, but an urgent necessity in the global effort to combat antimicrobial resistance⁵.

One of the most promising strategies in this effort is the bioprospecting of microorganisms from extreme or unique environments⁶. Ecosystems such as peatlands, characterized by anaerobic conditions, acidic pH and high organic matter content, foster the abundance of a unique and diverse microbiota^{7,8}. Previous research successfully isolated a *Lysinibacillus* sp. bacterium from peat soil in South Kalimantan. The CFS of this isolate demonstrated highly impressive antibacterial activity *in vitro*, exhibiting a broad spectrum against both Gram-positive and Gram-negative bacteria. This activity included clinical MDR isolates such as MRSA and ESBL-producing *E. coli*⁹⁻¹¹. Notably, this activity even surpassed that of the control antibiotic chloramphenicol in inhibiting the growth of MRSA¹².

A critical step in any drug development pathway is *in vivo* safety evaluation. Acute toxicity, which measures the adverse effects following a single dose administration of a substance, is a fundamental safety assessment that provides preliminary information on a drug candidate's potential hazards¹³. Without a clear understanding of its toxicity profile, the therapeutic potential of a compound cannot be adequately evaluated. Therefore, this study was designed to conduct an initial acute toxicity evaluation of *Lysinibacillus* sp. The CFS in a mouse animal model. The main objective of this research is to assess the impact of a single oral dose of CFS on the survival, behavior and macroscopic integrity of vital organs in mice.

MATERIALS AND METHODS

Study area and duration: This study was conducted at the Microbiology and Biopharmacy Laboratory, Universitas Sari Mulia, Banjarmasin, Indonesia. The experimental facility is located at Jalan Pramuka No. 02, Banjarmasin 70238, Indonesia. All procedures, including CFS preparation, animal handling, oral administration, clinical observations and necropsy, were performed within the same institution. The total duration of the study was approximately 6 weeks (42 days). The study was carried out between August and September 2025.

CFS Preparation: The preparation used in this study was CFS obtained from a culture of *Lysinibacillus* sp. isolated from South Kalimantan peat soil. *Lysinibacillus* was subcultured in Tryptic Soy Broth (TSB; Sigma-Aldrich, Germany) at 37°C, pH 6, for 120 hrs and then centrifuged at 4000 rpm for 10 min. The supernatant was separated from the crude extract and subsequently filtered through a 0.22 µm membrane filter to ensure complete removal of bacterial cells and sterility of the cell-free supernatant. The filtrate was stored at -20°C as the test preparation.

Animals model and acclimatization: The animals model used were mice (*Mus musculus*) of the Swiss Webster strain, aged 8-10 weeks, with a body weight of 24-28 g. A total of 15 mice were used in this study (n = 3 per group for five treatment groups). The mice were obtained from a local commercial breeder, Banjarbaru, South Kalimantan, Indonesia. Before treatment, the mice were acclimatized for seven days in an experimental room under controlled environmental conditions, including a temperature of 22±2°C, relative humidity of 50-60% and a 12 hrs light-dark cycle (light from 06:00 to 18:00). During the acclimatization and study period, the mice were provided with standard feed and drinking water *ad libitum*. Mice cages were maintained clean with rice husk bedding changed regularly.

Experimental design and dosing¹⁴: The test animals were randomly divided into five treatment groups, each consisting of three mice (n = 3). Group P1 received 52 mg/kg of cell-free supernatant (CFS), P2 received 130 mg/kg of CFS and P3 received 260 mg/kg of CFS. Group P4 was given 130 mg/kg of a 0.5% Sodium Carboxymethyl Cellulose (Na-CMC) suspension as a negative control, while group P5 received 130 mg/kg of nutrient broth (NB) as a media control. The administration volume for each group was adjusted according to the body weight of the mice. Both the CFS and control solutions were administered orally.

Clinical observation and body weight measurement:

Following the single-dose administration, all mice were intensively observed for signs of toxicity and mortality. Observations were conducted at regular intervals: 30 min, 1, 2, 4 and 8 hrs post-treatment on day one and then once daily on days 3, 7 and 14. Parameters observed included behavioral changes (lethargy, hyperactivity, aggressiveness), motor activity, response to stimuli and physical signs such as changes in skin, fur, eyes and respiratory tract. The body weight of each mouse was measured on day 0 (before treatment [D0]), day 3 (D3), day 7 (D7) and day 14 (D14) to monitor for significant weight loss as an indicator of systemic toxicity.

Necropsy and macroscopic organ evaluation: At the end of the observation period (day 15), all mice were euthanized using the cervical dislocation method. Immediately following euthanasia, a necropsy was performed to macroscopically examine the condition of vital organs. The organs examined included the heart (*cor*), lungs (*pulmo*), kidneys (*rena*), liver (*hepar*), brain (*encephalon*), intestines and trachea. Observations focused on identifying abnormalities such as color changes (pallor, cyanosis, hemorrhage), swelling (edema), changes in size, erosion, or other lesions visible to the naked eye. Pathological findings were documented using written descriptions and photography for further analysis.

Ethical consideration: Ethical approval for the animal research was obtained from the Research Ethics Commission of Universitas Sari Mulia, Banjarmasin, Indonesia, with ethical approval number No. 090/KEP-UNISM/VII/2025.

Statistical analysis: Statistical analysis was performed using SPSS version 26 (IBM Corp., Armonk, NY, USA). Body weight data measured on days 0, 3, 7 and 14 were analyzed using general linear model (GLM) repeated measures to evaluate the effect of multiple treatments and observation periods on body weight changes. Normality of the data was assessed using the Shapiro-Wilk test (p-values ranged from 0.115 to 0.668, indicating normal distribution). Mauchly's Test of Sphericity was used to test the homogeneity of variances across observation periods ($p = 0.448$, indicating that the sphericity assumption was met). The Bonferroni *post-hoc* test was applied for pairwise comparisons between observation periods when significant differences were detected¹⁵⁻¹⁸. For all statistical analyses, a significance level of $p < 0.05$ was considered statistically significant. Data are presented as Mean $\pm$ standard deviation (SD).

RESULTS

Clinical observation and survival: Over the 14-day observation period, no cases of mortality were found in any of the mice groups, including the group that received the highest CFS dose (1.0 mL). All mice survived until the end of the study. Generally, the behavior of the mice across all groups did not show significant changes. No signs of severe stress, diarrhea, convulsions, or paralysis were observed. Eating and drinking activity remained normal. The average body weight did not show a significant difference between the administered dose treatments. Weight gain during the observation period showed a statistically significant increase. This indicates that, within the tested volume and dose range, the *Lysinibacillus* sp. The CFS did not cause mortality or severe acute clinical toxicity observable from the animals' general behaviour, shown in Table 1.

Macroscopic organ evaluation: The organ-wise pathological observations across experimental groups (P1-P5) indicate variable degrees of tissue alterations, with most organs remaining largely normal except for specific lesions observed in the kidney, heart, lungs and trachea shown in Table 2.

In the kidney, predominantly normal morphology was observed in P1, P2, P4 and P5 groups, with 2/3 or 3/3 samples showing no visible lesions. However, edema was recorded in P3 (3/3), along with minor occurrences in P1 and P2 (1/3 each), indicating localized fluid accumulation in certain treatments.

The heart exhibited edema as the primary alteration, particularly prominent in P1 (3/3), while P2, P3 and P4 mostly showed normal architecture (3/3 or 2/3). Mild edema was also observed in P4 (1/3) and P5 (2/3), suggesting inconsistent cardiac involvement across groups.

In the lungs, most samples appeared normal (3/3) in P1 and P4; however, hemorrhagic lesions were evident in P2 (2/3), P3 (3/3) and to a lesser extent in P5 (1/3). Additionally, pale pink discoloration with hemorrhage was noted in P2, indicating possible vascular compromise.

The trachea showed marked linear hemorrhage in P1 (3/3), whereas P2 and P3 exhibited pale appearance (2/3 and 3/3, respectively). Normal tracheal structure was maintained in P4 and P5 (3/3 each), suggesting recovery or absence of pathological insult in these groups.

The gastrointestinal tract remained largely unaffected, with normal observations (3/3) across most groups except for pallor in P3 (3/3), which may indicate reduced blood supply or mild pathological stress.

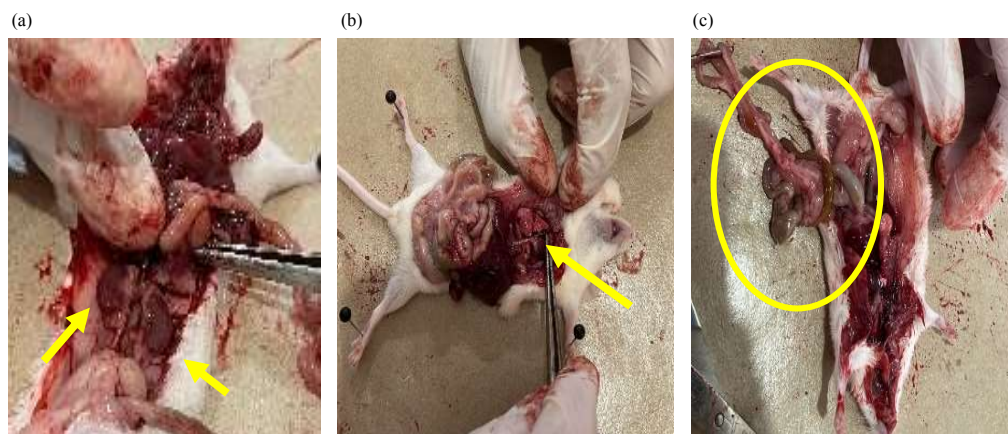


Fig. 1(a-c): Macroscopic abnormalities observed during organ examination (a) Swelling in the kidney organ, (b) Hemorrhage in the lungs and (c) Abnormal digestive tract characterized by pale mucosa

Table 1: Average change in mouse body weight

Treatment	Observation period (days)				Mean per dose	p-value*
	D0	D3	D7	D14		
P1	25.33±0.58	26.67±0.58	28.33±0.58	29.33±2.08	27.42	<0.01 [†]
P2	26.67±1.15	28±1	28.67±0.58	31±1	28.58	0.088 ^{ab}
P3	27.67±0.58	24.33±1.53	26.67±0.58	29±1.73	26.92	
P4	24.67±0.58	26.33±1.53	29.67±0.58	32±1.73	28.17	
P5	24.67±0.58	25±2	25.67±3.06	27.67±3.51	25.75	
Mean per period	25.8 ^a	26.067 ^a	27.8 ^b	29.8 ^c		

*GLM Repeated Measures, [†]observation period, ^{ab}CFS dose, ^{ab}Bonferroni test, means followed by different letters indicate a significant difference. D0, D3, D7 and D14 represent days 0, 3, 7 and 14 of observation, respectively P1-P5 indicate treatment groups

Table 2: Macroscopic pathology of mouse organs

Organ	P1	P2	P3	P4	P5
Kidney	Normal (2/3) Edema (1/3)	Normal (2/3) Edema (1/3)	Edema (3/3)	Normal (3/3)	Normal (3/3)
Hearth	Edema (3/3)	Normal (3/3)	Normal (2/3) Edema (1/3)	Normal (3/3)	Normal (1/3) Edema (2/3)
Lungs	Normal (3/3)	Pale pink with Hemorrhage (2/3)	Hemorrhage (3/3)	Normal (3/3)	Hemorrhage (1/3)
Trachea	Linear Hemorrhage (3/3)	Pale (2/3)	Pale (3/3)	Normal (3/3)	Normal (3/3)
Gastrointestinal	Normal (3/3)	Normal (3/3)	Pale (3/3)	Normal (3/3)	Normal (3/3)
Brain	Normal (3/3)	Normal (3/3)	Normal (3/3)	Normal (3/3)	Normal (3/3)
Liver	Normal (3/3)	Normal (3/3)	Normal (3/3)	Normal (3/3)	Normal (3/3)

Values in parentheses indicate the number of animals exhibiting the observed finding out of a total of three animals per group, P1: Received CFS at a dose of 0.2 mL, P2: Received CFS at a dose of 0.5 mL, P3: Received CFS at a dose of 1.0 mL, P4: Served as the vehicle control (Na-CMC) and P5: Served as the negative control (NB)

No observable pathological changes were recorded in the brain and liver, as all groups consistently showed normal morphology (3/3), indicating that these organs were not significantly impacted under the experimental conditions.

Overall, the findings suggest that pathological effects were organ-specific and varied among treatment groups, with the most notable alterations being edema in the kidney and heart and hemorrhagic changes in the lungs and trachea.

Macroscopic examination of organs in the P1 group showed that the kidneys were normal in two out of three mice, as indicated by uniform shape and coloration. However,

in the third replicate, an abnormal finding was observed in the form of swelling of the left kidney. The heart exhibited macroscopic abnormalities in all mice, characterized by tissue swelling, suggesting possible congestion or edema. Examination of the trachea revealed linear hemorrhage in all mice, indicating vascular damage or irritation of the respiratory tract. In contrast, the gastrointestinal tract (stomach and intestines), brain and liver appeared normal, with no significant morphological alterations. Representative abnormal findings in this group are shown in Fig. 1a.

One out of three mice in the P2 group, exhibited abnormal kidney findings, characterized by edema of the left kidney. The heart appeared normal in all mice. However, abnormalities were observed in the lungs of two out of three, presenting as pale pink discoloration accompanied by hemorrhage. Macroscopic observation of the trachea showed pale coloration in two out of three mice. The gastrointestinal tract, brain and liver remained macroscopically normal across all replicates. Representative abnormal findings for this group are presented in Fig. 1b.

Toxic effects were more consistent and pronounced shown in the P3 group. All mice exhibited kidney edema, indicating a uniform pathological response. In the heart, two out of three mice showed normal morphology, while one exhibited tissue swelling. Macroscopic examination of the lungs revealed hemorrhage in all. In addition, the mucosal surfaces and gastrointestinal tract appeared pale in all. Despite these findings, the brain and liver remained macroscopically normal. The abnormal findings observed in this group, are illustrated in Fig. 1c.

The vehicle control group administered Na-CMC showed normal macroscopic appearances of all vital organs (kidneys, heart, lungs, liver, brain and gastrointestinal tract) in all three mice. No signs of degeneration, necrosis, hemorrhage, or swelling were observed, indicating that Na-CMC did not induce pathological alterations. Similarly, in the P5 (negative control) group, the major organs were generally well preserved. The kidneys, mucosal surfaces, gastrointestinal tract, lungs and liver appeared normal. However, mild alterations were noted, including cardiac swelling in two out of three mice and pulmonary hemorrhage in one mouse. These findings suggest that NB may exert mild organ-specific toxicity, although the effects were less pronounced than those observed in the CFS-treated groups.

DISCUSSION

This study provides the first *in vivo* safety assessment of CFS derived from *Lysinibacillus* sp., an Indonesian peat soil isolate with previously reported broad-spectrum antibacterial activity¹⁵⁻¹⁷. Although no mortality or significant clinical behavioral changes were observed during the 14-day observation period, macroscopic necropsy revealed clear pathological effects in several vital organs. The presence of cardiac and renal swelling, along with hemorrhage in the trachea and lungs, indicates that the CFS contains bioactive compounds that, while effective against pathogenic bacteria, may also exert deleterious effects on mammalian tissues¹⁹. The

present findings are consistent with fundamental principles of pharmacology and toxicology, which recognize that most bioactive compounds can induce toxic effects at certain concentrations or doses. This concept is encapsulated in the principle of Paracelsus, which states that all substances are poisons and there is none that is not a poison; the dose determines whether a substance is a poison or not²⁰.

At the lowest dose (52 mg/kg), cardiac edema and linear tracheal hemorrhage emerged as the primary findings, suggesting that the cardiorespiratory system may represent an early and principal target of toxic compounds present in the CFS. Cardiac edema-manifesting as cardiomegaly or myocardial edema may arise from multiple mechanisms, including direct cellular injury, disruption of ionic homeostasis, or inflammatory responses triggered by exogenous compounds^{21,22}. At higher doses, the occurrence of hemorrhage in the trachea and lungs indicates compromised vascular integrity within the respiratory system²³⁻²⁵. The vulnerability of these organs may be attributed to their high degree of vascularization and direct exposure to systemically distributed compounds absorbed through the gastrointestinal tract and circulated via the bloodstream. Furthermore, the consistent increase in renal swelling observed at the highest dose (260 mg/kg) suggests that the kidneys, as the primary organs of xenobiotic excretion, are also major targets of toxicity. The kidneys play a critical role in filtering and eliminating foreign compounds; repeated exposure or high doses of nephrotoxic agents can induce renal tubular injury and fluid accumulation, leading to oxidative stress and mitochondrial dysfunction, which may manifest macroscopically as kidney swelling^{26,27}. Overall, this organ-specific toxicity pattern provides valuable insights into the potential mechanisms of action of toxic compounds present in the CFS and helps guide subsequent diagnostic investigations, including histopathological evaluation and mechanistic studies.

One important aspect that warrants careful consideration in this study is the findings observed in the control groups, particularly P5, which received NB. The P4 group, administered 0.5% Na-CMC, exhibited normal macroscopic appearances across all examined organs, confirming that the vehicle was safe and did not contribute to the toxic effects observed in the treated groups. In contrast, the P5 group showed cardiac swelling in two out of three mice and pulmonary hemorrhage in one mouse. These findings are noteworthy, as NB served as the basal growth medium for *Lysinibacillus* sp. during CFS production. Although the abnormalities observed in the NB control group were less severe than those detected

in the CFS-treated groups, their presence suggests that components of the medium itself, either in its original form or following fermentation, may possess mild toxic potential. Such effects may be attributed to the accumulation of metabolic by-products, alterations in pH, or elevated salt concentrations inherent to NB composition²⁸.

Comparison between the CFS-treated groups and the NB control group clearly demonstrated a significant increase in toxicity that can be specifically attributed to metabolites produced by *Lysinibacillus* sp. Both the incidence and severity of lesions increased in a dose-dependent manner with increasing volumes of CFS administration. At the lowest dose (52 mg/kg), cardiac swelling was observed in 100% of the animals, a finding that was not detected in the NB control group. At a dose of 130 mg/kg, pulmonary hemorrhage became more prominent, while at the highest dose (260 mg/kg), renal swelling occurred in 100% of the tested animals—an effect that was also absent in the NB control. These findings indicate the presence of additive or synergistic effects between NB components and the secondary metabolites produced by the bacterium, resulting in a more complex and severe toxicity profile. Antimicrobial compounds produced by *Bacillus* and closely related genera such as *Lysinibacillus* are frequently lipopeptides (e.g., surfactin, iturin and fengycin) or peptide-based compounds such as bacteriocins²⁹⁻³¹. Several of these molecules have been reported to exhibit cytotoxic or hemolytic activity toward mammalian cells, which may mechanistically explain the hemorrhagic lesions and organ damage observed in the present study³²⁻³⁴. Therefore, future studies should prioritize the purification and identification of bioactive compounds within the CFS to determine whether the metabolites responsible for antibacterial activity are also those driving toxicity, or whether additional contaminating or co-produced compounds contribute to the observed effects.

The implications of these findings are highly relevant for the further development of *Lysinibacillus* sp. The CFS as a potential antibiotic candidate. A logical and urgent next step is the application of bioassay-guided fractionation, involving stepwise separation and purification of CFS components, followed by systematic evaluation of antibacterial activity and toxicity for each fraction. This approach aims to isolate therapeutically desirable compounds while separating and eliminating toxic constituents. In parallel, more comprehensive toxicity assessments are required. The present study evaluated only acute oral toxicity; thus, subchronic and chronic toxicity studies are necessary to assess the effects of prolonged exposure. Additionally, alternative routes of administration, such as intravenous or intraperitoneal delivery, should be examined, as antibiotics for systemic infections are

commonly administered parenterally. Organ-specific toxicity evaluations, including liver function tests (AST and ALT), kidney function markers (creatinine and urea) and detailed histopathological analyses of affected organs, would provide deeper insight into the mechanisms and severity of tissue injury.

This study provides the first *in vivo* safety assessment of CFS derived from *Lysinibacillus* sp. isolated from Indonesian peat soil. Although the findings indicate signs of mild toxicity, they do not preclude the potential development of this isolate as a source of novel antibiotics. Rather, these results serve as a critical guide for future research directions. Efforts should shift from the use of crude CFS toward the isolation, characterization and chemical modification of specific antimicrobial compounds responsible for therapeutic activity. With a careful and systematic approach including purification to remove toxic components and chemical optimization to enhance bacterial selectivity the substantial potential of unique microbes from Indonesian peatland ecosystems can be effectively harnessed. Overall, this study underscores the importance of early and comprehensive toxicity evaluation in the drug discovery pipeline, ensuring that only the most promising and safest candidates advance to subsequent stages of development, thereby contributing to the global effort to combat antimicrobial resistance.

CONCLUSION

The cell-free supernatant of *Lysinibacillus* sp. from Indonesian peat soil did not cause mortality or clinically observable behavioral changes in mice at the tested doses, indicating the absence of acute lethal toxicity. However, dose-dependent macroscopic abnormalities in vital organs were detected, suggesting that the crude supernatant contains bioactive compounds with potential off-target effects on mammalian tissues. These findings raise a safety concern that must be addressed before further development. While the isolate remains a promising source of novel broad-spectrum antibiotics, future efforts should prioritize the purification and characterization of its active metabolites to separate therapeutic efficacy from toxicity.

SIGNIFICANCE STATEMENT

This study provides the first *in vivo* acute toxicity assessment of cell-free supernatant (CFS) derived from *Lysinibacillus* sp. isolated from Indonesian peat soil, a bacterium previously reported to possess broad-spectrum antibacterial activity against multidrug-resistant pathogens.

The findings demonstrate that although no mortality or severe behavioral toxicity was observed, dose-dependent organ-specific abnormalities occurred, highlighting important safety considerations for future therapeutic development. This research contributes valuable preliminary evidence for balancing antibacterial efficacy with toxicity risk and supports further purification and characterization of bioactive metabolites from peatland-derived microorganisms as potential novel antibiotic candidates.

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