

ORIGINAL ARTICLE

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Synergistic effect of probiotic and postbiotic on attenuation of PM2.5-induced lung damage and allergic response

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Abstract: To date, few studies have been conducted on the relationship between postbiotics and air pollution, and there is limited knowledge if postbiotic and probiotic have synergistic effects. Therefore, we created a PM-induced lung inflammation mice model and demonstrated the effect of probiotic, postbiotic, and their combination treatment on attenuation of PM2.5-induced lung damage and allergic response. The mice were intratracheally given PM2.5 triggering conditions of acute lung damage and allergic response. Our results showed that individual treatment of probiotic and postbiotic reduced body weight loss by 47.1% and 48.9%, but the results did not show any effect on polarizing IFN- γ /IL-4 ratio. In addition, PM2.5-induced overactive expression of IgE treated by probiotic and postbiotic was reduced by 33.2% and 30.4%, respectively. While combination treatment of probiotic and postbiotic exerted a synergistic effect, especially considerably on improving IgE reduction by 57.1%, body weight loss by 78.3%, and IFN- γ /IL-4 ratio boost by 87.5%. To sum up the above functionality, these research findings may help establish a novel platform for postbiotic application, formulation, and mechanistic selection with regard to PM2.5-induced lung injury.

KEYWORDS

IgE, immunomodulation, PM2.5, postbiotics, probiotics, Th1/Th2

Practical Application: Allergic inflammation caused by PM2.5 is not like common allergens (ex. Pollens, ovalbumin, dust mites), which simply skewing Th1/Th2 polarization to Th2. Thus using probiotics screened by Th1-skewing criteria might not be the best choice to treat on PM2.5-induced symptoms. This research proposed a combination of probiotics and postbiotics on modulating immunity homeostasis, and consequently attenuating complications of PM2.5-induced lung damage. These research findings may help establish a novel platform for postbiotic application, formulation and mechanistic selection with regard to PM2.5-induced lung injury.

1 | INTRODUCTION

Constituents of PM include fine solids or liquid droplets such as dust, smoke, aerosols, fogs, or mixture of them able to be suspended in the air for a long time (You et al., 2018). More recently, a positive correlation has been shown between exposure to PM and numerous chronic diseases, especially heart attack, hypertension, skin/eye allergy, bronchitis, pneumonia, asthma, and other respiratory disorders (Kwon et al., 2020). How PM affects our health? After being inhaled, part of PM_{2.5} (aerodynamic diameter less than 2.5 μm) is able to escape from capture of lymphatic systems, and further gets into the blood circulation via diffusion due to small size (Schraufnagel, 2020). This is why syndromes caused by PM are mostly subjected to cardiovascular, respiratory, and immunity diseases. Moreover, there have been studies showing that PM_{2.5} can also enter the fetus, resulting in negative effect on fetus development (Bai et al., 2020).

Helper T cells are differentiated into two subtypes known as Th1 helper cells (Th1 cells) and Th2 helper cells (Th2 cells). Th1 cells lead to cell-mediated response, characteristically against infected virus and bacteria. They are triggered by the polarizing effector cytokines mainly IFN- γ and IL-12. However, Th2 cells lead to humoral immune response, typically against parasites. Their effector cytokines are primarily IL-4 and IL-5 (Raphael et al., 2015). Th2 hyperresponsiveness to antigen causes IgE-mediated allergy. As a result, Th2 cell-dependent induction of airway inflammation is proposed as a hallmark of asthma (Sahin et al., 2016). To date, there is no consensus on the issue as to whether PM_{2.5}-induced lung damage is categorized as Th1 or Th2 overactivation. Numerous studies have shown that PM_{2.5} causes elevated IL-4 and IgE (Th2 polarization), while other studies have revealed that IFN- γ also increased (Th1 polarization) when exposed to PM_{2.5}. Due to the counteractive roles of Th1 and Th2 cells, PM_{2.5} induces an atypical inflammation symptom which displays elevated Th1 and Th2 polarization simultaneously (Du et al., 2019; Ophir et al., 2019; Yang et al., 2019). This might be the reason why PM_{2.5} causes not only asthma (Th2-dependent), but also pneumonia (Th1-dependent).

Microorganisms can be recognized by host immune cells, giving rise to systemic cytokine release. Several lines of evidence indicates that host-microorganism interaction reaches far beyond the local site (He et al., 2017). Bioactive compounds of microbes or systemic cytokine releases may be absorbed into circulation and further influence responses in peripheral tissues. The gut-lung axis is proposed and addressed on the actions how gut microbes shape cell functionality, immune responses, and disease development of lung.

Probiotics have long been researched for their immunomodulatory function. Probiotics induced endotoxin tolerance, which is a crucial self-defense mechanism that avoids the excessive triggering of innate immune responses. Martinez (2014) points out that the onset and termination of the host immune responses are tightly controlled by our gut microbiome, and the concept of gut-immunity axis is popular and well known based on evidence established. Zhang et al. (2018) tried to use *Lactobacillus rhamnosus* GG to induce protective effects on allergic airway inflammation, and the results have shown that the efficacy is associated with gut microbiome. Juan et al. (2017) also yielded the similar positive consequence on releasing ovalbumin-induced allergic airway inflammation by *Clostridium butyricum* CGMCC0313-1. Hence, use of probiotics inducing immunity tolerance against PM_{2.5} caused lung inflammation, and investigation on the underlying mechanism is our primary goal.

In addition to probiotics, postbiotics refer to dead probiotics (most of them are heat-killed lactic acid bacteria), cell components (lipoteichoic acids, peptidoglycan, genomic DNA, etc.), and metabolites (peptides, enzymes, extracellular polysaccharides, etc.) that exert beneficial functions, such as regulation of metabolic, gastrointestinal, and immunity homeostasis (Wegh et al., 2019). Viability is important to probiotics, but it is no longer the most imperative subject for postbiotics. Postbiotic functions result from their metabolites, cell wall components, or other complex molecules that are produced inside or outside the cells (Rad et al., 2020). Among them, the most famous and well-studied substance might be short-chain fatty acids, which has been reported in numerous clinical trials. Postbiotics have drawn attention due to their safety in the product at a high dose, long shelf life, and ease at transport. What is more, they can also be used in some dosage form on which conventional live probiotics hard to apply (Homayouni Rad et al., 2020).

To date, there have been abundant literatures suggesting that postbiotics improved airway inflammation. Asama et al. (2017) reported that heat-killed *Lactobacillus kunkeei* YB38 improves pneumonia by enhancing IgA production. In addition to oral administration, Tomosada et al. (2013) conducted nasal administration of heat-killed *L. rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 to modulate respiratory antiviral immune responses against respiratory syncytial virus infection. As a result, postbiotic effect on PM_{2.5} challenge is also explored in this research.

In the study, probiotic, postbiotic, and their combination treatment were orally administered to mice that were given PM_{2.5}. After mice were euthanized, the lung tissues were collected and subjected to IFN- γ , IL-4, and IgE quantification by ELISA and histological analysis. Our result

TABLE 1 Histological scoring system for characteristics of peribronchial infiltrates and alveolar involvement

Score	Peribronchial infiltrate	Alveolar involvement
0	None	None
1	Mild (infiltrate ≤ 4 cells thick)	Mild (patchy increased cellularity/ thickening)
2	Moderate (infiltrate 5–10 cells thick)	Moderate (25%–50% visualized lung with increased cellularity/thickening)
3	Severe (25%–50% visualized lumens)	Severe (>50% visualized lung with increased cellularity/thickening)
4	Diffuse (>50% visualized lumens)	

showed that both probiotic and postbiotic attenuated the pathological responses induced by PM2.5 as evidenced by modulation of inflammatory cytokine and IgE level, as well as maintenance of normal body weight. Furthermore, it was observed that combination treatment of probiotic and postbiotic exerted synergistic effects and was possibly via regulation of Th1/Th2 paradigm in the lung.

2 | MATERIALS AND METHODS

2.1 | Source of materials

Probiotic (*Bacillus coagulans* DSM33893) and postbiotic (heat-killed *Lactobacillus plantarum* DSM33894) were commercial products purchased from Chambio Co., Ltd., Taichung, Taiwan. Identification was done by Bioresource Collection and Research Center of Food Industry Research and Development Institute (Hsinchu, Taiwan). The Standard Reference Material of PM2.5 (NIST[®] SRM[®] 2783) was purchased from Sigma-Aldrich Co., Ltd (St. Louis, MO, USA).

2.2 | Animal study

A total number of 50 male C57BL/6 mice (purchased from BioLASCO Co., Ltd, Taipei, Taiwan) were used in this study. Mice were acclimatized under a standard laboratory condition for 15 days before experiments. C57BL/6 mice were randomly divided into five groups: (1) control group, (2) PM2.5 challenge group, (3) probiotic group, (4) postbiotic group, and (5) combination treatment of probiotic and postbiotic group. The control group was healthy mice only orally treated with RO water (100 μ l/day). The PM2.5 challenge group was mice treated with RO water (100 μ l/day) for 15 days by gavage feeding and then intratracheally challenged by PM2.5 (200 μ g/day) for 3 consecutive days. The probiotic group (6.8×10^6 /day), the postbiotic group (1.0×10^7 /day), or combination of probiotic (3.4×10^6 /day) and postbiotic (5.0×10^6 /day) group were mice orally administered with bacterial suspensions in RO water (100 μ l/day) for 15 days. Afterward, the mice were intratracheally given PM2.5 (200 μ g/day) for 3 consecutive days.

2.3 | Preparation of probiotics and postbiotics

A review of dose responses of probiotics in human studies published by Ouwehand (2017) showed that average clinical dosage range was set from 1.0×10^9 to 5.0×10^9 . Although the specific actions of postbiotics rely on definite dosage level, most studies have adopted a specific dose alike probiotics at 10^9 cell level to investigate the beneficial effects. According to a clinical review published by Mu and Cong (2019), human dosage of *B. coagulans* mostly ranges from 1.0×10^9 to 2.0×10^9 per day. As a result, the animal equivalent dose calculation of probiotic and postbiotic is on the basis of the body surface area by conversion of the human dose at 2.6×10^9 and 3.8×10^9 per day, respectively. Probiotic enumeration was operated by plate count methods using De Man, Rogosa and Sharpe (MRS) agar. Postbiotic counting is examined by fluorescent microscope using 4',6-diamidino-2-phenylindole (DAPI) staining.

2.4 | Tissue analysis

After mice were euthanized, the lung tissue was collected and divided into two parts. One was for IFN- γ , IL-4, and IgE ELISA assay (BD Biosciences, San Jose, CA, USA), and the other was for histological analysis (H&E staining). For the part of histological analysis, lung tissue was fixed in paraffin and then stained with hematoxylin and eosin (H&E staining). Ten areas of the lung tissue sections were graded independently and blindly by two well-trained investigators for the status of peribronchial infiltrates and alveolar involvement using an adapted histological scoring system (Table 1; Bayes et al., 2016). Lung lobes were frozen by liquid nitrogen and homogenized at 4°C. Lung homogenates were centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatants of lung homogenates were transferred for subsequent ELISA assay according to the user manual.

2.5 | Statistical analysis

All values are expressed as mean $\pm$ standard deviation (SD). Each value is the mean of three separate

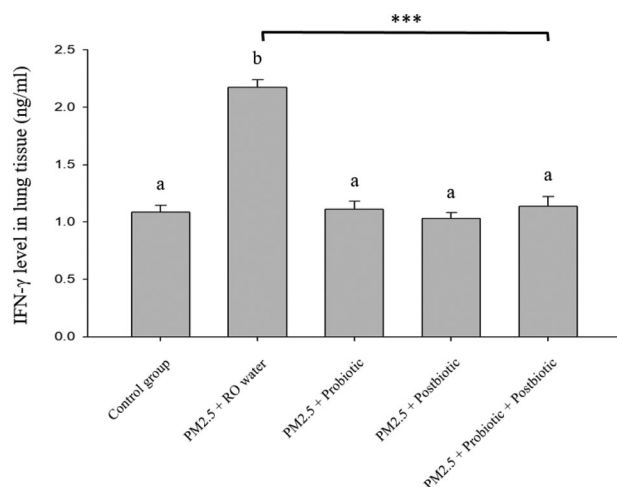


FIGURE 1 IFN- γ level in lung tissue (ng/ml) was assayed by ELISA. Each value is expressed as mean $\pm$ standard deviation ($n = 3$). Means with different letters are considered statistically significant ($p < 0.05$). Statistical significance is presented relative to combination group (PM2.5 + Probiotic + Postbiotic) as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

experiments in each group. Statistical comparisons were carried out by analysis of variance followed by Tukey's range test. $p < 0.05$ was considered significantly different.

3 | RESULTS

3.1 | Probiotic and postbiotic treatment ameliorated PM2.5-induced pro-inflammatory cytokine secretions

As Figure 1 shows, after PM2.5 challenge for 3 consecutive days, increased IFN- γ level (more than twofold) in lung tissue was observed, and this phenomenon is similar to the study by Guan et al. (2019). PM2.5 induces excessive pro-inflammatory cytokine secretions and might cause systemic inflammation and oxidative stress in an animal model. No significant changes were observed in the group of probiotic, postbiotic, and their combination treatment compared to that of control (healthy mice). For Th2 cytokine (IL-4) analysis (shown in Figure 2), PM2.5 challenge caused considerably elevated IL-4 level by 2.2-fold, while in the group of probiotic and postbiotic treatment, the IL-4 expression returned to the same level as the control group. Surprisingly, in the group of combination treatment of probiotic and postbiotic, the IL-4 expression significantly decreased to a lower level (by 75.2%) than the group of probiotic (by 58.1%) and postbiotic (by 58.2%).

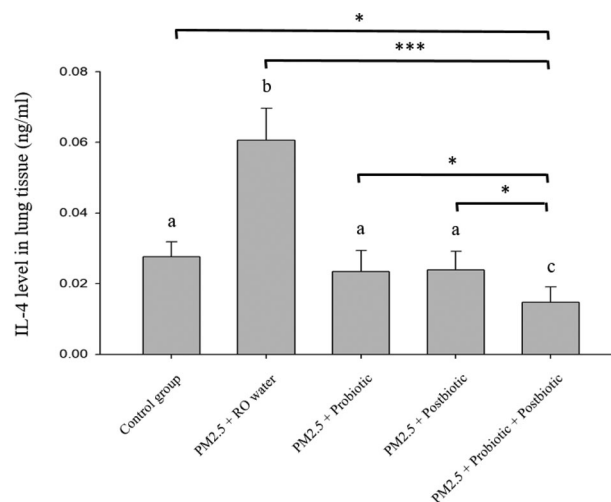


FIGURE 2 IL-4 level in lung tissue (ng/ml) was assayed by ELISA. Each value is expressed as mean $\pm$ standard deviation ($n = 3$). Means with different letters are considered statistically significant ($p < 0.05$). Statistical significance is presented relative to combination group (PM2.5 + Probiotic + Postbiotic) as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

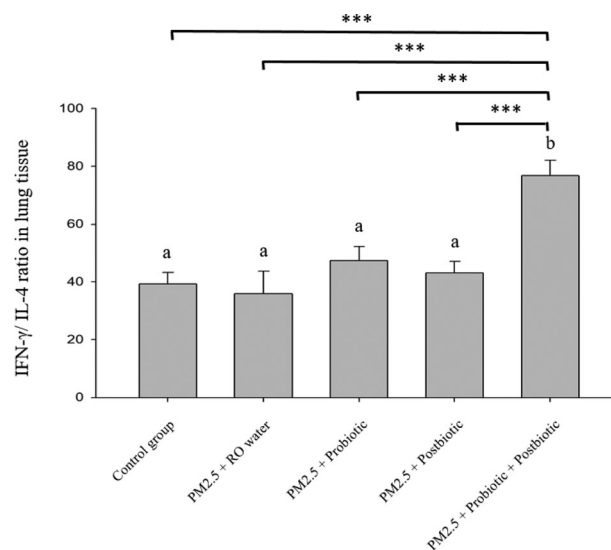


FIGURE 3 IFN- γ /IL-4 ratio (Th1/ Th2) in lung tissue was calculated by means of IFN- γ level divided by means of IL-4 level in each group. Statistical significance is presented relative to combination group (PM2.5 + Probiotic + Postbiotic) as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

3.2 | Combination treatment of probiotic and postbiotic increased Th1/ Th2 ratio

In order to elucidate the effect of PM2.5 exposure on Th1/ Th2 homeostasis, the ratio of IFN- γ / IL-4 was calculated, and the result (Figure 3) showed that the group of combination treatment of probiotic and postbiotic raised the ratio to approximately 80, which is nearly twofold higher

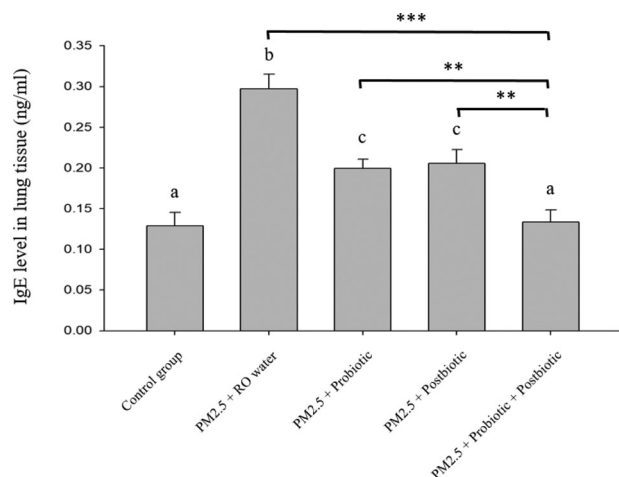


FIGURE 4 IgE level in lung tissue (ng/ml) was assayed by ELISA. Each value is expressed as mean $\pm$ standard deviation ($n = 3$). Means with different letters are considered statistically significant ($p < 0.05$). Statistical significance is presented relative to combination group (PM2.5 + Probiotic + Postbiotic) as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

than that of other groups. However, the group of probiotic or postbiotic treatment alone seems not to launch substantially increased ratio.

3.3 | Probiotic and postbiotic treatment decreased IgE production

Since IFN- γ and IL-4 cytokine expressions showed a simultaneous increase and were difficult to conclude the Th1/Th2 polarization in some groups, IgE secretion is an important marker for evaluating whether PM2.5 challenge caused the characteristic symptoms of allergy or not. As Figure 4 shows, the group of PM2.5 treatment showed increased IgE secretion in lung tissue, and among three treatment groups, combination treatment of probiotic and postbiotic exerted the most significant improvement with regard to IgE reduction by 57.1%. Probiotic or postbiotic treatment alone, although effective (33.2% and 30.4%, individually), did not reduce as well as combination treatment.

3.4 | Probiotic and postbiotic treatment released PM2.5-induced lung tissue injury and body weight reduction

The acute exacerbation of pulmonary infiltration which might cause obstruction of airflow and pneumonia (due to airborne PM) was exhibited by H&E staining (Figure 5) and histological scoring system (Figure 6) for inflamma-

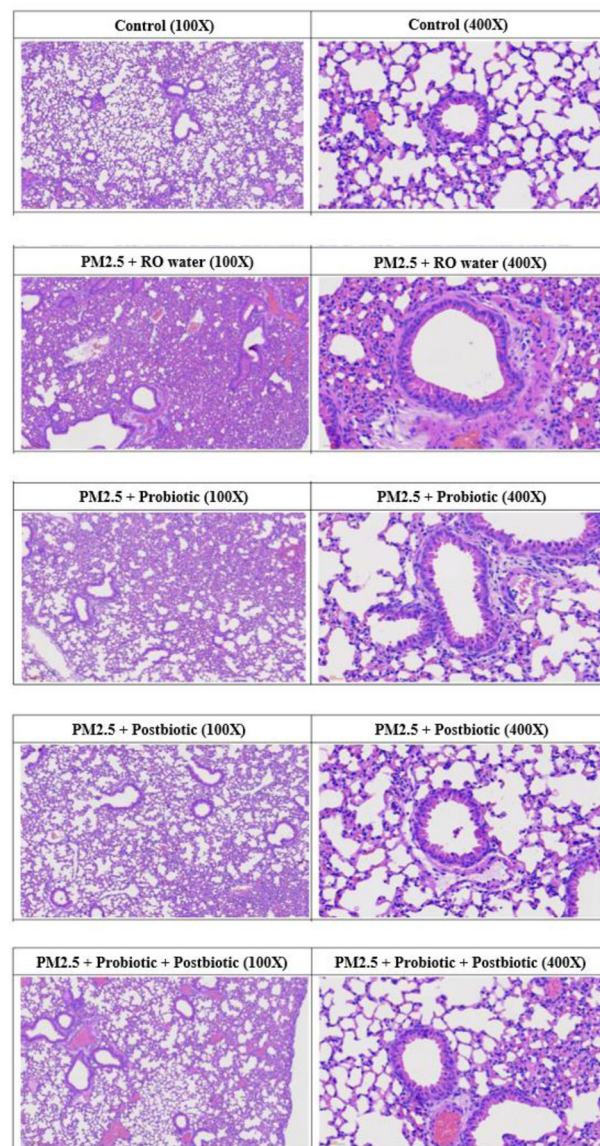


FIGURE 5 Hematoxylin and eosin (H&E) staining was applied on dehydrated lung tissues. The tissue slices are under microscopic examination (100 $\times$ and 400 $\times$ total magnification)

tion in lungs of mice. Characteristics examined contain peribronchial infiltrates and alveolar involvement. H&E-stained lung tissue of control mice displayed normal lung histological features of alveolar septa and bronchiolar epithelium. In the PM2.5 challenge group, lung section showed diffuse alveolar damage characterized by inflammatory cell infiltration around bronchioles and alveolar lining cells. While the histopathological lesion in lung was observed improvement in group of probiotic, postbiotic and combination treatment of them, as the pneumocytes in alveolar spaces were less tightly stacked, and the extents to septal congestion, endothelial cell adhesion and bronchiole swollen were all released compared to group of PM2.5 challenge. Jia et al. (2021) investigated

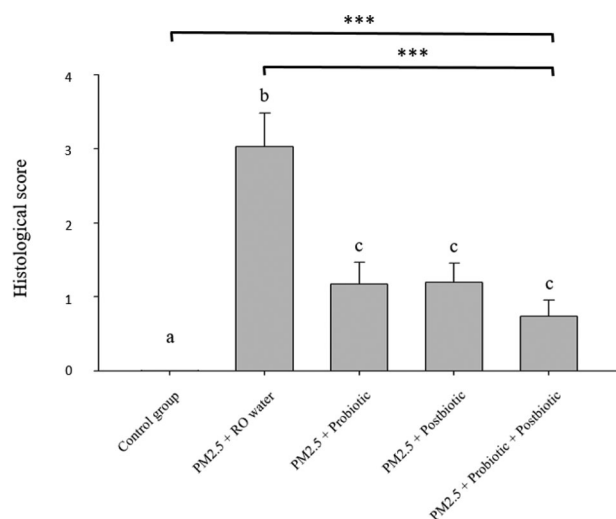


FIGURE 6 Histological scoring system for inflammation in lungs of mice treated with PM_{2.5} particles. Semi-quantitative characteristics examined contain peribronchial infiltrates and alveolar involvement. Means with different letters are considered statistically significant ($p < 0.05$). Statistical significance is presented relative to combination group (PM_{2.5} + Probiotic + Postbiotic) as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

white blood cell counts in bronchoalveolar lavage fluid (BALF) and found an increase of cell numbers in neutrophils, macrophages, lymphocytes, and eosinophils from the mice treated with PM_{2.5}. There are also other scientific results supporting that enhanced numbers of neutrophils, macrophages, lymphocytes, and eosinophils in BALF are triggered by PM_{2.5} exposure, thus giving rise to inflammatory cell infiltration around bronchioles and alveolar lining cells (Ogino et al., 2017; Zhou et al., 2019).

The body weight of PM_{2.5} challenge mice is shown in Figure 7. The result shows that treatment of probiotic and postbiotic improved reduction of body weight loss by 47.1% and 48.9%, respectively. However, combination treatment of probiotic and postbiotic gave rise to reduction of body weight loss by 78.3%. Although the H&E stain results among the three treatment groups were improved to a great degree, body weight changes also played an important role in quantitative evaluation of physiological state. According to our data, the body weight of mice in the combination treatment group improved better than probiotic or postbiotic treatment along.

4 | DISCUSSION

Preceding research has shown that high concentration of PM carries bacterial pathogens, and some respiratory diseases are caused by these airborne bacteria (Li et al., 2020; L. Wang et al., 2019). To analyze the taxonomic compo-

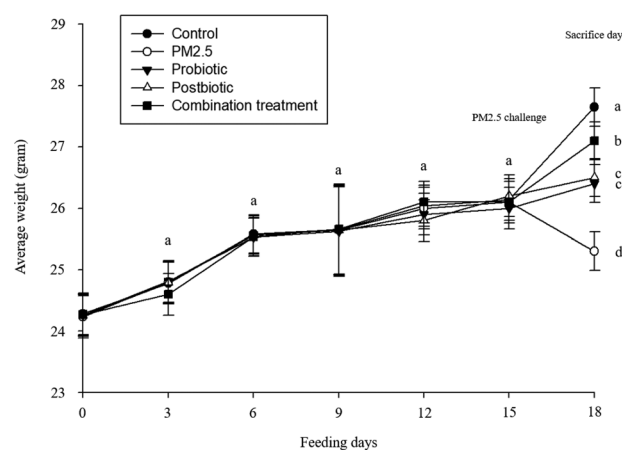


FIGURE 7 The survival rates were recorded once a day, and the body weights were measured once per 3 days. Each value is expressed as mean $\pm$ standard deviation ($n = 3$). Means with different letters are considered statistically significant ($p < 0.05$)

sition alternation of microbiota induced by PM_{2.5}, Qin et al. (2019) used high-throughput sequencing to probe the V3-V4 regions of the 16S rRNA gene and found that PM_{2.5} exposure is highly related to human pharyngeal dysbiosis. Additionally, it has been revealed that exposure to ambient PM_{2.5} alters the composition of not only nasal but gastrointestinal microbiota and causes gut dysbiosis (Liu et al., 2019; Mariani et al., 2018; W. Wang et al., 2018). These results suggest that the negative effects of atmospheric PM_{2.5} are not restricted to the respiratory tract, but also comprise the gastrointestinal tract. The human gastrointestinal tract harbors massive and complex microbiota, which have coevolved with hosts and are involved in the modulation of the immune system (Halloran & Underwood, 2019; Joukar et al., 2020). The observation that germ-free mice have impaired immune responses to pathogens has indicated that the intestinal microbiota contribute to both pro- and anti-inflammatory responses. Furthermore, germ-free mice also lacked innate immune tolerance and might pose an exaggerated pro-inflammatory action once exposed to bacterial endotoxin (Leshem et al., 2020; Singhvi et al., 2020).

PM_{2.5}-induced lung inflammation is very different from those of lipopolysaccharide (LPS)-induced acute lung injury model and ovalbumin (OVA)-induced allergic airway inflammation model (Tsuchiya et al., 2012). LPS induces IFN- γ but not IL-4, while OVA induces IL-4 but not IFN- γ . Our result showed that PM_{2.5} challenge simultaneously elevated levels of IFN- γ and IL-4 in lung tissue. In general, it is believed that raising Th1 cytokines (such as IFN- γ , IL-12) can counteract the generation of Th2 cytokines (such as IL-4, IL-5). It has been shown in the PM_{2.5} challenge group that the level of IgE was far higher than other groups, indicating that mice are in a

severe status of allergic airway inflammation. Nevertheless, in this case of PM2.5 exposure, elevated levels of IFN- γ seem not to result in suppression of allergic responses. For analysis of Th1/Th2 ratio, the PM2.5 challenge group did not show substantial change compared to the control group. This is because of simultaneously elevated levels of IFN- γ and IL-4. It is worth noting that IgE-reducing effect is related to the result of increased IFN- γ /IL-4 ratio in the group of combination treatment of probiotic and postbiotic. Only combination treatment showed improved IFN- γ /IL-4 ratio, and this might be the underlying mechanism contributing to the most significant IgE-reducing effect.

A question arises as to whether PM2.5 triggers Th1 or Th2 polarization and how our body responds to such foreign antigens. Ma et al. (2017) pointed that exposure to PM2.5 induced macrophage-dependent inflammation, featured by elevated Th1/Th17 cytokine secretion. Similar results were also yielded by Gu et al. (2017) and Gao et al. (2020), both studies showing that PM2.5 exposure polarizes Th1/Th17 but not Th2 and promotes the overactivation of the Notch signaling pathway. In contrast, some reports observed that PM2.5 polarizes Th2 (X. Wang et al., 2017; Zhao et al., 2012; Zhou et al., 2019). These reports also proposed that raising Th1 cytokines helps suppress excessive Th2 cytokine expression.

The composition of PM is quite complex, and at least in part comprises heavy metal, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), and some biological contaminants, such as fungal spore, bacteria, viruses, endotoxin, and pollens. In their detailed exploration, Merker et al. (2006) reported that styrene-7,8-oxide (a species of VOCs) induces Th1 cytokines IFN- γ and IL-12 production, but not Th2. Moreover, there have been several lines of research showing that fungal metabolites and pollens stimulate bronchial allergy and asthma (Ghosh et al., 2015; Moss, 2005; Palma-Carlos et al., 1995) via inducing Th2 cytokines and IgE releasing. In addition to one-way driving Th1 or Th2 polarization, Cheng et al. (2020) revealed that PAH exposure induces cytokine storm in preschool children. In this study, increased PAH exposure results in at least nine cytokine level elevations (IL-1 β , IL-4, IL-10, IL-12, IL-18, IL-22, IL-23, TNF- α , and IFN- γ) mediated by AhR and NLRP3 signal transduction. The situation of cytokine storm or systemic inflammatory response syndrome (SIRS) induced by PAHs (one of the main components of PM2.5) is similar to our result which displayed simultaneous increase of Th1 and Th2 cytokines. It is our body's responses to infectious or non-infectious challenges, and there is abnormal regulation of various pro- and anti-inflammatory cytokines that might cause sharp variation of body temperature, heart rate,

respiratory rate, and leucocyte count. To conclude, the immune responses to PM2.5 are multifaceted and this might be due to fluctuated and complex composition of PM2.5 which comes from different sources and districts (Park et al., 2018). In our study, combination treatment of probiotic and postbiotic showed better effects on improving Th1/Th2 ratio, IgE over-production, and weight loss. Probiotics have multiple mechanisms of action. Some are able to promote Th1/Th17 polarization to drive immature dendritic cells (DCs) into mature ones. Others are capable of inducing Treg subsets (IL-10 and TGF- β) and mediate immature DCs to become tolerogenic ones. It has been well studied that differentiation of immature DCs into mature or tolerogenic ones can be regulated by probiotics (Fu et al., 2017; Kim et al., 2018; Rutten et al., 2011). As a result, broad-spectrum probiotic, prebiotic, synbiotic strategy, or cocktail therapy with a combination of antibiotics are often tried in those systemic inflammatory diseases with unclear and multifactorial pathogenies (Sartor, 2003; Schultz et al., 2003; Shimizu et al., 2013). To our knowledge, there has far been relatively little research into the area of probiotic or postbiotic-induced tolerance to PM2.5. Most published studies have focused on suppressing Th2 polarization (Panebianco et al., 2019; X. Wang et al., 2017; Zheng et al., 2020). Since the human immunity responses to PM2.5 and the typical composition of PM2.5 are diverse, the efficacy of probiotic or postbiotic treatment on airway inflammation needs to be investigated in depth because the pathogenesis of PM2.5-induced inflammation is not limited to Th2 overactivation.

5 | CONCLUSIONS

In this study, PM2.5 induced Th1, Th2 cytokines, IgE, and weight loss. Probiotic and postbiotic treatment reduced Th1 and Th2 overactivation, but not affected the ratio of Th1/Th2. While combination treatment yielded significant elevation on Th1/Th2 ratio, its IgE reduction and weight loss improvement effects are even better than probiotic and postbiotic treatment alone. As discussed above, the constituents of PM2.5 vary from different sources. Hence, more sophisticated investigations on proper formulation for different resolutions to PM2.5-caused multifaceted inflammation responses are necessary.

AUTHOR CONTRIBUTIONS

Investigation, methodology, and writing—original draft: **Yi-Heng Chiu**. Investigation, methodology, project administration, and writing—review and editing: **Hung-Pin Chiu**. Supervision and writing—review and editing: **Meei-Yn Lin**.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

All procedures including animal use were approved by the Agricultural Research Institute, Council of Agriculture, Taiwan. The principles of the 3Rs (Replacement, Reduction and Refinement) were reviewed.

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