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A study on the production of tyndallized aloe vera fermented powder with lactic acid bacteria and its immunostimulatory effects

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Abstract:
Background: Immunity is a vital biological defense mechanism that protects the body against infections. Enhancing immunity through dietary supplements is a key strategy to improve health outcomes. Aloe vera and tyndallized lactic acid bacteria are known for their potential immunomodulatory properties. This study aimed to evaluate the immune-enhancing effects of Tyndallized Aloe vera Fermented Powder with Lactic Acid Bacteria (TAVFP-LAB), a novel formulation created by fermenting Aloe vera polysaccharide extract with lactic acid bacteria, followed by tyndallisation and drying.
Materials and Methods: TAVFP-LAB was prepared by fermenting Aloe vera polysaccharide extract with pre-cultured lactic acid bacteria. The fermented product was then tyndallized and dried into a powder. The immunostimulatory activity of TAVFP-LAB was assessed in vitro using mouse spleen cells. Various concentrations of the powder were applied to the spleen cells, and their proliferation was measured using the MTT assay. Additionally, the secretion levels of immune-related cytokines, Interleukin-2 (IL-2) and Interleukin-4 (IL-4), were quantified using ELISA.
Results: The results demonstrated that TAVFP-LAB significantly increased spleen cell proliferation in a concentration-dependent manner, achieving an absorbance of 3.4 ± 0.05 at 100 μg/mL. Moreover, compared to spleen cells treated with concanavalin A (Con A) and lipopolysaccharide (LPS), those treated with TAVFP-LAB exhibited enhanced proliferation and greater secretion of cytokines IL-2 and IL-4. Time and concentration also influenced the viability of splenocytes, with higher concentrations and longer exposure times correlating with increased immune cell proliferation and viability.
Conclusion: The findings suggest that TAVFP-LAB enhances immune function by promoting the proliferation of immune cells and the secretion of cytokines. These effects underscore the potential of TAVFP-LAB as an effective immunostimulant and its applicability as a functional food or dietary supplement aimed at boosting immune function.
Key Word: Tyndalization, Aloe vera, Lactic acid bacteria, Immunity, Splenocyte, Proliferation.

I. Introduction

Immunity is defined as the body's defense against threats, such as disruption of homeostasis or invasion by pathogens. Since 2020, the emergence of new infectious diseases such as COVID-19 around the world has increased the importance of immunity[1]. Innate immunity functions as an initial response when a pathogen enters the body by directly responding to the infection through cells and inducing and maintaining an optimal adaptive immune response[2]. Natural substances are being actively studied to identify cellular activators of these immune functions[3].

Aloe is defined as a Crassulacean Acid Metabolism (CAM) plant, a perennial herbaceous plant in the Liliaceae family with succulent, fleshy leaves, and there are approximately 600 species worldwide. The main constituents of aloe are anthraquinones, including aloin, aloe-emodin, aloenin, and aloesin, and also include polymeric polysaccharides, glycoproteins, amino acids, and minerals[4]. In particular, the total amount of polysaccharides in aloe juice is approximately 10% of the dry weight. The molecular weight of these

polysaccharides varies widely, with an average molecular weight of more than 2 million daltons[5]. These polysaccharides are known to have various bioactive properties. In particular, aloe vera is one of the most widely used natural materials as a pharmaceutical or cosmetic ingredient, and it has been used as an immune material due to its medically proven anti-inflammatory and anti-tumor effects on human and animal gut health and immune activity[6].

Lactic Acid Bacteria (LAB) are beneficial bacteria that ferment sugars to obtain energy, produce large amounts of lactic acid, and prevent the spoilage of more than 50% of the sugars consumed without producing metabolites. In general, the consumption of LAB has been shown to improve digestive health by increasing the abundance of beneficial bacteria that help the gut microbiome digest, absorb, break down, and excrete food. Recent studies have reported a variety of nutritional and health-promoting effects through the synthesis of useful substances through fermentation of lactic acid bacteria, including a variety of nutritional effects, such as a stomachic effect, immune enhancement, liver cirrhosis improvement, anticancer effect, and skin beauty effect[7]. In recent years, technologies have been continuously developed to ferment natural food materials to enhance their functional enhancement properties and convert them into useful ingredients with high bioavailability[8]. However, to date, few technologies have been reported to enhance immune activity by inoculating aloe with LAB followed by fermentation.

Tyndallization is the process of killing a substance by heating it multiple times and holding it in that state. This process is used to inactivate microorganisms without physically damaging the cells[9]. Tyndalized cells have their cellular structure and cell walls remain unaltered and are considered physiologically intact. These cells are much more immunologically active than cells that have not undergone the tyndalization process. In particular, the lactobacilli cells obtained through the tyndalization process retain their immunostimulatory properties specific to gut-associated lymphoid tissue (GALT), completing the immune system of a strong mucosal-associated lymphoid tissue immune system that is responsible for mucosal defense against pathogen attack in the primary and secondary responses of immune system sites present at the level of the digestive tract (especially the intestine)[10].

In this study, based on the 'Method for Manufacturing Tyndalized Aloe Lactic Acid Bacteria Fermented Powder and Immune-boosting Composition Containing the Same' disclosed in Korean Patent No. 10-2021-0049751, TAVFP-LAB, an immune-boosting composition, was manufactured by inoculating LAB into aloe, a natural material, and then fermenting the mixture using the tyndalization process. Therefore, the effect of the combination of aloe and LAB with tyndalization process on improving immune function was evaluated.

II. Material And Methods

Materials and Reagents

The Aloe vera used in this study was obtained from the Kim Jeong Moon Aloe Jeju Farm, which has 3-5-year-old raw leaves that were subsequently washed three times with purified water and peeled[11]. After that, the obtained aloe was liquefied through the process to obtain a viscous aloe polysaccharide extract for the LAB fermentation process. Furthermore, all media and reagents used in this study except those specifically mentioned were purchased from Sigma Aldrich (USA).

Fermentation of TAVFP-LAB using aloe polysaccharides and LAB

1) Preparation of LAB-fermented liquid of aloe polysaccharide extract

The LAB-fermented liquid of aloe polysaccharide extract was prepared using an aloe polysaccharide extract fermentation medium and LAB pre-culture. First, the pH of the aloe polysaccharide extract was corrected to 6.0, and then the concentration was adjusted to 10 ~ 20° Brix. Then, the aloe polysaccharide extract obtained in Method 1 was supplemented with MRS medium components (1.00% Pancreatic digest of gelatin, 0.80% Beef extract, 2.00% Dextrose, 0.20% Dipotassium phosphate, 0.10% Polysorbate 80, 0. 50% Sodium acetate, 0.20% Ammonium citrate, 0.02% Magnesium sulfate, 0.01% Manganese sulfate) were added and autoclaved at 121℃ for 15 minutes to prepare and cool the aloe polysaccharide extract fermentation medium.

Furthermore, to prepare the LAB pre-culture, the LAB pre-culture medium was prepared as MRS medium (1.00% Pancreatic digest of gelatin, 0.80% Beef extract, 2.00% Dextrose, 0.20% Dipotassium phosphate, 0. 10% Polysorbate 80, 0.50% Sodium acetate, 0.20% Ammonium citrate, 0.02% Magnesium sulfate, 0.01% Manganese sulfate, 95.18% Water) was prepared and sterilized at 121℃ for 15 minutes and then cooled[12]. Then, one LAB strain selected from Lactobacillus helveticus (Bifido, Korea) or Bifidobacterium bifidum (BGN4, Bifido, Korea) was inoculated into the medium and incubated at 37°C, 90 rpm for 28 hours with shaking.

The cooled aloe polysaccharide extract fermentation medium was inoculated with 1-5% of the LAB pre-culture and then incubated at 37°C and 90 rpm for 68-76 hours with shaking to prepare the LAB-fermented liquid of aloe polysaccharide extract (Fig. 1).

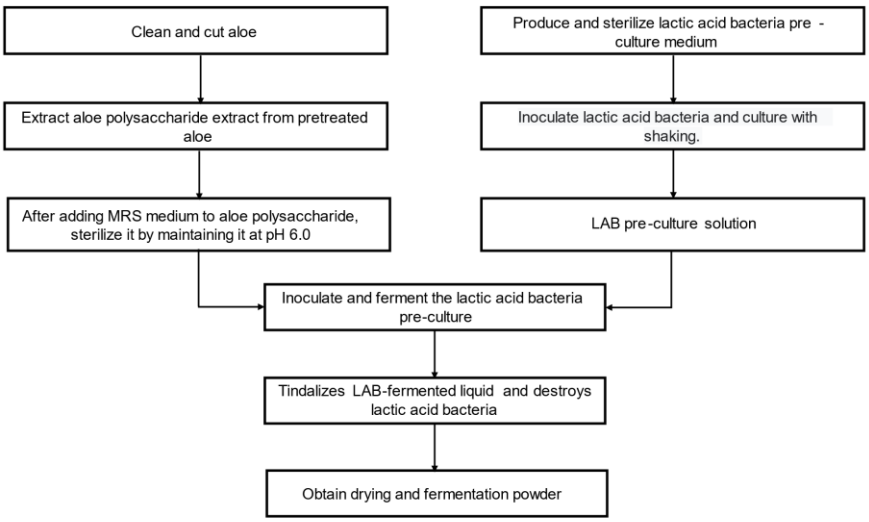

Fig. 1. Flowchart showing the manufacturing process of TAVFP-LAB. The chart shows the overall manufacturing process of the TAVFP-LAB.

2) Preparation of TAVFP-LAB

Tyndalization of LAB-fermented liquid is a method of tindalizing live lactic acid bacteria after fermentation, and the LAB-fermented liquid prepared in Method 2.1 was used to prepare Tyndalized aloe lactic acid bacteria. The LAB-fermented liquid was subjected to the tyndalization process by intermittent sterilization by heating at 150℃ for 30 minutes and rapid cooling to 40℃ or less for degradation. The primary heat treatment killed the trophozoites in the LAB-fermented liquid and promoted lactic acid bacteria inactivation during a 24-hour incubation period. This process was repeated at least twice to kill the live lactic acid bacteria in the LAB-fermented liquid, resulting in tyndalized LAB with inactivated replication and enzymatic capabilities.

The LAB-fermented liquid prepared in Method 2.1 was subjected to lactic acid bacteria body crushing when the total number of lactic acid bacteria reached 5 to 8×109 CFU/ml or more, and the LAB-fermented liquid was connected to a continuous centrifuge (Continuous Centrifuge, J-075AT, MDM Co., Ltd, Korea) at 8,000 rpm for 30 min, and the fermented liquid and bacteria were separated after centrifugation. The isolated bacteria were diluted with 5 ~ 10 times sterile water and crushed under the condition of 40% amplitude, pulse on 20sec, off 10sec, 15℃, 25min (Vibracell, VCX2500, SONICS, USA). The digest was centrifuged again in the same way to remove cell debris and the supernatant was obtained as the final LAB crushed mixture.

The tyndalized aloe LAB - fermented liquid and the LAB crushed mixture were mixed and concentrated under reduced pressure to 0.01 to 0.1% by weight and then freeze dried or spray dried using a binding agent such as maltodextrin. The dried powder was then prepared as the final TAVFP-LAB sample (Fig. 1).

Evaluation of Mouse Splenocyte Proliferation Activity

To evaluate mouse splenocyte proliferation activity against splenocytes, which are cells that play an important role in the immune system, spleens harvested from BALB/c specific pathogen-free mice (Samtako, Korea) were incubated with Roswell Park Memorial Institute (RPMI) 1640 medium (Biolegend, USA). Then the spleens were crushed using a stainless mesh (0.038 aperture), and the splenocyte crush was centrifuged to obtain a precipitate. RBC hemolysis buffer (Red Blood Cell, Biolegend, USA) was added to the obtained sediment to hemolute the red blood cells contained in the sediment. After centrifugation to obtain splenocytes with the red blood cell hemolysate layer removed, the splenocytes were washed, suspended in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS, ThermoFisher, USA), and cultured. Different concentrations (0, 25, 50, and 100 μg/ml) of TAVFP-LAB treated and incubated for 72 hours, and then the number of cultured splenocytes was counted and compared using a hemocytometer and trypan blue exclusion method[13].

Assessment of Cell Proliferation Using the MTT Assay

To determine the changes in the proliferation of immune cells according to the treatment concentration of TAVFP-LAB, a 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. First, cultured splenocytes were suspended in RPMI 1640 medium, and the suspension was inoculated into 96-well plates at 1×10^6 cells/well. Then, they were treated with different concentrations (0, 15, 25, 50 and 100 μg/ml) of TAVFP-LAB, concanavalin A (Con A) or lipopolysaccharide (LPS), and incubated at 5% CO2 and 37℃ for 72

hours to obtain respective cultures. To each culture obtained, 20 µl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, 5 mg/ml) was added and allowed to react for 4 hours, after which the reaction was centrifuged to obtain a precipitate. To this precipitate, 100 µl of eluent was added to dissolve the formazan crystals produced by the cells contained in the precipitate, and the absorbance was measured at 570 nm[14].

Evaluation of Immune-Related Cytokine Secretion Levels

The secretion of immune-related cytokines was evaluated using the enzyme-linked immunosorbent assay (ELISA) kits. The same method as in Method 4 was performed to obtain the respective cultures, except that the prepared TAVFP-LAB and Con A or LPS were treated in combination at various concentrations (0, 15, 25, 50 and 100 µg/ml). The culture supernatants of the cultures were then subjected to an ELISA kit to measure the secretion of immune-related cytokines (interleukin-2 (IL-2) or interleukin-4 (IL-4)) to determine the changes in the amount of immune-related cytokines (IL-2 or IL-4) secreted by each cultured splenocyte[15]. IL-2 is mainly involved in the proliferation and activation of T cells and promotes cell-mediated immune responses, while IL-4 is mainly involved in the proliferation and differentiation of B cells, antibody production, and promotes humoral immune responses[16].

Assessment of Immunoenhancement Activity

The immune-enhancing activity was evaluated by measuring cell proliferation levels. Briefly, cell proliferation levels were measured for viability in the same way as in Method 4 by treating cells with different concentrations (0, 25, 50 and 100 µg/ml) of TAVFP-LAB. As a control, splenocytes cultured without TAVFP-LAB treatment were used.

Statistical analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics, NY, USA) and Student's t-test (*p value* < 0.05*, 0.01**, 0.001***).

III. Result

Number of LAB in the Pre-Cultured Supernatant

The fermented LAB pre-culture was prepared by preparing MRS medium, sterilizing and cooling it, and inoculating it with the LAB strain Bifidobacterium bifidum (strain accession number: KCCM12754P) and shaking it. The prepared lactobacilli pre-culture contained sufficient lactobacilli numbers of lactobacilli (at least 1×10^{10} to 1×10^{12} UFC/ml)[17]. This pre-culture was subsequently used for the fermentation of the aloe polysaccharide extract, which played an important role in improving the quality and effectiveness of the final ferment. The high LAB count facilitated the fermentation process and contributed to maximizing the fermentation efficiency of the aloe polysaccharide extract[18].

Number of Immune Cells Proliferated by TAVFP-LAB

To evaluate the effect of TAVFP-LAB on splenocyte proliferation, splenocytes were counted and compared using a hemocytometer and trypan blue exclusion method. The results showed that the number of splenocytes gradually increased with the treatment concentration of TAVFP-LAB (0, 25, 50, and 100 µg/ml) to 2.7 ± 0.05, 2.9 ± 0.1, 3.1 ± 0.05, and 3.4 ± 0.05, respectively (Fig. 2). This suggests that TAVFP-LAB significantly promoted the proliferation of splenocytes in a concentration dependent manner.

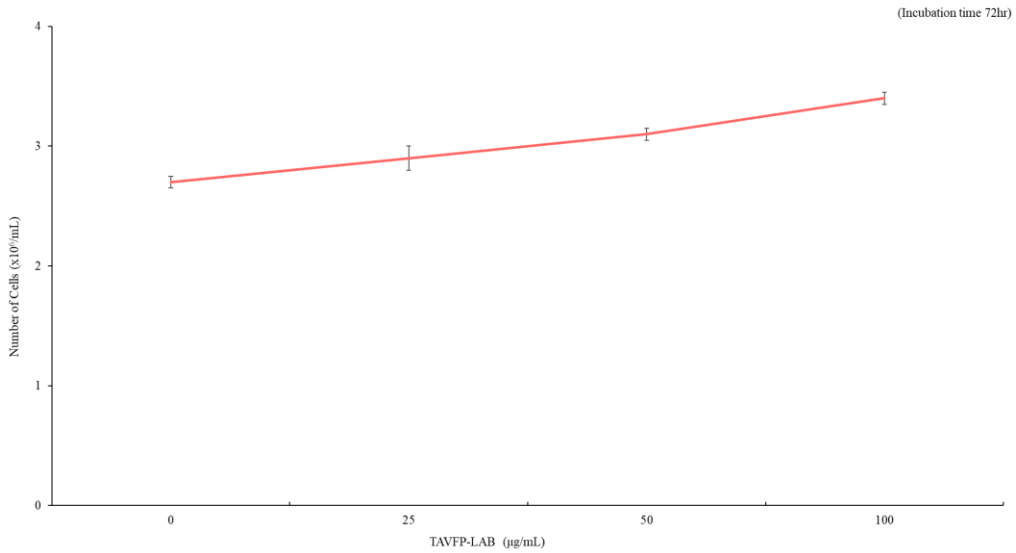

Fig. 2. Number of cells after 72 hours at different treatment concentrations TAVFP-LAB. An increase in the number of immune cells indicates a change in the level of proliferation of immune cells. The graph shows that the number of cells increased with increasing concentration of TAVFP-LAB.

Comparison of Immune Cell Proliferation Activity

Using splenocytes and the MTT assay, we evaluated whether TAVFP-LAB affected the promotion of cell proliferation. The absorbance at 570 nm was measured, and as shown in Fig. 3, the proliferation level of immune cells increased with the concentration of TAVFP-LAB (0, 15, 25, 50, and 100 μg/ml). The concentration of TAVFP-LAB showed much greater proliferative activity than Con A and LPS. In particular, the cell proliferation level gradually increased with the concentration of TAVFP-LAB to 1.2 ± 0.1, 1.5 ± 0.1, and 3.2 ± 0.1 at 25, 50, and 100 μg/ml, respectively. This indicates that TAVFP-LAB promoted the proliferation of immune cells in a concentration-dependent manner, showing the positive effect of increasing the concentration of TAVFP-LAB on immune cell proliferation.

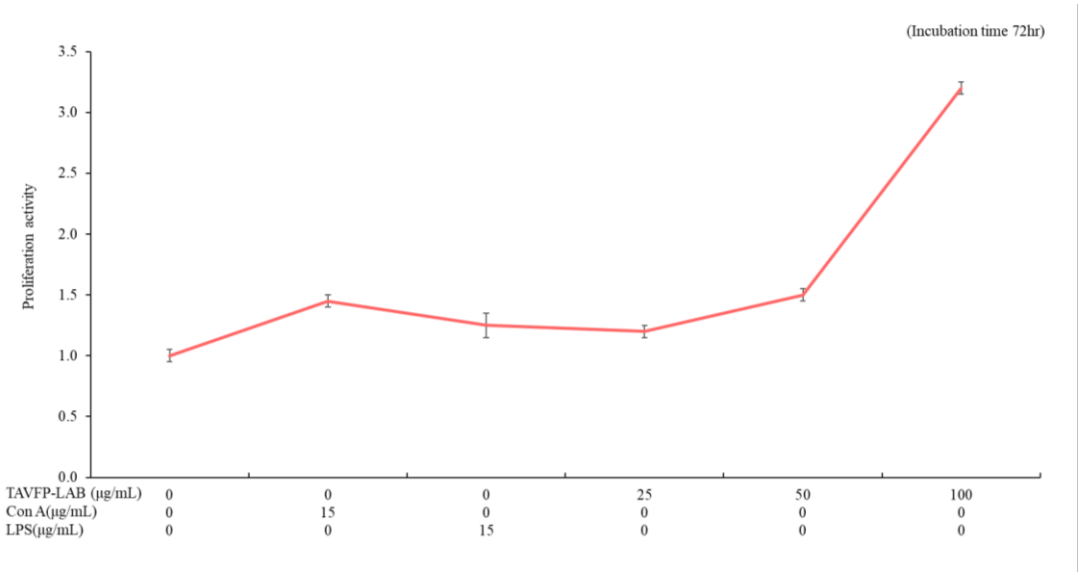

Fig. 3. Proliferation activity of splenocytes in mice after 72 hours as a function of treatment concentration of TAVFP-LAB. Proliferation activity increased significantly with increasing concentration of TAVFP-LAB compared to Con A or LPS.

Cytokine Secretion by TAVFP-LAB

The secretion of immune-related cytokines (IL-2, IL-4) from splenocytes was assessed using ELISA kits. The levels of cytokines secreted by splenocytes tended to be affected by Con A or LPS treatment, but generally increased as the treatment concentration of the TAVFP-LAB increased. In particular, the increase in secretion of

IL-4 was greater than that of IL-2 (Fig. 4). This suggests that TAVFP-LAB may be more effective in promoting the secretion of IL-4, which may be involved in B-cell proliferation and differentiation and antibody production.

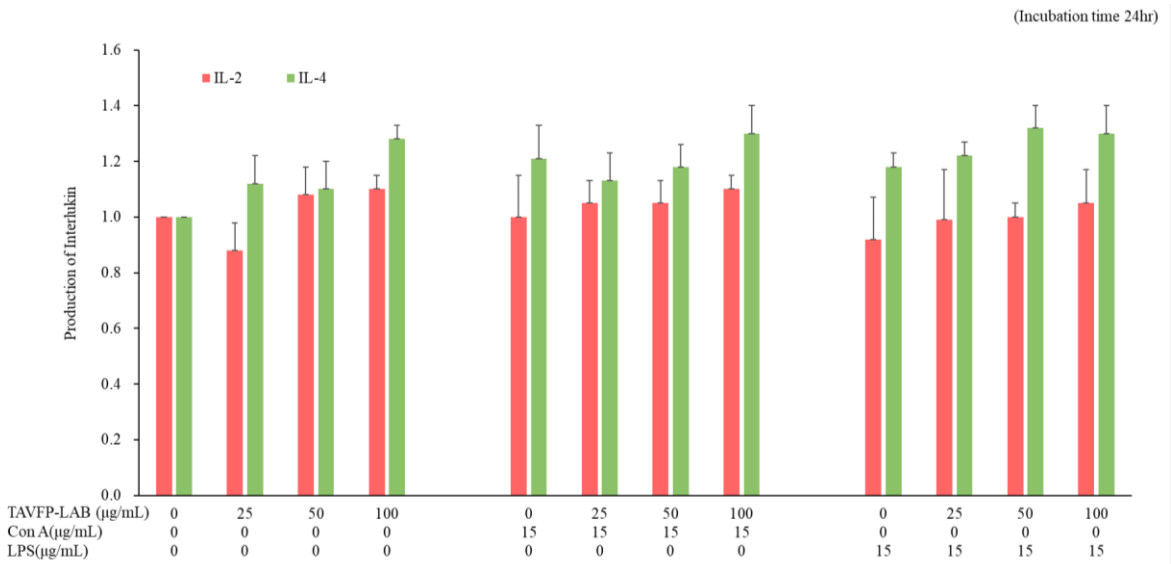

Fig. 4. Production of Interlukin levels secreted by splenocytes after 24 hours in response to different concentration combinations of TAVFP-LAB. Con A or LPS. Red color indicates IL-2, green color indicates IL-4. Production of interleukins increased overall as affected by the concentration of TAVFP-LAB, although not as affected by Con A or LPS. The graph shows a tendency for IL-4 to increase more than IL-2.

Immunoenhancement Activity Over Time

The immunostimulatory activity was assessed by measuring the level of cell proliferation. Different concentrations (0, 25, 50, 100 μg/ml) of TAVFP-LAB were treated and cell proliferation levels were measured using the MTT assay. According to Fig. 5, splenocytes cultured for 24 to 72 hours after treatment with TAVFP-LAB increased as follows compared to the control: 1.2-fold at 25 μg/ml, 1.5-fold at 50 μg/ml, and 2 to 3-fold at 100 μg/ml. This indicates that TAVFP-LAB improves the viability of splenocytes, and the viability increases with concentration and time.

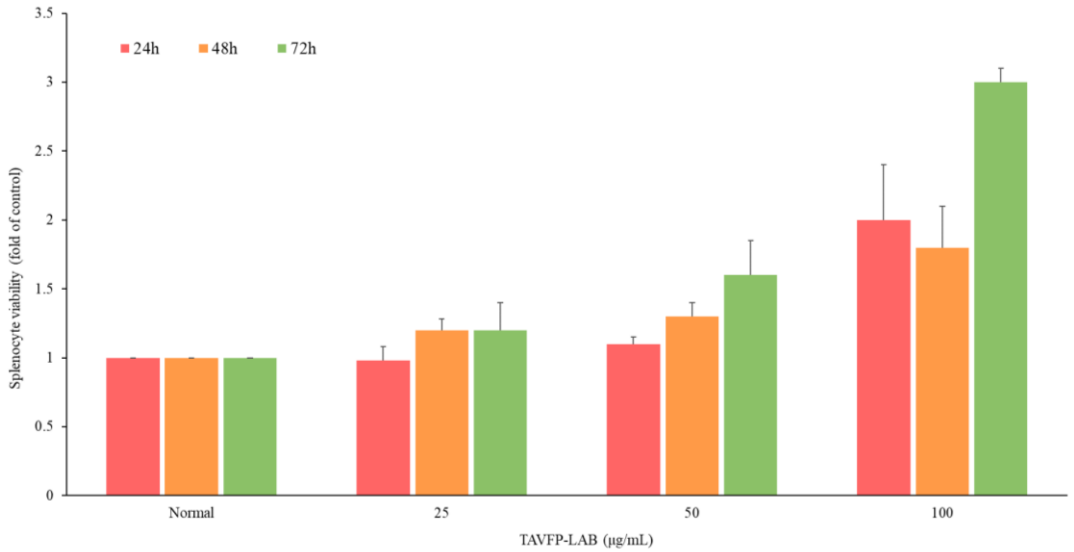

Fig. 5. Splenocyte viability of TAVFP-LAB a function of treatment level and treatment time. Red indicates after 24 hours, orange after 48 hours, and green after 72 hours. Splenocyte viability increased with time and concentration compared to the control.

IV. Discussion

This study aimed to enhance immunity by producing a TAVFP-LAB through the use of a fermentation medium containing Aloe polysaccharide extract and LAB pre-culture, and subsequently evaluating its immuno-enhancing effects. Previous studies have confirmed that polysaccharides extracted from aloe vera gel have potent immunomodulatory activity in vivo[19]. In addition, the killing of LAB, which regulate innate immunity, through the process of tyndalization, has been shown to modulate immune responses by inducing immune-enhancing substances[10,20]. However, little research has been done on fermented powders that combine these two substances. Previous studies have confirmed an increase in antioxidant activity in probiotic fermented juices prepared with aloe vera, but have not addressed some of the challenges of using live LAB and their effect on immune enhancement [10,21]. Therefore, this study is significant as it is the first attempt to determine the effects of a TAVFP-LAB combining the benefits of aloe vera and LAB on immunity.

The spleen plays an important role in innate immunity, and an increase in splenocytes implies that it can modulate immune responses[22,23]. In the present study, we investigated the immune-enhancing effect of TAVFP-LAB through the proliferation of splenocyte numbers. The results showed that TAVFP-LAB increased the number of splenocytes in a concentration-dependent manner. The proliferation level of splenocytes treated with TAVFP-LAB was higher than that of the Con A and LPS treatment groups and increased continuously in a concentration-dependent manner, reaching 2-3 times higher than the initial level. This suggests that TAVFP-LAB is a potential immune enhancer that can enhance immunity by promoting the proliferation of immune cells in a concentration-dependent manner. The levels of immune-related cytokines IL-2 and IL-4 were measured using an ELISA kit. The results indicated that as the concentration of TAVFP-LAB increased, the levels of IL-2 and IL-4 secreted by splenocytes generally rose, with a particularly notable increase in IL-4 secretion. This demonstrates that TAVFP-LAB can affect the activation of B cell proliferation, antibody production, and class switching in the context of immune responses[24]. These observations indicate that TAVFP-LAB has a positive effect on immune cell activation and modulation of the immune response. Finally, immunopotentiating activity was evaluated, and the proliferation of splenocytes treated with TAVFP-LAB increased approximately 1.2- to 3-fold from 24 to 72 hours compared to that of control. The results showed that TAVFP-LAB improved the viability of splenocytes in a concentration- and time-dependent manner. The increase in viability over time is consistent with the ability of L. acidophilus to stimulate immune responses over time and supports the observation that TAVFP-LAB increased splenocyte numbers[25]. These findings provide evidence that TAVFP-LAB has the potential to enhance immunity by promoting splenocyte viability and proliferation.

These results are different from those of previous studies. For example, Lactobacillus strains from aloe vera possess probiotic properties such as antimicrobial properties and intestinal viability, but they were not combined and did not undergo the tyndalization process, leading to problems with the use of live bacteria[26]. This study addresses these issues and presents a safe and effective immune enhancer through the process of tyndalization.

Taken together, these results confirmed that TAVFP-LAB can enhance immune function by promoting the proliferation of splenocytes and the secretion of immune-related cytokines, thereby regulating immune responses. In particular, the enhancement of immune cell proliferation and survival rate as a function of the concentration and time of TAVFP-LAB suggests the possibility of utilizing it as a new food and dietary supplement for functional food and immunity enhancement. Compared to previous studies, TAVFP-LAB is an immune enhancer is safe and effective, and is expected to be used in various applications in the future.

V. Conclusion

This study successfully developed Tyndallized Aloe vera Fermented Powder with Lactic Acid Bacteria (TAVFP-LAB) and demonstrated its significant immunostimulatory effects. TAVFP-LAB enhanced splenocyte proliferation and increased the secretion of cytokines IL-2 and IL-4 in a concentration-dependent manner, indicating its potential as an immune-boosting functional food or dietary supplement. These findings highlight TAVFP-LAB's promise in enhancing immunity, warranting further in vivo validation and exploration for broader applications.

Ethical Statements

This study was approved by the Animal Care and Use Committee of Konkuk University (KKUASP (SE)-23-025). The animals were divided into 11 test groups, each containing 8 mice. Each test group was sacrificed 3 days after treatment to evaluate the effects.

Acknowledgements

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