

Effect of zinc and probiotics supplementation on IL-6 and tissue neutrophil levels in rats exposed to cigarette smoke

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ABSTRACT

Introduction: Cigarette smoke exposure can cause inflammation, inducing the release of acute phase cytokines, such as IL-6, that will then trigger the recruitment of neutrophils, which are mostly phagocytic cells. Zinc and probiotics are known to have beneficial effects against inflammation. This study was conducted to investigate the effect of zinc and probiotics supplementation on IL-6 and tissue neutrophil levels in rats exposed to cigarette smoke. **Methods:** In a randomised, experimental study with post-test control group design, thirty 2 to 3-month-old male Wistar rats, each weighing 180-220 g, were divided into five groups: control group without treatment (C); exposed to cigarette smoke [C (-)]; exposed to cigarette smoke and received zinc (Z); exposed to cigarette smoke and received probiotics (P); and exposed to cigarette smoke and received a combination of zinc and probiotics (ZP). **Results:** Mean tissue neutrophil levels in Z, P, and ZP groups were 43.43 ± 2.01 , 34.67 ± 1.32 , and 29.77 ± 5.05 cells, respectively. There were significant differences between supplementation intake and tissue neutrophil levels in each group compared to C (-) group ($p < 0.05$). Meanwhile, only IL-6 level in the ZP group (6.02 pg/mL) decreased significantly compared to C (-) group (10.61 pg/mL). **Conclusion:** These results suggest that a combination of zinc and probiotics have an anti-inflammatory effect as measured by IL-6 and neutrophil levels.

Keywords: cigarette smoke, IL-6, neutrophils, probiotics, zinc

INTRODUCTION

Cigarette smoke emitted from burning cigarettes contains thousands of chemicals such as nicotine, hydrogen

cyanide, formaldehyde, arsenic, benzene, carbon monoxide, tobacco-specific nitrosamines (TSNAs), and polycyclic aromatic hydrocarbons (PAHs)

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doi: <https://doi.org/10.31246/mjn-2022-0116>

(Oberg *et al.*, 2010). These particulates may cause inflammation and oxidative stress (American Cancer Society, 2021; Padmavathi *et al.*, 2018). There are three types of cigarette smoke inhaled through passive smoking: mainstream smoke, sidestream smoke, or a combination of both (second-hand smoke). Mainstream smoke is smoke exhaled by active smoking, meanwhile sidestream smoke is smoke from the end of the cigarette. Mainstream smoke contains 3-11% carbon monoxide, 15-43% particulates, and 1-9% nicotine. In a previous study, sidestream smoke was reported to be more dangerous than mainstream smoke because it contained two times more nicotine and carbon monoxide, fifteen times more formaldehyde, and was more toxic (Oberg *et al.*, 2010).

Health effects of cigarette smoke includes chronic obstructive pulmonary disease (COPD), hypertension, cardiovascular disease, cancer, low birth weight, and death (Oberg *et al.*, 2010; CDC, 2018). More than 80% of the 1.3 billion people worldwide use tobacco in low- and middle-income countries. Tobacco causes 8 million deaths annually, with over 7 million mortalities due to direct tobacco, whereas 1.2 million is the result of passive smoking being exposed to second-hand smoke (WHO, 2021). According to Riskesdas (2018), the prevalence of cigarette smoking in Indonesia was 28.8%, while the prevalence of cigarette smoking among 10-18 years old was 9.1%, which meant that there was an increase compared to the data in 2013 (7.2%). Generally, smoking behaviour is found to be higher among males (62.9%) aged 15 years or older (Kementerian Kesehatan RI, 2018). More than 57.8% of students in Indonesia are exposed to cigarette smoke at home, 66.2% in closed public areas, and 67.2% in the open public areas (WHO, 2020).

Cigarette smoke may cause oxidative stress, an imbalanced condition

between the number of free radicals and antioxidants in our body. Oxidative stress may cause tissue damage and induce an inflammatory response that releases pro-inflammatory cytokines such as tumour necrosis factor (TNF), Interleukin-1 (IL-1), Interleukin-6 (IL-6), granulocyte macrophage-colony stimulating factor (GM-CSF), and monocyte-colony stimulating factor (M-CSF). IL-6 is an acute pro-inflammatory cytokine produced by macrophages and monocytes. The production of IL-6 during acute inflammation triggers a subset of chemokines and adhesion molecules to be activated by the endothelial cells, smooth muscle cells, and fibroblasts, which in turn triggers the recruitment of neutrophils. Neutrophils are predominant phagocyte cells during acute inflammation. They are the first cells to reach the site of infection and the first line of defence. Additionally, it has been demonstrated that IL-6 inhibits neutrophil death, extending the neutrophil's lifespan (Padmavathi *et al.*, 2018; Choy & Rose-John, 2017).

Zinc is a micronutrient that plays a role in cell replication, growth, immune responses, antioxidant, and anti-inflammation. Zinc functions as an anti-inflammatory agent by inhibiting the activation of nuclear factor kappa B (NF- κ B) (Prasad & Ananda, 2014). Previous studies have shown that zinc has an anti-inflammatory effect by decreasing leukocyte, IL-6, and TNF- α levels (Anggraeni, Adji & Murwanti., 2015; Utomo *et al.*, 2020). Probiotics, such as *Lactobacillus sp.*, *Bifidobacterium sp.*, *Lactococcus sp.*, *Bacillus sp.*, and yeasts, are live microorganisms that in adequate amounts confer a benefit to human health (Azad *et al.*, 2018). Probiotics act as anti-inflammatory agents by suppressing cytokines production in the intestinal or the extra-intestinal region. Basically, probiotics work in the intestine, but can also affect

other systems, such as the respiratory system through the gut-lung axis, and the nervous system via the gut-brain axis. In the respiratory system, when there is an imbalance of microbiota in the gut, known as dysbiosis, it will affect the immunity of the lungs. Contrarily, the respiratory system has its own microbiota, and intestine dysbiosis can result from lung inflammation and vice versa (Luminturahardjo, 2021). This study aimed to investigate the effect of zinc and probiotics supplementation on IL-6 and tissue neutrophil levels in rats exposed to cigarette smoke.

MATERIALS AND METHODS

Figure 1 shows the flow of the experimental study. The study had a randomised design with post-test control groups. Thirty 2 to 3-month-old male Wistar rats, each weighing approximately 180-220 g, were purchased from FMIPA UNNES and

divided into five groups (six rats in each group) and were group-housed in cages with a three-day acclimation period. They were maintained under controlled temperature (28-32°C) and light (12/12-hour light/dark cycle). (C) was the control group without treatment; [C (-)] was exposed to cigarette smoke from two cigarettes per day for seven days; (Z) was exposed to cigarette smoke from two cigarettes per day and received 5mg/kg body weight/day zinc for seven days; (P) was exposed to cigarette smoke from two cigarettes per day and received one sachet of probiotics per day for seven days; and (ZP) was exposed to cigarette smoke from two cigarettes per day and received a combination of zinc and probiotics for seven days.

The animals were placed in the chamber with holes on the top and side, and given two cigarettes per day. The full procedure was conducted twice a day (in the morning and afternoon). A cigarette

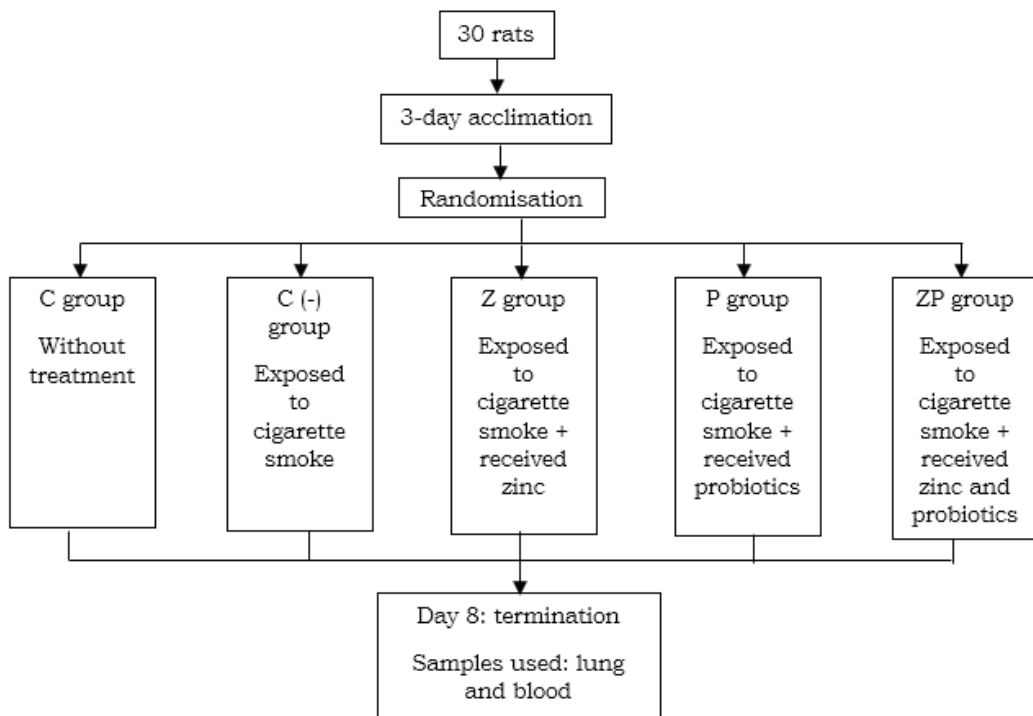


Figure 1. Flow diagram

was aspirated with a syringe and puffs of smoke were expelled into the chamber. Each cigarette took six minutes to burn completely. Our study used zinc sulphate and L-bio probiotics®, which contained *Lactobacillus casei*, *Lactobacillus salivarius*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Bifidobacterium lactis*, *Bifidobacterium infantis*, and *Lactococcus lactis* in every sachet ~ $\pm 2.5 \times 10^9$ CFU. Zinc sulphate (5mg/kg body weight/day) and probiotics (1 sachet ~ $\pm 2.5 \times 10^9$ CFU) were given by oral gavage once daily, 30 minutes before being fed.

On day 8, blood samples were collected by medial canthus sinus orbitalis puncture and put in an ethylenediamine tetraacetic acid (EDTA) tube. The blood samples were centrifuged at 1000 xg for 15 minutes. Plasma IL-6 was measured by the Enzyme-linked immunosorbent assay (ELISA) method using Rat IL-6 ELISA Kit Elabscience®. Absorbance at 450 nm was measured using an ELISA microplate reader (ELx800, BioTek Instruments, USA). Next, the rats were terminated by cervical dislocation and their right lungs collected from all groups. Lung tissue sections (4- μ m thick) were fixed and embedded in phosphate-buffered formalin (pH 7.0). Lung sections were stained with haematoxylin and eosin (H&E). Histological examination was done under a microscope to identify neutrophils (magnifications: 400x). All procedures were carried out as approved by the Health Research Ethics Commission (KEPK) of Diponegoro University (Permit number:15/EC/H/FK-UNDIP/III/2022).

Normality testing was completed using Shapiro-Wilk test. One-way analysis of variance (ANOVA), followed by Games-Howell post-hoc test were used for analysis. All statistical analyses were performed using the IBM SPSS Statistics for Windows version 26.0 (IBM corp, Armonk, New York). Results were

expressed as mean \pm standard deviation (SD).

RESULTS

IL-6 levels in C, C (-), Z, P, and ZP groups were 5.04, 10.61, 8.69, 9.21, and 6.02 pg/mL, respectively. Statistical analysis showed significant differences in IL-6 levels between the groups ($p=0.002$). Mean IL-6 level in C (-) group (10.61 pg/mL) was higher than C group (5.04 pg/mL), and it was significantly different ($p=0.011$). Mean IL-6 levels in Z and P groups were lower than in C (-) group, but no significant differences were detected. Meanwhile, IL-6 level in ZP group was lower and had a significant difference than C (-) group ($p=0.022$), but almost the same as (C) group (Figure 2).

Mean tissue neutrophil levels in groups C, C (-), Z, P, and ZP were 10.77, 63.97, 43.43, 34.67, and 29.77 cells, respectively. Statistical analysis showed significantly different tissue neutrophil levels between the groups ($p<0.001$). Tissue neutrophil level in C (-) group was higher and significantly different than in C group. Mean tissue neutrophil levels in Z, P, and ZP groups were lower and significantly different than in C (-) group (Figures 3 & 4).

DISCUSSION

Cigarette smoke releases danger signals, which act as ligands for toll-like receptors (TLR). The binding of ligand to its receptor induces cytokines and chemokines release like IL-6, IL-8, IL-1 β , TNF- α , GM-CSF, monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule 1 (ICAM-1), which cause innate immune response (Yudhawati & Prasetyo, 2018). Cigarette smoke also activates transcription factor NF- κ B through degradation of I κ B so that NF- κ B translocates into the nucleus. This process causes an increase in the amount of lung

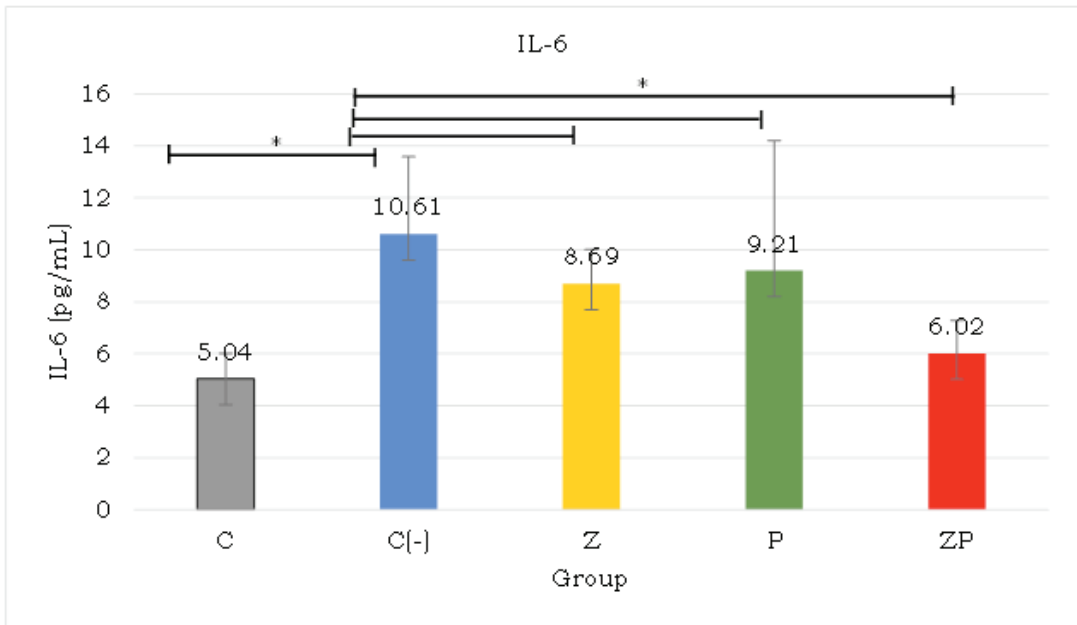


Figure 2. IL-6 levels in different treatment groups; * $p < 0.05$: statistically significant difference

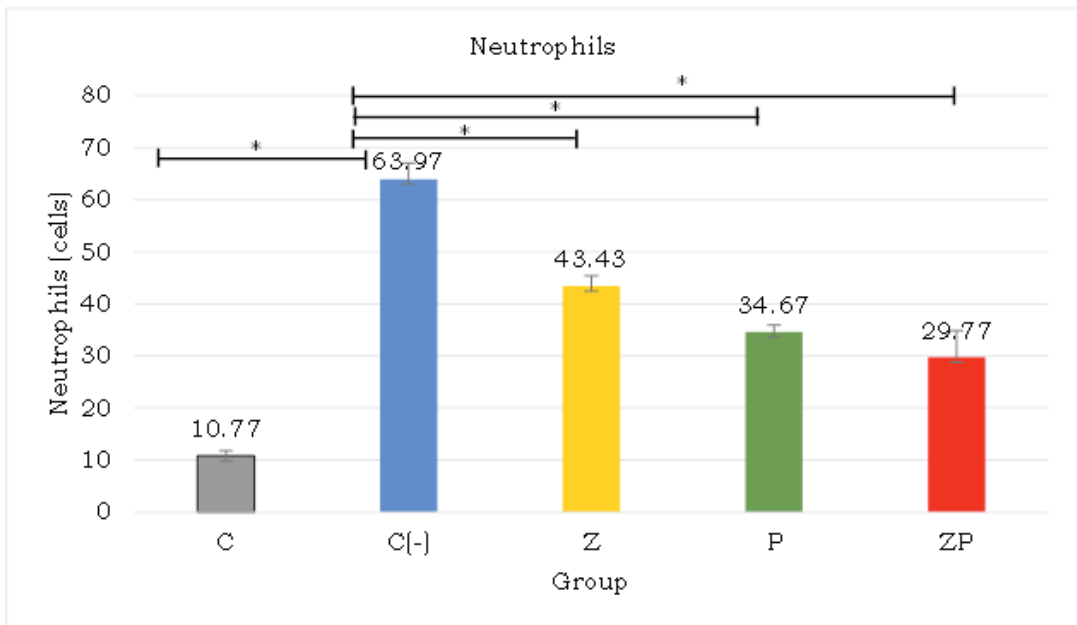


Figure 3. Tissue neutrophil levels in different treatment groups; * $p < 0.05$: statistically significant difference

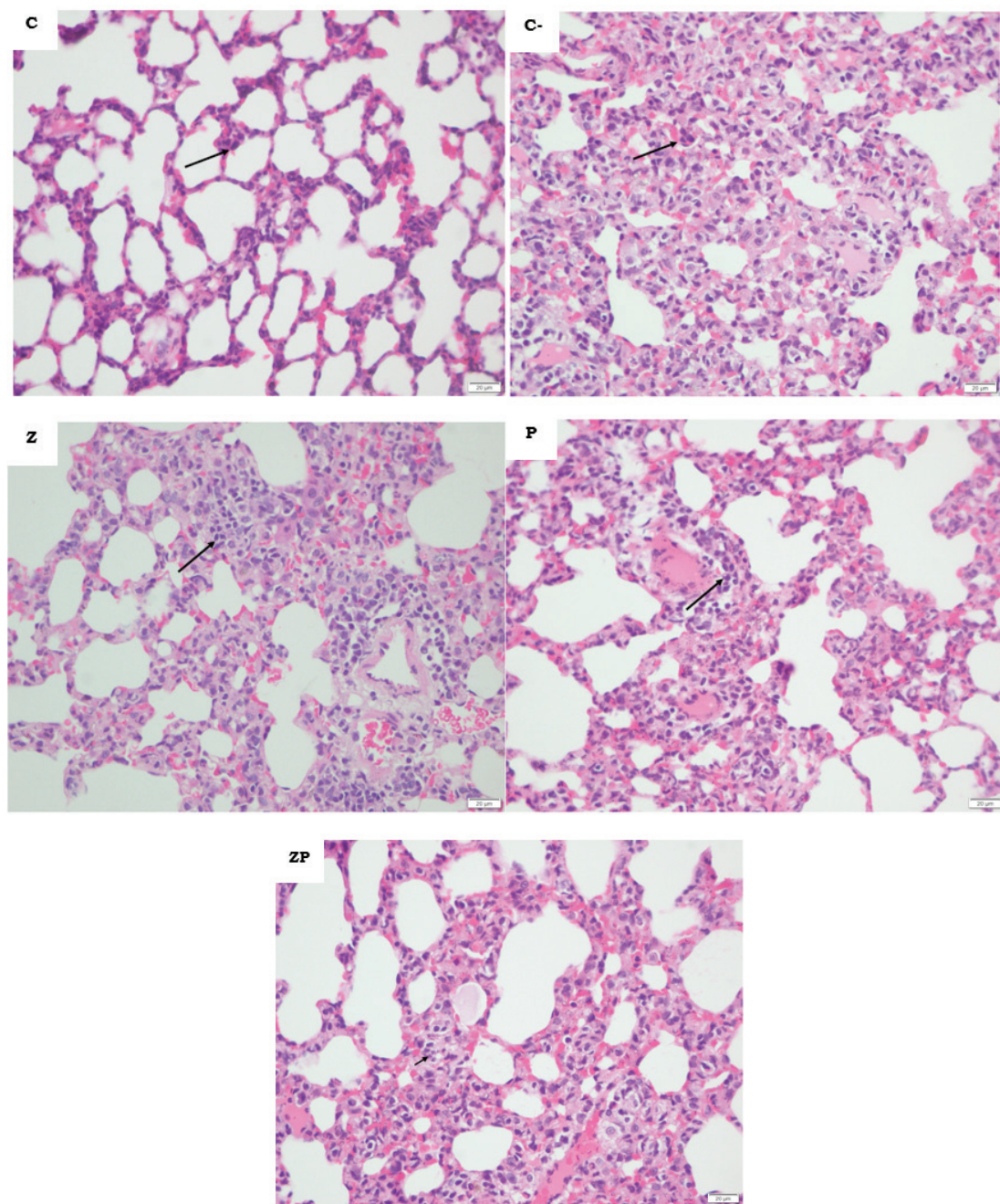


Figure 4. Tissue neutrophils histology. C = control group without treatment; C (-) = exposed to cigarette smoke from two cigarettes per day for seven days; Z = exposed to cigarette smoke from two cigarettes per day and received 5mg/kgbw/day zinc for seven days; P = exposed to cigarette smoke from two cigarettes per day and received one sachet probiotics per day for seven days; ZP = exposed to cigarette smoke from two cigarettes per day and received a combination of zinc and probiotics for seven days. H&E staining was performed on 4 μ m sections. Representative images were obtained by a 400x magnification. Black arrow: neutrophils.

and airway macrophages, leading to inflammation (Lu, Gottlieb & Rounds, 2018). Increased tissue neutrophil levels also occur because of released GM-CSF on inflammation. Studies showed that accumulation of neutrophils on cigarette smoke exposure was caused by increase of neutrophil recruitment and suppression of neutrophil apoptosis. Moreover, nicotine and acrolein delay neutrophil spontaneous death by suppressing Akt deactivation (Xu *et al.*, 2013; Heijink *et al.*, 2015). In this study, rats exposed to cigarette smoke [C (-)] showed an increase in the level of IL-6 (10.61 pg/mL) compared to the C group (5.04 pg/mL) ($p=0.011$). In the lung histology, rat lung sections from C (-) group were presented with an elevation of neutrophils.

Rats exposed to cigarette smoke and receiving zinc (Z group) presented a decrease in IL-6 level (8.69 pg/mL) compared to the C (-) group (10.61 pg/mL) without a significant difference; while tissue neutrophil level in the Z group (43.43 cells) was lower and significantly different than in the C (-) group (63.97 cells). Utomo *et al.* (2020) reported that zinc supplementation is a cytokine regulator, showing that zinc can decrease pro-inflammatory cytokines like IL-6 and TNF- α . The mechanism of zinc decreasing IL-6 levels is by increasing the expression of A20, an anti-inflammatory protein and peroxisome proliferator-activated receptor α (PPAR- α), a mediator involved in lipid metabolism, inflammation, and glucose homeostasis. Furthermore, zinc inhibits IKK β kinase activity, keeps sequestering NF- κ B in the cytoplasm, and inhibits inflammation (Gammoh & Rink, 2017). Anggraeni *et al.* (2015) reported that topical zinc can decrease leukocyte levels in incision wounds. It showed that zinc plays a role in wound healing as a zinc-dependent matrix

metalloproteinases cofactor, which may reduce the risk of infection (Anggraeni *et al.*, 2015). Cahiadewi, Santosa & Suprihati (2016) also reported that zinc supplementation can decrease lung eosinophil numbers in ovalbumin-sensitized mice via intraperitoneal injection and inhalation. Zinc deficiency impairs neutrophil's ability to phagocyte, increases NETs release, and increases neutrophil degranulation. A previous study showed that zinc decreased NETs release through inhibiting citrullination of histone H3 (Kuzmicka *et al.*, 2021). Zinc may also contribute to maintaining the integrity of the membrane and acts as a cytoprotective and anti-apoptotic agent (Liu *et al.*, 2022).

The group exposed to cigarette smoke and received probiotics (P) showed a decrease in IL-6 level (7.31 pg/mL) compared to C (-) group (5.04 pg/mL) with no significant difference. This may be because the 7th day was still the peak time of IL-6 in inflammation (Wang *et al.*, 2010). However, tissue neutrophils level in the P group (34.67 cells) was lower and significantly different than in the C (-) group (63.97 cells). Several studies reported that probiotics strains could modulate immune response through metabolite compounds that produce antimicrobial agents or short-chain fatty acids (SCFAs), such as butyrate, acetate, and propionate, which play a role as immunomodulators in the intestines. Butyrate stimulates anti-inflammatory signalling through GPR109A on colonic macrophages and dendritic cells to induce IL-10 production. Butyrate also stimulates T cells differentiation to T effectors. This mechanism not only affects the intestinal system, but also the respiratory system via the gut-lung axis (Dang & Marsland, 2019). Target sites of probiotics affect NF- κ B. *Lactobacillus casei* could inhibit degradation of I κ B; while *Bifidobacterium*

lactis could inhibit degradation of I κ B and activation of NF- κ B (Bhardwaj et al., 2020). *Bifidobacterium longum* was reported to decrease pro-inflammatory response by decreasing neutrophils recruitment and accumulation in rat lungs induced by *Klebsiella pneumonia* (Lajqi et al., 2020). Another study showed that probiotics contained in Prato cheese – *Lactococcus lactis* and *Lactobacillus casei* O1 decreased total leukocytes in the bronchoalveolar lavage (BAL) of rats exposed to cigarette smoke from 12 cigarettes per day compared to the control group and the group which received conventional cheese containing *Lactococcus lactis* only (Vasconcelos et al., 2019).

Rats exposed to cigarette smoke and receiving zinc and probiotics (ZP group) presented a decrease in IL-6 level (6.02 pg/mL) compared to C (-) group (10.61 pg/mL), with a significant difference ($p=0.022$), though its level was almost the same as in (C) group (5.04 pg/mL). Tissue neutrophils level in the ZP group (29.77 cells) was lower and significantly different than in the C (-) group (63.97 cells). Park et al. (2018) reported that a combination of probiotics complex, rosavin, zinc, and prebiotics could decrease TNF- α , IL-6, IL-1 β , and IL-17 levels. Therefore, a combination of zinc and probiotics could decrease pro-inflammatory cytokines like IL-6. *Lactobacillus* and *Bifidobacterium* are the most common strains of probiotics. *Lactobacillus acidophilus* has been known to reduce STAT3 and phosphorylated STAT3 in mice induced with colitis. Furthermore, *Lactobacillus casei* and *Bifidobacterium lactis* could repair mucosal and liver injuries on colitis in rats (Park et al., 2018). Previous studies showed that probiotics could decrease inflammatory response in Crohn's disease and food allergy by increasing anti-inflammatory cytokines

and decreasing pro-inflammatory cytokines; while zinc supplementation in tuberculosis patients could increase their nutritional status, haemoglobin level, and plasma zinc, and also increase immunity in human immunodeficiency virus (HIV) patients. A combination between zinc and probiotics increases the immune system by increasing lymphocyte levels and decreasing levels of monocytes and neutrophil-to-lymphocyte ratio (NLR) (Widiastuti, Darmono & Sofro, 2019; Setiyaningrum, Darmono & Sofro, 2016). Another study showed that the combination of zinc and probiotics was more effective in resolving diarrhoea in children under five years old than in children who received zinc supplementation only (Surono et al., 2014). Therefore, the combination of zinc and probiotics has a synergistic effect (Park et al., 2018).

CONCLUSION

The repeated consumption of a combination between zinc and probiotics in rats exposed to cigarette smoke was able to reduce inflammatory responses, as indicated by decreased IL-6 and tissue neutrophil levels.

Authors' contributions

Putu GA, principal investigator, conceptualised and designed the study, prepared the draft of the manuscript and led the data collection; Endang M, advised on the data analysis and interpretation, and reviewed the manuscript; Kusmiyati T, reviewed the manuscript; Yan WP, reviewed the manuscript; Neni S, reviewed the manuscript; Hermawan I, conducted data analysis and interpretation.

Acknowledgement

We thank the animal laboratory, GAKY laboratory, and pathology anatomy laboratory for providing animals, reagents, and histopathological analysis in this study.

Conflict of interest

Authors declare no conflict of interest.

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