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Corneal Inflammatory Responses Following Topical Exposure to a Commercial Bacillus thuringiensis-Based Bioinsecticide in Wistar Rats

Respons Inflamasi Kornea Setelah Paparan Topikal Bioinsektisida Berbasis Bacillus thuringiensis *Komersial pada Tikus Wistar*

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Abstrak

Bacillus thuringiensis (Bt) secara luas digunakan sebagai agen mikroba dalam formulasi bioinsektisida dan umumnya dianggap aman untuk organisme non-target. Namun, laporan klinis tentang ulserasi kornea setelah paparan okular terhadap produk berbasis Bt telah menimbulkan kekhawatiran mengenai potensi toksisitasnya terhadap mata. Penelitian ini bertujuan untuk mengevaluasi integritas epitel kornea, perubahan histopatologis, infiltrasi neutrofil, dan ekspresi penanda inflamasi dan stres oksidatif, termasuk *tumor necrosis factor-alpha* (TNF-α) dan *malondialdehyde*(MDA), pada tikus Wistar setelah paparan topikal bioinsektisida berbasis Bt komersial. Desain kelompok kontrol pasca-uji (*post-test-only control group design*) diterapkan menggunakan 24 tikus Wistar jantan yang dibagi menjadi satu kelompok kontrol dan tiga kelompok perlakuan yang menerima konsentrasi Bt 8 g/L, 10 g/L, dan 12 g/L. Defek epitel kornea dinilai menggunakan pewarnaan *fluorescein*, sementara pemeriksaan histologis dan pengukuran semi-kuantitatif neutrofil dilakukan pada mata yang dienukleasi. Kadar TNF-α diukur menggunakan ELISA, dan kadar MDA ditentukan melalui uji asam tiobarbiturat (TBA). Ditemukan adanya deskuamasi epitel, peningkatan ketebalan epitel, dan infiltrasi neutrofil yang lebih tinggi pada kelompok perlakuan dibandingkan dengan kontrol, meskipun parameter-parameter ini tidak mencapai signifikansi statistik. Khususnya, kadar TNF-α meningkat secara signifikan pada kelompok perlakuan 10 g/L ($p = 0,023$), menunjukkan aktivasi inflamasi lokal. Kadar MDA menunjukkan tren peningkatan yang tidak signifikan. Paparan topikal terhadap bioinsektisida berbasis Bt komersial mungkin berhubungan dengan respons inflamasi kornea, seperti yang ditunjukkan oleh peningkatan kadar TNF-α, bahkan tanpa adanya kerusakan struktural yang signifikan. Temuan-temuan ini menyoroti pentingnya meminimalkan paparan okular yang tidak disengaja selama penanganan produk bioinsektisida berbasis Bt.

Kata Kunci: *Bacillus thuringiensis*; TNF-α; MDA; toksisitas okular; bioinsektisida

Abstract

Bacillus thuringiensis (Bt) is widely used as a microbial agent in bioinsecticide formulations and is generally regarded as safe for non-target organisms. However, clinical reports of corneal ulceration following ocular exposure to Bt-based products have raised concerns regarding its potential toxicity to the eye. This study aimed to evaluate corneal epithelial integrity, histopathological changes, neutrophil infiltration, and the expression of inflammatory and oxidative stress markers, including tumor necrosis factor-alpha (TNF-α) and malondialdehyde (MDA), in Wistar rats following topical exposure to a commercial Bt-based bioinsecticide. A post-test-only control group design was applied using 24 male Wistar rats divided into one control and three treatment groups receiving Bt concentrations of 8 g/L, 10 g/L, and 12 g/L. Corneal epithelial defects were assessed using fluorescein staining, while histological

examination and semi-quantitative measurements of neutrophils were performed on enucleated eyes. TNF-α levels were measured using ELISA, and MDA levels were determined via the thiobarbituric acid (TBA) assay. There was epithelial desquamation, increased epithelial thickness, and higher neutrophil infiltration in the treatment groups compared to controls, although these parameters did not reach statistical significance. Notably, TNF-α levels increased significantly in the 10 g/L treatment group ($p = 0.023$), suggesting local inflammatory activation. MDA levels showed a non-significant upward trend. Topical exposure to a commercial Bt-based bioinsecticide may be associated with corneal inflammatory responses, as suggested by increased TNF-α levels, even in the absence of significant structural damage. These findings highlight the importance of minimizing accidental ocular exposure during the handling of Bt-based bioinsecticide products.

Keywords: *Bacillus thuringiensis*; TNF-α; MDA; ocular toxicity; bioinsecticide

Introduction

The use of bioinsecticides as a more environmentally friendly alternative to chemical pesticides has grown significantly over the past two decades. Among the various biological agents employed, *Bacillus thuringiensis* (Bt) is among the most widely used due to its ability to produce δ-endotoxins, which are highly effective at killing insect pests, particularly during their larval stages. In Indonesia, approximately 80% of commercially available bioinsecticides utilize Bt as the primary active ingredient, indicating a high level of adoption in the agricultural sector (Mazid *et al.,* 2011; Horizon Agro Analytic, 2023).

Although Bt is generally considered safe for humans and vertebrate animals, clinical reports of corneal ulcers following exposure to Bt-based bioinsecticides have raised new concerns regarding its potential ocular toxicity (Samples *et al.,* 1983; UNECEE, 2019). Bt formulations are known to have an acidic pH, ranging from 4.1 to 7, which poses a risk of chemical trauma to the ocular surface (Kompa *et al.,* 2005; Ilyas *et al.,* 2015). Chemical injuries, particularly those caused by acidic substances, can damage the corneal epithelium and trigger inflammatory responses, which, if left unmanaged, may lead to permanent visual impairment (Avetisov *et al.,* 2015; Dua *et al.,* 2020).

Corneal inflammation caused by exposure to irritants or toxins is typically characterized by increased expression of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) (Agbanoma *et al.,* 2012; Peterson *et al.,* 2019), and the infiltration of immune cells such as neutrophils(Pengal *et al.,* 2006; Margraf *et al.,* 2020). This inflammatory response is often accompanied by oxidative stress, which can damage cellular lipids through the formation of malondialdehyde (MDA), a well-established biomarker of oxidative stress (Maurya *et al.,* 2021; Riaz *et al.,* 2023). As epithelial cells are damaged, desquamation and epithelial thickening occur as part of the tissue's response to injury.

To date, no experimental studies have systematically evaluated the effects of topical exposure to Bt-based bioinsecticides on the integrity of corneal tissue in animal models. This is particularly concerning, given that the eye is an organ highly susceptible to irritation and damage from direct contact with environmental chemicals, including those found in agricultural products.

Material and Method

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals. All experimental procedures were approved by the Ethics Committee of the Faculty of Medicine, University of Jember, Indonesia (Approval No. 2018/UN25.11/ET/2020). A post-test-only control group design was employed, utilizing 24 male Wistar rats divided into control and treatment groups (8 g/L (P1), 10 g/L (P2), and 12 g/L (P3) of Bt). The concentrations of 8, 10, and 12 g/L were selected based on a previously published pilot study conducted by our group, which evaluated multiple Bt exposure methods and concentrations ranging from 0.5 to 10 g/L (Komariah *et al.,* 2026). In that preliminary study, corneal epithelial defects were observed only after repeated topical exposure to concentrations around 10 g/L, suggesting that this concentration range may be biologically relevant for inducing ocular surface responses. Therefore, 8 g/L, 10 g/L, and 12 g/L around the concentration range associated with corneal epithelial alterations in the pilot study. The control group received no exposure to *Bacillus thuringiensis* (Bt), while the

three treatment groups received topical Bt solutions at concentrations of 8 g/L (P1), 10 g/L (P2), and 12 g/L (P3). A commercial *Bacillus thuringiensis* subsp. *kurstaki* formulation (Dipel® WG; Nufarm Indonesia) was used in this study. According to the product label, Dipel® WG contains 54% *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351 as the active ingredient (Registration No. RI 01010120134857). The study was designed to evaluate ocular responses following exposure to the commercial formulation rather than to characterize its microbiological composition. Therefore, parameters such as spore viability, purity, colony-forming unit (CFU) concentration, and Cry toxin concentration were not independently assessed. The Bt solutions were prepared from commercial wettable granules, dissolved in sterile distilled water, and homogenized using a magnetic stirrer at 400 rpm for 5 minutes. The pH of the prepared solutions ranged from 4.2 to 4.4, as previously reported in our pilot study (Komariah *et al.,* 2026). Each day for seven consecutive days, 3 mL of the test solution was gradually instilled onto the right ocular surface over approximately 2 minutes under intraperitoneal ketamine anesthesia (75–100 mg/kg body weight). This exposure protocol was adapted from our previously published pilot study, which demonstrated that repeated topical exposure using this model produced detectable corneal epithelial alterations (Komariah *et al.,* 2026). The seven-day exposure period was also based on findings from our preliminary study, in which shorter exposure durations failed to produce detectable corneal alterations, whereas repeated exposure for seven consecutive days resulted in observable corneal epithelial defects. After instillation, the eyelid was sealed with sterile adhesive tape (Hipafix) to ensure retention of the solution on the corneal surface. The control group received an identical procedure using saline solution (Komariah *et al.,* 2026).

Bt solution was applied to the anesthetized right eyes daily. Corneal damage was assessed via fluorescein staining and histopathology. Inflammatory markers TNF-α (ELISA) and oxidative stress marker MDA (TBA) were analyzed.

On day eight, corneal epithelial defects were evaluated using a fluorescein staining test (0.1% fluorescein solution) under cobalt blue light. Subsequently, the animals were humanely euthanized, and the eyeballs were enucleated for histopathological analysis. Corneal tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) according to standard ocular histopathological procedures (Dua *et al.,* 2020; Rauchman *et al.,* 2023). Histological evaluation was performed in a double-blind manner by two independent observers. Parameters assessed included epithelial desquamation, epithelial thickness, and neutrophil infiltration. epithelial thickness was measured from the superficial squamous layer to the epithelial basement membrane in five representative fields under 400× magnification, and neutrophils were quantified as the number of cells per high-power field as previously described in ocular surface inflammation studies (Dua *et al.,* 2020).

To evaluate inflammatory and oxidative stress markers, TNF-α levels were measured from corneal homogenates using a commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kit according to the manufacturer's instructions and established protocols for ocular inflammatory biomarker assessment (Maurya *et al.,* 2021). Malondialdehyde (MDA) levels were determined using the thiobarbituric acid reactive substances (TBARS) assay with spectrophotometric detection, as previously described for the assessment of lipid peroxidation and oxidative stress (Ohkawa *et al.,* 1979; Maurya *et al.,* 2021).

Numerical data were tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test). Parametric data were analyzed using One-Way ANOVA followed by LSD post hoc tests, while non-parametric data were analyzed with the Kruskal-Wallis test. Non-parametric tests (Kruskal–Wallis) were applied when data did not meet the assumptions of normality or homogeneity. Categorical variables, such as the presence of epithelial defects, were analyzed using Chi-Square or Fisher's Exact tests when necessary. All statistical analyses were conducted at a 5% significance level ($p < 0.05$). Statistical analyses were conducted following established biostatistical methods described by Motulsky (2022).

Results and Discussion

Corneal Epithelial Defects

After seven days of topical Bt bioinsecticide exposure, fluorescein staining was used to detect corneal epithelial defects. All control group samples showed negative results for epithelial defects. In

contrast, among the treatment groups, one positive case was observed in Group P1 (8 g/L) and two positive cases in Group P3 (12 g/L), while Group P2 (10 g/L) showed no epithelial defects.

Statistical analysis using Fisher's Exact test revealed no significant difference among the groups ($p = 0.546$), indicating that Bt exposure within this concentration range did not result in statistically significant macroscopic epithelial damage. Representative fluorescence staining images are shown in Figure 1.

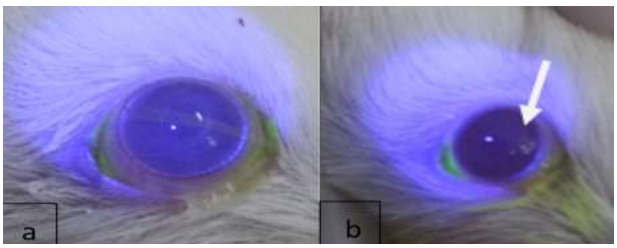

Figure 1. Fluorescein staining images: (a) Negative epithelial defect; (b) Positive epithelial defect (white arrow).

Histopathological Findings

Histopathological examination showed an increased incidence of epithelial desquamation in the treatment groups compared to the control. Desquamation was observed in 2 control samples, 4 samples in P1, and 5 samples each in P2 and P3. Although there was a trend of increasing desquamation with higher Bt concentrations, Fisher's Exact test indicated no statistically significant difference ($p = 0.192$). Microscopic images of epithelial desquamation are presented in Figure 2.

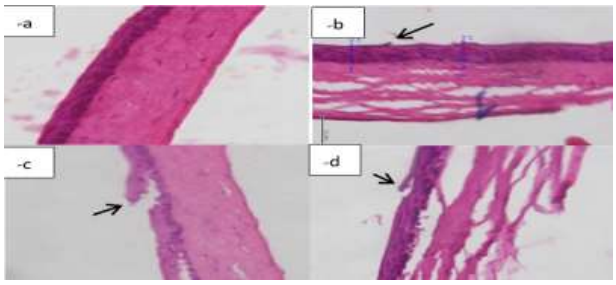

Figure 2. Microscopic images (400×) of corneal epithelial desquamation (black arrows): (a) Control (no desquamation); (b) P1; (c) P2; (d) P3.

Corneal Epithelial Thickness

Epithelial thickness was measured microscopically and analyzed using ANOVA. The average thickness in the control group was 6.13 ± 1.7 µm. Groups P1, P2, and P3 had average thicknesses of 6.21 ± 1.9 µm, 5.87 ± 3.0 µm, and 7.12 ± 5.3 µm, respectively. Despite these fluctuations, ANOVA analysis yielded a p-value of 0.713, indicating no significant differences among the groups. This suggests that Bt exposure did not consistently or significantly affect epithelial thickness. Representative images are shown in Figure 3.

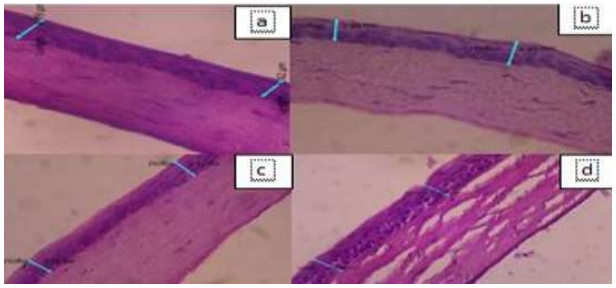

Figure 3. Microscopic views (400×) of corneal epithelial layers (blue line): (a) Control; (b) P1 (8 g/L); (c) P2 (10 g/L); (d) P3 (12 g/L).

Neutrophil Infiltration

Figure 4 illustrates neutrophil infiltration levels, revealing an increase in inflammatory cell presence in the treatment groups, especially in P1 (0.53 ± 0.49 cells/field) compared to the control group (0.17 ± 0.16). Due to non-normal distribution in some groups, the Kruskal-Wallis test was used, yielding a p-value of 0.111, indicating that the increase in neutrophil count was not statistically significant.

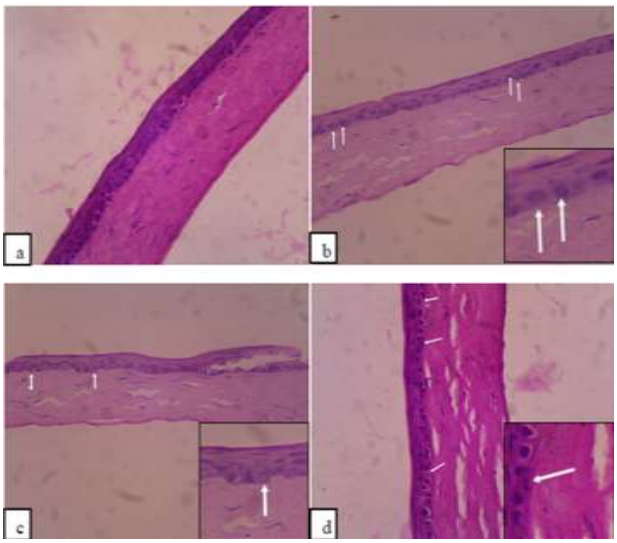

Figure 4. Microscopic images (400×) of neutrophils in the cornea (white arrows): (a) Control; (b) P1; (c) P2; (d) P3. These demonstrate the presence of neutrophils migrating into corneal tissue, particularly in the P1 and P3 groups.

TNF-α Levels

Analysis of TNF-α levels as an inflammatory marker revealed a significant difference among groups ($p = 0.023$, ANOVA). Post hoc LSD analysis showed that Treatment 2 (10 g/L) had significantly

higher TNF-α levels compared with Treatment 1 (8 g/L). Meanwhile, the Control group and Treatment 3 (12 g/L) did not show significant differences from either Treatment 1 or Treatment 2.

Table 1. Mean TNF-α levels across treatment groups

Group	TNF-α (ng/L ± SD)
Control	347.83 ± 121.42^{ab}
Treatment 1	259.08 ± 45.97^{a}
Treatment 2	421.17 ± 68.49^{b}
Treatment 3	367.42 ± 77.42^{ab}

Values are mean ± SD. Different superscripts indicate significant differences among groups ($p = 0.023$, ANOVA; LSD post hoc test). Groups sharing at least one superscript letter are not significantly different.

MDA Levels

MDA levels, as an indicator of oxidative stress, varied among groups, with the highest levels observed in Treatment 2 (7.03 ± 5.4 nmol/g) and the lowest in Treatment 1 (4.75 ± 1.6 nmol/g). Since the data did not meet the assumptions of normality, the non-parametric Kruskal–Wallis test was applied. The analysis yielded a p-value of 0.722, indicating no statistically significant differences in MDA levels across groups. These results suggest that although Bt exposure tended to increase oxidative stress markers, the variation was not sufficient to reach statistical significance.

Table 2. Mean MDA levels across treatment groups

Group	MDA (nmol/g ± SD)
Control	6.33 ± 2.7
Treatment 1	4.75 ± 1.6
Treatment 2	7.03 ± 5.4
Treatment 3	5.94 ± 4.6

Discussion

Considering the results indicating that topical exposure to a commercial *Bacillus thuringiensis*-based formulation over seven days was associated with increased corneal TNF-α levels, this study suggests that local inflammatory activation may occur even in the absence of overt morphological damage. It is important to note that commercial Bt-based formulations may contain, in addition to bacterial spores and crystal proteins, other formulation ingredients intended to enhance product stability and application. These components could potentially contribute to ocular irritation (Raucman *et al.,* 2023; Raymod *et al.,* 2023). Because the detailed composition of these ingredients was not independently characterized in the present study, their potential contribution to the observed inflammatory response cannot be excluded. However, previous studies have demonstrated that Bt-derived proteins, including Cry toxins and S-layer proteins, are capable of activating corneal innate immune pathways via TLR2/4 and NF-κB signaling. Therefore, both microbial components and formulation ingredients may potentially contribute to the observed inflammatory response. Because the present study did not evaluate these components separately, the relative contribution of each factor remains uncertain.

The elevation of TNF-α levels suggests activation of innate immune pathways, despite no observable microscopic epithelial damage. Recent in vivo and in vitro studies (2021–2024) have shown that Bt surface proteins (e.g., S-layer proteins and endotoxins) can activate TLR2/4 and downstream MyD88/NF-κB signaling pathways, leading to increased expression of TNF-α, IL-1β, IL-6, and chemokines such as CXCL1/IL-8, even without direct bacterial invasion (Cordiano *et al.,* 2023; Fichan *et al.,* 2023; Zeng *et al.,* 2024).

Histological images (Fig. 4) clearly demonstrate the presence of neutrophils migrating into corneal tissue following Bt exposure, particularly in P1 and P3 groups. Although statistical significance was not reached, the microscopic evidence of neutrophil extravasation suggests an early stage of tissue infiltration, consistent with subclinical inflammatory activation. The observation that neutrophil infiltration and epithelial damage were not statistically significant aligns with prior reviews on general ocular toxicity (including that of pesticides), which emphasize that mild to moderate inflammation may occur without overt histological alterations (Mursalin *et al.,* 2025; Pahwa *et al.,* 2025). This suggests that superficial corneal exposure can still initiate local inflammation, even in the absence of structural changes.

Despite elevated TNF-α levels, neutrophil counts did not increase significantly. This finding is consistent with the principle that robust neutrophilic infiltration typically requires additional chemokine activation and neovascularization—processes that may not be fully initiated during short-term topical exposure. The observation that TNF-α peaked at the intermediate concentration (10 g/L) and declined at 12 g/L may reflect immunological feedback

regulation. Literature reports suggest that repeated exposure to TLR ligands can lead to receptor downregulation or induction of anti-inflammatory mediators such as IL-10 (Cordiano *et al.,* 2023).

To our knowledge, this study is the first experimental investigation evaluating ocular responses to topical exposure of a Bt-based bioinsecticide, using TNF-α and MDA as biomarkers of inflammation and oxidative stress. This highlights the potential for ocular inflammatory responses following exposure to commercial Bt-based formulations, even in the absence of visible tissue damage. Our results indicate that the intermediate concentration (10 g/L, P2) was most effective in stimulating TNF-α production, with significantly higher levels compared to P1. These findings are valuable for policymakers and agricultural practitioners, particularly for workers at risk of direct ocular exposure, by emphasizing the importance of eye protection and safe handling of Bt-based products. Although most morphological parameters, such as epithelial defects, desquamation, and thickness, did not differ significantly between groups, a clear trend of increased inflammatory response was observed, especially at the 10 g/L concentration. This was further supported by the statistically significant rise in TNF-α levels ($p = 0.023$), indicating local inflammatory activation in the corneal tissue.

Our results indicate that the intermediate concentration (10 g/L, P2) was most effective in stimulating TNF-α production, with significantly higher levels compared to P1. This suggests a possible non-linear dose–response relationship, with 10 g/L representing the concentration associated with the highest TNF-α levels observed in the present study. The significance of this finding lies in its demonstration that Bt, despite being generally considered systemically safe, can activate local immune responses when applied topically to the eye, even without inducing visible tissue damage. This highlights the biological potential of Bt to cause irritation or inflammation in non-target tissues such as the eye. The study also reveals that Bt, while primarily targeting insects, contains active components capable of stimulating inflammatory mediators in vertebrate tissues.

The novelty of this research lies in its experimental approach, which specifically addresses the ocular safety of Bt bioinsecticides, an area that remains largely underexplored in toxicological literature. Furthermore, the use of a Wistar rat model and the assessment of biomarkers such as TNF-α and MDA provide a robust basis for concluding that Bt exposure may exert biologically relevant effects, even in the absence of infection or direct microbial invasion.

Although no statistically significant epithelial damage or oxidative stress was observed, the 8 g/L group exhibited lower TNF-α levels than the 10 g/L group. This observation is consistent with our previous pilot study, which identified corneal epithelial defects following repeated exposure to concentrations around 10 g/L (Komariah *et al.,* 2026). Nevertheless, the present findings are insufficient to establish a definitive ocular safety threshold, and further studies with larger sample sizes, longer exposure durations, and additional inflammatory markers are required to better characterize the dose–response relationship of Bt-based bioinsecticide exposure.

A limitation of this study is that independent microbiological characterization of the commercial Bt-based formulation was not performed. Although the product label identified the active ingredient as *Bacillus thuringiensis* subsp. *kurstaki* strain ABTS-351, parameters such as spore viability, purity from other microorganisms, colony-forming unit (CFU) concentration, Cry toxin concentration, and batch-specific characteristics were not independently verified. Consequently, the observed ocular responses should be interpreted as being associated with exposure to the commercial formulation as a whole rather than exclusively to *Bacillus thuringiensis*, its toxins, or other individual formulation components. Future studies incorporating microbiological characterization and additional control groups may help clarify the relative contribution of these factors to the observed inflammatory responses.

Conclusion

Topical exposure to a Bt-based bioinsecticide may be associated with corneal inflammatory responses, as suggested by the observed increase in TNF-α levels, even in the absence of significant structural damage. Because the present study evaluated a commercial formulation, the relative contributions of Bt spores, Bt-derived toxins, and formulation additives could not be determined. Further studies are needed to elucidate the specific factors involved and the molecular mechanisms underlying these responses. Future investigations

incorporating additional control groups, including untreated controls, purified Bt cultures, and formulation-additive controls, may help clarify the specific components responsible for the observed inflammatory effects.

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