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Regulation of the immune system by administering lactic acid bacteria to suppress the effects of aflatoxin B1 in mice (*Mus musculus*)

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ABSTRACT

Aflatoxin B1 (AFB1), which is a toxic compound produced by the filamentous fungus *Aspergillus* sp., is highly carcinogenic, damages vital organs, and may cause death. Prevention of aflatoxin poisoning through proper food storage and physical treatment is an added cost, thus there is a need to identify alternative methods including treatment with probiotic bacteria. We evaluated the effect of *Lactobacillus bulgaricus* on activating immune cells in mice exposed to Aflatoxin B1. The study used a post-test control design consisting of five treatment groups including a negative control, positive control, and T1, T2, and T3 groups treated with lactic acid bacteria at doses of 105 colony forming unit (CFU)/ml, 107 CFU/ml, and 109 CFU/ml, respectively, administered on days 7–28 and AFB1 at a dose of 0.2 mg/kg bw orally on days 15–28. The relative number of lymphocytes consisting of CD11c+transforming growth factor-beta (TGF-β)+, CD4+CD8+, and B220+IgG+, was measured using flow cytometry. The data were analyzed using a one-way analysis of variance test. The results indicated that *L. bulgaricus* bacteria increased the relative number of CD11c+TGF-β+, B220+IgG+, and CD4+CD8+ cells in mice exposed to the mycotoxin. *Lactobacillus bulgaricus* may function as an immunostimulator against mycotoxins by inducing the humoral and cellular immune response.

INTRODUCTION

Mycotoxins, such as aflatoxin B1 (AFB1), are secondary metabolites produced from filamentous fungi that are toxic, carcinogenic, and immunosuppressive to animals and humans. The estimated world production of feed contaminated with fungus is approximately 5%–10% (Oswald *et al.*, 2005; Qian *et al.*, 2012; Tomkova *et al.*, 2001). Aflatoxin reportedly caused up to 100,000 turkey deaths following the consumption of contaminated peanut mushrooms. Broiler feed contaminated with a mycotoxin mixture (3.5 mg/kg diet as 79% of AFB1, 16% AFG1, 4% AFG2, and 1% AFB2)

can lead to weight loss and inflammation of the liver and kidneys. Aflatoxin B1 is a potent agent that causes immunosuppression in pigs at a dose of 140–280 µg/kg of feed by inhibiting DNA synthesis and immune cells, such as lymphocytes, but does not affect the humoral immune response (Perczak *et al.*, 2018; Pierron *et al.*, 2016).

Aflatoxin B1 suppresses the cellular immune system, in particular T lymphocytes, because of decreased complement production by the liver, phagocytosis by macrophages, and neutrophil activity (Perdigon *et al.*, 2001). T lymphocytes affected by the toxin, as well as other lymphoid cells, such as cytotoxic T cells and natural killer cells, can promote tumor cell function directly or indirectly. The cellular components of the immune system produce cytokines for protection against tumor progression; however, cytokines may also play a role in the inflammation mechanism that causes damage to various organs (Ibrahim, 2013).

Methods such as heating, chemical treatment, or radiation can destroy and eliminate mycotoxin (Zain, 2011);

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however, the cost is prohibitive and it may impair the nutritional value of the feed. The decline in fungal growth may increase during feed production or storage (Munoz *et al.*, 2010). Lactic acid bacteria (LAB) may exhibit antifungal activity, especially *Lactobacillus* sp (Sadiq *et al.*, 2019). *Lactobacillus pentosus* and *Lactobacillus brevis* bacterial strains at a concentration of 3.5 × 108 colony forming unit (CFU)/ml can bind and release aflatoxin B1 by 17.4% and 34.7%, respectively, in liquid media as measured by ELISA (Hamidi *et al.*, 2013).

Microorganisms, such as *Saccharomyces cerevisiae* and LAB, may be used as biopreservatives in feeds, so it is possible to extend the shelf-life and increase food safety with microflora supplementation. Antimicrobial products of microorganisms also have potential as probiotics and may improve health (Tran *et al.*, 2020); however, there is limited data demonstrating the immunostimulating effects of LAB. Therefore, we evaluated the effect of LAB on the cellular and humoral immunity profile of mice.

MATERIAL AND METHODS

Preparation of animal

Male mice (*Mus musculus*), strain Balb/c aged 8–12 weeks (*n* = 25), with a body weight of 25–30 g, were obtained from Brawijaya University's bioscience laboratory and divided into five groups (five mice/group). The treatment groups consisted of a positive control (mice induced with AFB1 0.2 mg/kg bw on days 15–28), negative control (healthy mice), and treatment groups, T1, T2, and T3 in which mice were administered 1 × 105 CFU/ml, 1 × 107 CFU/ml, and 1 × 109 CFU/ml of LAB on days 7–28, respectively.

Bacterial suspension preparation

Lactobacillus bulgaricus (LAB) was obtained from the Microbiology Laboratory of the Faculty of Medicine, Universitas Brawijaya, and confirmed by biochemical tests and Gram staining. The bacteria were grown on de Man, Rogosa, and Sharpe (MRS) agar media at 37°C for 24 hours. A bacterial suspension was prepared using MRS broth media and the bacterial concentrations were measured using a spectrophotometer. The bacteria were diluted with phosphate buffer saline (PBS) for the experiments.

Aflatoxin B1 preparation

Aflatoxin B1 (Sigma Company catalog: A6636®) is potent with respect to acute toxicity, mutagenicity, and carcinogenicity, and one vial of AFB1 contained 5 mg of powder. AFB1 (0.2 mg/kg BW) was diluted in 1 ml PBS, pH 7.2 (Qian *et al.*, 2012).

Flow cytometry

The mice were sacrificed on day 29 by cervical dislocation. Spleens were harvested, placed into a petri dish containing sterile PBS, and crushed with the base of a syringe. 10 ml homogenates of the sample were prepared in a volume of 10 ml, centrifuged, and the pellets were resuspended in 1 ml PBS by pipetting (Ardiana and Rifa'i, 2015).

Then, 100 µl of the suspension was placed into a microtube; 500 µl of PBS was added; and the mixture was centrifuged at 2,500 rpm for 5 minutes at 4°C. Then, 50 µl of PE/Cy5 conjugated rat anti-mouse Cd11c, PECy5 conjugated rat

anti-mouse transforming growth factor-beta (TGF-β), fluorescein isothiocyanate (FITC) conjugated rat anti-mouse CD4, PE conjugated rat anti-mouse CD8, FITC conjugated rat anti-mouse B220, and PECy5 conjugated rat anti-mouse IgG (Biolegend®, San Diego) were added to the cells and incubated for 20 minutes in 4°C (8). Afterward, 50 µl of cytofix (BD Biosciences Pharmingen) was added and incubated for 20 minutes in 4°C; wash perm solution (BioLegend®, USA) was added; and the mixture was centrifuged at 2,500 rpm at 10°C for 10 minutes. The cells were analyzed by flow cytometry (BD FACSCalibur, USA) using BD Cellquest ProTM software.

Statistical analysis

The data are presented as the relative number of immune cells (CD11c+TGFβ+, CD4+CD8+, and B220+IgG+). Data were analyzed statistically using one-way analysis of variance (ANOVA) with an error level of α = 0.05, followed by Tukey's test.

RESULTS

CD11c⁺TGF-β⁺ cells

The results indicated that supplementation of the mice with LAB induced by AFB1 increased the relative number of CD11c+ cells that produce TGFβ+ (Fig. 1). In the positive control group, the relative number of CD11c+ expressing molecule TGF-β+ (1.75%) was different, but no significance was observed when compared with the negative control (0.91%). All treatment groups (T1, T2, and T3) administered LAB at a 105–109 CFU/ml concentration showed an increase in the relative number of CD11c+TGF-β+ cells by 2.05% 3.14%, and 3.06%, respectively, when compared to the negative and positive controls, as shown in Figure 1.

CD4⁺CD8⁺ cells

The results showed that the supplementation with LAB in mice induced with AFB1 increased the relative number of CD4+CD8+ cells in all treatment groups, but the amount did not significantly differ and was similar to that of the negative control, as shown in Figure 2. The negative control was higher when compared with the positive control.

B220⁺IgG⁺ cells

The results showed significant differences in the relative number of B220+-expressing IgG+ cells in all the treatment groups following supplementation with LAB after induction with AFB1. The negative control group (24.56%) was significantly different from the positive control group (5.76%), which showed a relatively higher number of B220+IgG+ cells compared with the positive control. There was an increase in the relative number in all treatment groups (7.44%, 10.26%, and 7.67% for T1, T2, and T3, respectively), as shown in Figure 3.

DISCUSSION

Effect of LAB on the relative number of CD11c⁺TGF-β⁺ cells

The results indicated that the relative number of CD11c+ cells that expressed TGF-β was different, but there was no significant between the treatment groups; however, a 107 CFU/ml concentration of LAB increased the average of CD11c+TGF-β+
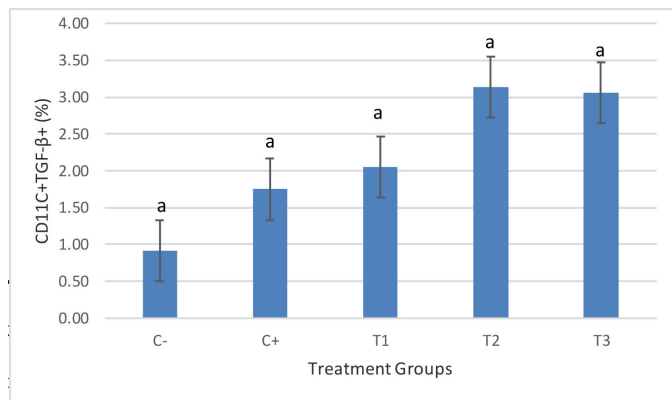
Figure 1. Flow cytometry analysis shows that giving LAB showed an increase in the relative number of CD11c+TGF-β+ cells ($p < 0.05$), but was not significantly different between treatments. There was an increase in the number when compared with the positive control. The highest average increase was seen in the T2 treatment. The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered with AFB1 and 1×10^5 CFU/ml, 1×10^7 CFU/ml, and 1×10^9 CFU/ml of LAB on days 7–28, respectively.
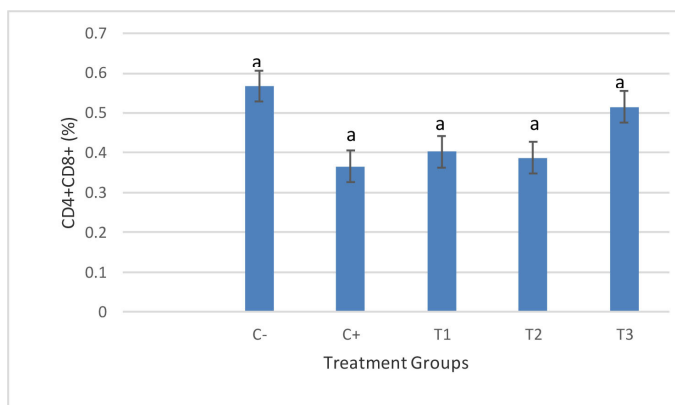

Figure 2. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered with AFB1 and 1×10^5 CFU/ml, 1×10^7 CFU/ml, and 1×10^9 CFU/ml of LAB on days 7–28, respectively.

cells in all treatments. Mycotoxin exposure decreased the relative amount of CD11c+TGF-β+ cells. Mycotoxin AF1 altered or decreased anti-inflammatory cytokine synthesis by inhibiting macrophage or T cell activation. In fact, mycotoxin inhibits the synthesis and proliferation of T cells, which prevents macrophage cells from producing anti-inflammatory cytokines. Mycotoxin is cytotoxic to lymphocytes by interfering with lymphocyte receptors or lymphocyte function (Tran *et al.*, 2020). A study by Murugesan *et al.* (2015) revealed that mycotoxin was not immunogenic. It could not induce an immune response to pathogens, but interfered with mitogen-activated protein kinase signaling to modulate cell growth, apoptosis, or the immune response. This could expose an individual to a high risk of infection.

Dendritic cells (DC) and CD11c markers are antigen-presenting cells (APCs) that are regulated specifically and nonspecifically by immune cells found in the lamina propria of the small intestine and gut-associated lymphoid tissues, such as
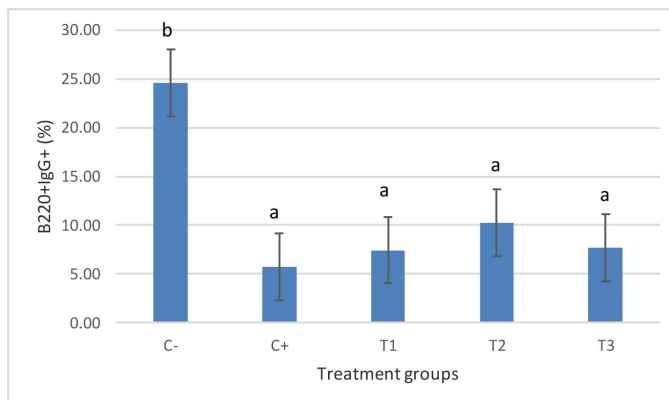

Figure 3. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered with AFB1 and 1×10^5 CFU/ml, 1×10^7 CFU/ml, and 1×10^9 CFU/ml of LAB on days 7–28, respectively.

the Peyer's patches. Most dendritic cells (DCs) are present in an immature condition and are less immunogenic because of the low expression of MHC costimulators. Contact with pathogen-associated molecular patterns (PAMPs) or other signals induces the pattern recognition receptor (PRR) signal and activates the NF-κB pathway, resulting in maturation and activation of DC cells (Wells, 2011). Mature DCs may then express high MHC levels, costimulatory molecules, and cytokines which attenuate APC activation and differentiation of T cells to cause inflammation (Mohamadzadeh *et al.*, 2005). DCs exposed to AFB1 cannot respond correctly to any invading microbes and fails to initiate antigen presentation to activated T cells that are susceptible to pathogenic agents (Mohammadi *et al.*, 2014).

The anti-inflammatory cytokine, TGF-β, inhibits the proliferation of fibroblast epithelial cells, dendritic cells, and macrophages to produce inflammatory cytokines and controls cell growth through adhesion and extracellular matrix formation (Hussain *et al.*, 2018). Exposure to mycotoxin could stimulate CD11c+ cells to activate inflammatory pathways that TGF-β may have suppressed. Oral exposure to LAB concentrations of 10⁵ CFU/ml could activate inflammatory cytokines, such as TGF-β, in DCs, which may inhibit CD11c+ cell activity. This was evident in the T1 and T2 group, prior to an observed decrease in the T3 group. According to Vindirelo and Alberto (2015), the higher the concentration of cell bacteria, the greater the binding capacity of AFB1 in liquid media *in vitro*. The concentration of bacteria that can bind AFB1 was 1010 CFU/ml for *L. rhamnosus* GG, *L. casei Shirota*, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Escherichia coli*. Probiotics have an immunomodulatory effect on the release of cytokines, interleukins, tumor necrosis factor, transforming growth factor, and chemokines from immune cells that play a role in the innate and adaptive immune systems. LAB may interact with enterocytes and DCs, Th1/Th2 cytokines, or T reg cells in the intestine to stimulate the adaptive immune response into a proinflammatory or anti-inflammatory action (Azad *et al.*, 2018; Mohamadzadeh *et al.*, 2005).

Mycotoxin AFB1 exerts toxicity because it is readily absorbed by the intestine and rapidly binds to serum protein. AFB1 is

genotoxic and immunogenic in animals (Zimmermann *et al.*, 2014). Mycotoxin can activate the microbial intestine, and mycotoxin adsorption–desorption is highly dependent on the intestinal environment and digestive enzymes. The LAB, *L. rhamnosus* RC007, stimulates pH, salts, enzymes, and peristalsis at each stage of AFB1 absorption in the digestive tract. Saliva secretion results in low adsorption and high AFB1 reabsorption. Gastric fluids and intestinal fluids do not decrease the AFB1 adsorption of LAB, rather they stimulate higher AFB1 adsorption (Sadiq *et al.*, 2019).

Metabolic LAB products inhibit aflatoxin biosynthesis. Heterofermented LAB, such as *L. bulgaricus*, produce a high level of acetic acid and propionic acid at acidic pH (Vinderola and Ritieni, 2015). The mechanism of action of LAB is to inactivate the fungal membrane and inhibit the absorption of amino acids and inactivated products from fungi, such as acetic acid (Perczak *et al.*, 2018). Bacteria and yeasts may neutralize mycotoxins in the body by reshuffling, transforming, and breaking them down into nontoxic metabolic products or inactive forms (Murugesan *et al.*, 2015). LAB binds mycotoxins to prevent further absorption by the intestine, which are then secreted with feces (Adilah *et al.*, 2018). LAB walls contain peptidoglycans that could interact with mutagenic compounds, including mycotoxins, through binding to reduce stability and bioavailability, and stimulate the secretion of anti-inflammatory cytokines by macrophages (Niderkorn *et al.*, 2009; Tabari *et al.*, 2018). LAB's capacity to bind mycotoxins would be optimal when the bacteria cells die due to a change in the cell surface. Live LABs require a long time to release mycotoxin from the body (Perczak *et al.*, 2018). Cell wall protein denaturation may function by creating a broader area to absorb mycotoxins (Tabari *et al.*, 2018). The proteins in the ribosomes, nucleus, chromosomes, cytosol, and cellular cytoskeleton components support the forming of the cell wall of bacteria in the exponential growth phase.

On the contrary, binding between the cell wall of LAB with mycotoxin takes place at the beginning of the end of the bacterial growth cycle (Moller *et al.*, 2021). AFB1 could bind to the cell wall β-d-glucan through hydrogen or van der Waals bonds. Absorption of AFB1 toxin depends on the availability of the number of binding sites on the surface of microbes, and the equilibrium constant [K (eq)], which could change as a result of genetic, physical, or chemical alterations (Sadiq *et al.*, 2019).

LAB may act as an anti-inflammatory agent, resulting in the reduction of oxidative stress from AFB1 exposure (Abbes *et al.*, 2016). Probiotics could also stimulate T cell subsets, humoral immune cells, epithelial-associated dendritic cells, and macrophages to increase anti-inflammatory cytokine products Braat *et al.*, 2004. The entry of LABs into the body may increase the capacity and phagocytic receptors of leukocyte cells, especially complement receptor 3 (CR3), for bluffing respiratory bursts (Bravo *et al.*, 2019).

Effect of LAB on the relative number of CD4+/CD8+ cells

Our results showed that the administration of LAB (*L. bulgaricus*) had an effect on the relative number of CD4+/CD8+ immunocompetent cells in mice induced with aflatoxin B1 in the treatment groups ($p > 0.05$) based on a Kruskal–Wallis test. The negative control group had a higher relative number of CD4+/CD8+ T cells than the positive group. This result is consistent with that of Qian *et al.* (2012), in which the administration of AFB1 to mice orally for 24 hours reduced CD3 T cells in the intestinal mucosa.

AFB1 caused a decrease in the cellular immune response to the specific dose and duration treatments (Zimmermann *et al.*, 2014). A low dose of mycotoxin could induce an inflammatory response if activated by enzymes, such as inflammatory inhibitors (Hussain *et al.*, 2018). Mycotoxin AFB1 may undergo systemic hydrolysis and further activate metabolism. Phase metabolism includes conjugation with glucuronic acid and sulfate by the whole-cell biotransformation system during immune cell communication (Tran *et al.*, 2020).

High LAB concentrations could increase naive T lymphocyte activation and proliferation as well as memory T cells (Wells *et al.*, 2011). Upon entering the body, an antigen is presented by DCs in the digestive tract mucosa (CD11c). Histocompatibility complex class II molecules combined with the expression of costimulatory molecules and cytokines (Gaudinoand Kumar, 2019) and activation of T lymphocytes. As a cellular defense, T cells may differentiate into a subset of T1 that activates macrophages. Macrophages and APCs induce T cells to secrete inflammatory cytokines that cause tissue damage (Mohamadzadeh *et al.*, 2005). The T1, T2, and T3 groups had a higher relative amount of CD4+/CD8+ T cells compared with the positive control group is shown in Figure 2.

LAB may act as an antifungal agent because it contains metabolites, such as organic acids, carboxylic acids, phenolic acids, cyclic dipeptides, hydrogen peroxide, and compounds that inhibit sporulation, which may decrease mycotoxin production (Sadiq *et al.*, 2019). LAB at concentrations of 105 CFU/ml could activate T lymphocyte cells in all groups compared with the positive control group. These results were consistent with that reported by Tsai *et al.* (2012) in which LAB activates the cellular adaptive immune response.

THE EFFECT OF LAB ON THE RELATIVE NUMBER OF B220+IGG+ CELLS

Our results showed that B220+ cells expressing IgG in the negative control group was higher compared with the positive group, whereas it was not significantly different among all of the treatment groups. The average of B220 cells expressing IgG increased after LAB administration. Mycotoxin can reduce immunoglobulin production in chicken feed supplemented with AFB1 (Nazarizadeh and Pourreza, 2019). LAB stimulates the humoral immune response by increased circulating antibodies and levels of plaque-forming cells in the host when exposed to mycotoxin (Abbes *et al.*, 2015). LAB can absorb and eliminate mycotoxin to prevent intestinal absorption and reduce liver damage, which is a target of mycotoxin. Upon entering the host orally, mycotoxin stimulates the secretion of immunoglobulin A (IgA) in the digestive mucosa and antibody IgG in the circulation. IgG represents a secondary response to protect the body against foreign antigens (Chen and Tsai, 2011).

In the normal intestinal epithelium, microflora bacteria act as a barrier antigen. However, when epithelial cells are degraded by infectious and noninfectious substances, alterations in intestinal permeability and inflammation of the intestinal mucosa occur. The inflammatory response that occurs in the intestine activates IgG antibodies and causes translocation of the microflora bacteria (Paludan *et al.*, 2020).

LAB plays a role in the body to activate the immune response by inducing the formation of Secretory IgA (SIgA) and producing vitamins (Wold, 2001). Antibody SIgA is dominant in the

mucous membrane, which is the first defense immune system against a dangerous environment. SIgA antibodies play a role in neutralizing toxins, viruses, salivary exotoxins, and eliminating pathogenic microbes (Hayati *et al.*, 2018). *Lactobacillus* bacteria and other probiotics are commensal microorganisms that interact with the mucosa or the immune cells. LAB stimulates specific functions of the mucosal immune system and produces secretory IgA. The presence of receptors, such as Toll-like receptors, nucleotide oligomerization domain-like receptors, and C-type lectin receptors may stimulate *Lactobacillus*. *Lactobacillus* associates with microbe-associated molecular patterns to activate APC and modulate their function through the expression of surface receptors, secretion of cytokines and chemokines, and other nonspecific immune effector cells (Mohamadzadeh *et al.*, 2005). Our results indicate that there was an increase in the relative number of IgG+ cells resulting from LAB induction in mice exposed to mycotoxin. This is consistent with the results obtained by Tran *et al.* (2020) in which IgG levels in the serum of Balb/c mice increased when *Lactobacillus* was administered for 7 days (infected with *Salmonella typhimurium* bacteria). DCs play a role in the adaptive immune response. LAB stimulates DC cells to activate specific immune responses in the intestinal mucosa to maintain homeostasis, protect against pathogenic microbes, and maintain intestinal permeability (Mohamadzadeh *et al.*, 2005). LAB increases the cellular and nonspecific humoral immune response in mice exposed to mycotoxin AFB1.

CONCLUSION

L. bulgaricus bacteria exhibited a potent effect as an immunostimulator resulting from exposure to mycotoxin AFB1.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the ethics committee (certified no. 012-KEP-UB-2020) of Institut BioSains.

COMPETING INTERESTS

The authors declare that there are no financial and nonfinancial conflicts of interest.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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AUTHORS' CONTRIBUTIONS

DQS obtained the funding; designed the study, analysis and interpretation of data; and was a major contributor in writing the manuscript. DQS, SM, and IAA analyzed the flow cytometer data, treated the animals, and collected the data. All authors read and approved the final manuscript.

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