Effect of multi-strain probiotics on enhancing apoptosis in lung cancer cells A549

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ABSTRACT

Introduction: Apoptosis is an important pathway for regulating cell proliferation and the occurrence of cancers such as lung cancer. The release of Caspase-9 and activation of Caspase-3 influence the process of apoptosis in lung cancer cells, making these proteins markers of apoptosis in lung cancer cells. Multi-strain probiotics show increased benefits due to the combined effects of different strains. The probiotic metabolites, such as short-chain fatty acids (SCFA), become important metabolites in lung immunity. This study aimed to examine the effectiveness of multi-strain probiotics in increasing apoptosis through increasing Caspase-3 and Caspase-9 expression in A549 cultures.

Material and Methods: The study was conducted in vitro using a cytotoxic assay to determine the IC_{50} value of multi-strain probiotics, followed by an In Cell Western assay to assess Caspase-3 and Caspase-9 expression.

Results: The cytotoxic assay IC_{50} result of the multi-strain probiotic was 58.952 ppm. Based on the result of the cytotoxic assay, the multi-strain probiotic in this study is not toxic to A549 lung cancer cells. In the Cell Western assay results, the multi-strain probiotic at concentrations of $2xIC_{50}$ and $0.5xIC_{50}$ showed the highest Caspase-3 and Caspase-9 expression, respectively, compared to the control group.

Conclusion: Multi-strain probiotics can stimulate the expression of Caspase-3 and Caspase-9 proteins in A549 lung cancer.

KEYWORDS:
Caspase-3; Caspase-9; lung cancer; multi-strain probiotics; apoptosis

INTRODUCTION

Lung cancer is one of the most fatal malignant cancers worldwide. According to GLOBOCAN 2020, it ranks second after breast cancer in terms of cancer incidence, with 2.2 million cases (11.4%). However, based on mortality rates, lung cancer ranks highest, causing 1.8 million deaths (18%), followed by colorectal cancer (9.4%) and liver cancer (8.3%).[1] Globally, Asia accounts for the largest proportion of lung cancer cases (58.2%), and Indonesia is one of the countries with the highest incidence, even ranking first in mortality.[2] Lung cancer risk factors fall into two main categories: genetic predisposition and environmental exposure. Environmental factors include smoking, chemical exposure, outdoor air pollution, exposure to metals, and ionising radiation. Smoking is the most dominant factor causing breast cancer and is responsible for 80% of cases in men and 50% in women.[3] Inhaled tobacco smoke contains approximately 4,000 chemical compounds, 69 of which are carcinogenic. Repeated exposure to tobacco and these carcinogenic agents can damage cells by affecting cell functions such as cell proliferation, apoptosis, and Deoxyribonucleic Acid (DNA) repair.[4]

Cellular disorders in lung cancer cause uncontrolled cell proliferation and impaired apoptosis. It begins with the release of cytochrome C due to changes in membrane permeability, and then activates apoptotic protease-activating factor-1 (Apaf-1). Apaf-1 will combine with proCaspase-9 to form an apoptosis complex that activates Caspase-9.[5] Caspase-9 will activate the Caspase-3 protein, and then the cytoplasmic endonuclease will be activated. This mechanism plays a role in degrading DNA chromosomes and the cytoskeleton. Then, nuclear fragmentation will form apoptotic bodies, which are phagocytosed by phagocytic cells.[6] However, in many cancers, such as lung, breast, prostate, colon, and colorectal cancer, apoptosis is suppressed due to overexpression of anti-apoptotic proteins like B-cell lymphoma 2 (Bcl-2) and downregulation of pro-apoptotic proteins like Bcl-2-associated protein X (BAX). Thus, it hinders Caspase activation and promotes resistance to therapy.[7] Lung cancer treatment involves several treatment modalities, including surgery, radiotherapy, systemic therapy (chemotherapy, immunotherapy, and targeted agents), radiological interventions, and palliative care.8 While chemotherapy is widely used, it often causes collateral damage to healthy cells and faces challenges due to cancer resistance, often caused by dysregulated apoptotic mechanisms.[9] Unfortunately, resistance to chemotherapy is an obstacle to treating lung cancer effectively.[8]

Probiotics have gained attention as potential adjuvant therapies in cancer management due to their

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antiproliferative and pro-apoptotic effects. Probiotics can downregulate nuclear factor-kappa B (NF-κB), reducing Bcl-2 expression and promoting apoptosis.[10] Many probiotics have shown benefits, including *Lactobacillus* spp., which are found in the gastrointestinal tract, vagina, oral cavity, respiratory tract, and skin. The use of probiotic *Lactobacillus* has been widely studied for its involvement in the treatment of lung cancer.[11-15] A single-strain study of *L. rhamnosus* administration to mice showed that the probiotic can inhibit B16 melanoma metastasis in the lungs.[12] In vitro test of L. casei administration showed a significant inhibitory effect on the proliferation of A549 lung cancer cells.[13] In other studies related to the administration of *L. casei*, it can increase antitumor activity against lung carcinoma in mice.[14] In addition, administration of *L. casei* reduces the side effects of chemotherapy so that it can improve the prognosis of lung cancer patients.[15] This suggests that some *Lactobacillus* may have the potential to be effective adjuvants for treating lung cancer. In recent years, many studies have reported the relationship between the gut and lungs, which is described as the gut-lung axis.[16-18] Some potential mechanisms for Lactobacillus to reach the lungs: *Lactobacillus* or its components and metabolites, such as short-chain fatty acids (SCFAs), which are in the intestinal lumen and then taken up by intestinal epithelial cells, enter the lungs through circulation, or by *Lactobacillus* or its components from the intestinal lumen reaching the lungs directly through micro breathing or oesophageal reflux.[19]

The use of multi-strains has its advantages because the characteristics of the different strains can increase colonization, so the chances of survival of the probiotic strain increase.[20] The use of multi-strain probiotics may show increased benefits from the preparation due to the combined effects of different strains in one preparation compared to a single strain. Having multiple strains can help maximize the scope of benefits and provide complementary benefits to each other.[21] Current research related to the use of multi-strain probiotics in lung cancer is still limited, so further in vitro research is needed to determine the anticancer activity of multi-strain probiotics against A549 lung cancer cells using the Resazurin assay method with PrestoBlue reagent to obtain the IC_{50} value. After obtaining the results of the cytotoxic test in the form of an IC_{50} value, it was continued with the In Cell Western Assay (ICW) test to see the effect of administering multi-strain probiotics in increasing apoptosis in A549 lung cancer cell cultures through the expression of Caspase-3 and Caspase-9.

MATERIALS AND METHODS

Cell Line A549 Culture

A549 cells were thawed in a 37°C water bath until fully melted. The cells were resuspended in Roswell Park Memorial Institute (RPMI) media, then centrifuged at 3000 rpm for 5 minutes. The pellet was resuspended and cultured in a 3 cm petri dish with 2.5 ml of media, incubated for 24 hours, and monitored for >80% confluence. Once confluence exceeded 80%, the media was removed, the dish was rinsed twice with PBS, and 0.2 ml of trypsin was added, followed by a 5-minute incubation. After adding 1.3 ml of media, the cells were divided into three microtubes and centrifuged. The pellet from the first tube was cultured in a 10 cm petri dish with 10 ml RPMI media for further analysis, while pellets from the second and third tubes were stored in cryotubes with media and 10% Dimethyl Sulfoxide (DMSO) as stock.

Cytotoxic Assay

Toxicity testing was carried out using the Resazurine method using Presto BlueTM reagent.[22] A549 cells that have been incubated in the reculture process and confluence >80% indicate that the cells are ready for seeding. The media in the reculture petri dish is removed, rinsed 3 times using PBS, and 1 ml of trypsin is added. Incubate for 5 minutes to detach the cells, followed by the addition of 1 ml of media. After homogenization, the cell suspension was centrifuged at 3000 rpm for 5 minutes. The pellet was resuspended with 1 ml of media, then 10 µl of the suspension was mixed with 10 µl of trypan blue. A 10 µl aliquot of this mixture was used for cell counting with a hemacytometer. Cell calculation uses the following formula [1]:

$$\text{Counted cell count (cell/ml)} = \frac{(\text{number of living cells})}{4} \times \text{dilution factor} \times 10.000 \quad [1]$$

Planting cells in a 96-well plate has a target of 75.000 cells/well, so the volume of cells that can be transferred can be calculated using the following formula [2]:

$$\text{Volume of cells transferred} = \frac{\text{Total number of cells required}}{\text{Counted cell count)}} \quad [2]$$

Cells that have been planted in 96-well plates will be incubated and their confluence observed before being given the treatment. Treatment was carried out by adding blanks, media, Doxorubicin (positive control), solvent control (Mili-Q), and samples with various concentrations. The tests were performed in triplicate. Incubation was carried out for 48 hours. Presto blue was added with a 1:9 ratio of presto blue and media. The addition of presto blue to each well is 100µl, then incubated for 2 hours. The plate will be read at a wavelength of 570 and 600nm to measure absorbance, which will then be converted into a percentage of viability using a multimode reader Tecan 200M pro.

IC50 calculation from the experimental results obtained from absorbance from 3 types of controls and test compounds, including cell control (culture media + cells), solvent control (cell culture media + milli-Q water), positive control (Doxorubicin), and test compound (culture media + cells + test compound). The IC_{50} calculation step using the percentage of cell viability uses the following formula [3]:[23]

$$\%\ \text{Living Cell} = \frac{(\text{Absorbance of Treatment-Absorbance of Control Media})}{(\text{Absorbance of Cell Control-Absorbance of Control Media})} \times 100\% \quad [3]$$

In Cell Western Assay

A549 cells with a confluent level of >80% were counted using a hemacytometer. Cells were seeded and treated with various concentrations of the samples and incubated for 48 hours. The tests were performed in triplicate. Reagents in the form of secondary antibodies, Cell-Tag 700, washing solution

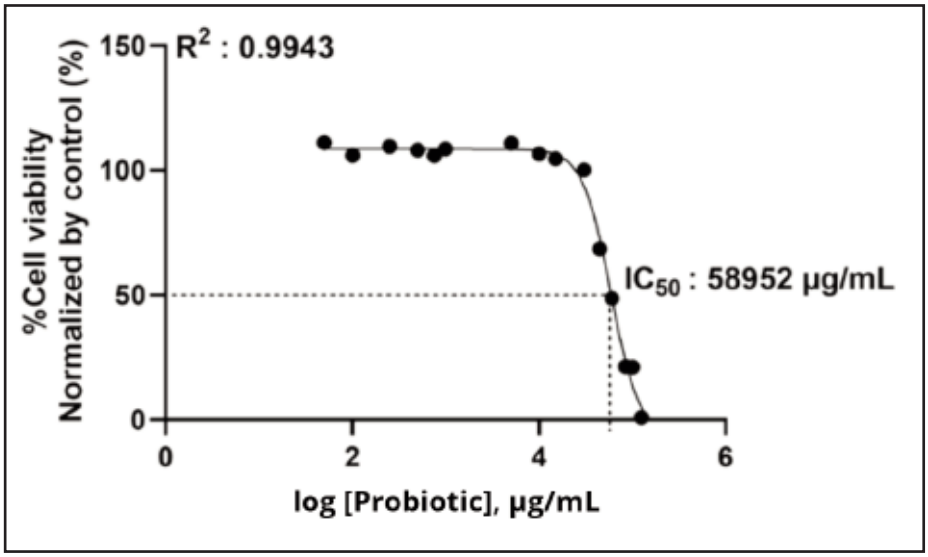

Fig. 1: Cytotoxic test result curve of multi-strain probiotic on A549 lung cancer cells.

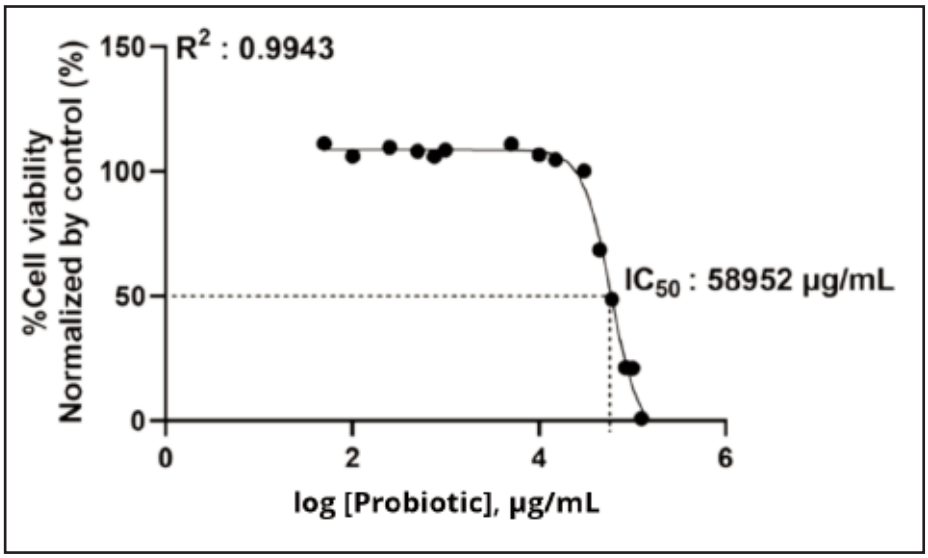

Fig. 1: Caspase-3 fluorescence results of multi-strain probiotic.

*A1-B1: Negative control background; C1-D1: Positive control background; A2-B2: Negative control; C2-D2: Positive control; A3-D3: Multi-strain Probiotic Background ½, 1, 1½, 2 x IC_{50}; A4-D4: Multi-strain Probiotic ½, 1, 1½, 2 x IC_{50}; A5-D5: Multi-strain Probiotic ½, 1, 1½, 2 x IC_{50}.

(Tween-20 + Tris buffer), fixation solution (37% formalin + Tris buffer), and permeabilization solution (10% Triton X-100 + Tris buffer) were prepared. A total of 150 µL of fixation solution was added to a 96-well microplate and incubated for 20 minutes on a rocker shaker, then the fixation solution was discarded. 150µL of permeabilization solution was added and incubated for 20 minutes, then the solution was discarded. 150µL of blocking solution was added, incubated for 30 minutes, and then the solution was discarded. A 50µL diluent solution was added to the background well, while 50µL of primary antibody was added to all other wells. The wells were then incubated overnight at 2–8°C. After incubation, the primary antibody solution was discarded, and 150µL of wash solution was added and incubated for 5 minutes; it was then discarded. This washing step was repeated four times. A 50µL secondary antibody without Cell-Tag 700 was added to the background well. In comparison, 50µL of secondary antibody with Cell-Tag 700 was added to the remaining wells and incubated for 1 hour, then discarded. Four more washes were performed with the wash solution. The 96-well microplate was then read using Odyssey® XF.

Statistical Analysis

The results obtained in this study were the percentage of living cells and the expression of Caspase-3 and Caspase-9. The living cell data obtained were analysed using statistical methods with the GraphPad Prism 9 program. The results of the analysis obtained with this program were in the form of

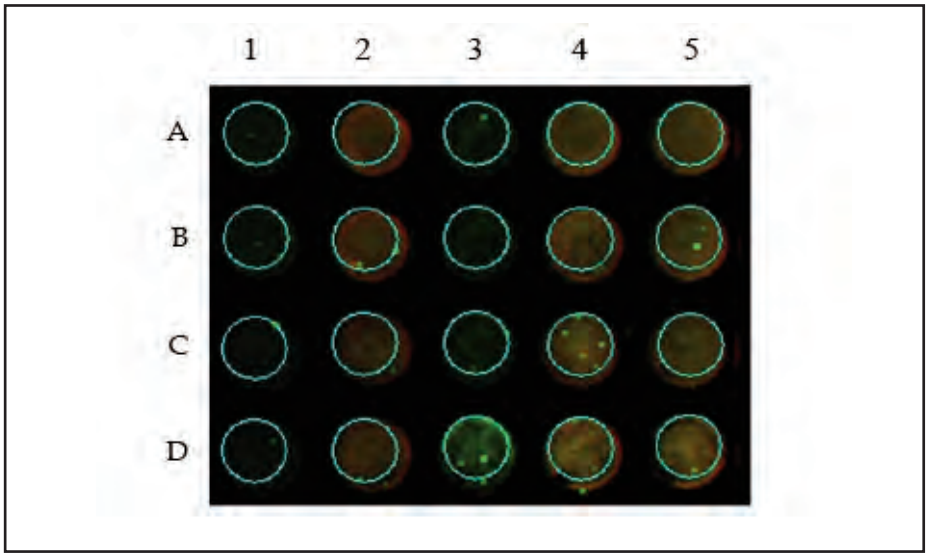

Fig. 3: Caspase-9 fluorescence results of multi-strain probiotic.

*A1-B1: Negative control background; C1-D1: Positive control background; A2-B2: Negative control; C2-D2: Positive control; A3-D3: Multi-strain Probiotic Background ½, 1, 1½, 2 x IC_{50}; A4-D4: Multi-strain Probiotic ½, 1, 1½, 2 x IC_{50}; A5-D5: Multi-strain Probiotic ½, 1, 1½, 2 x IC_{50}.

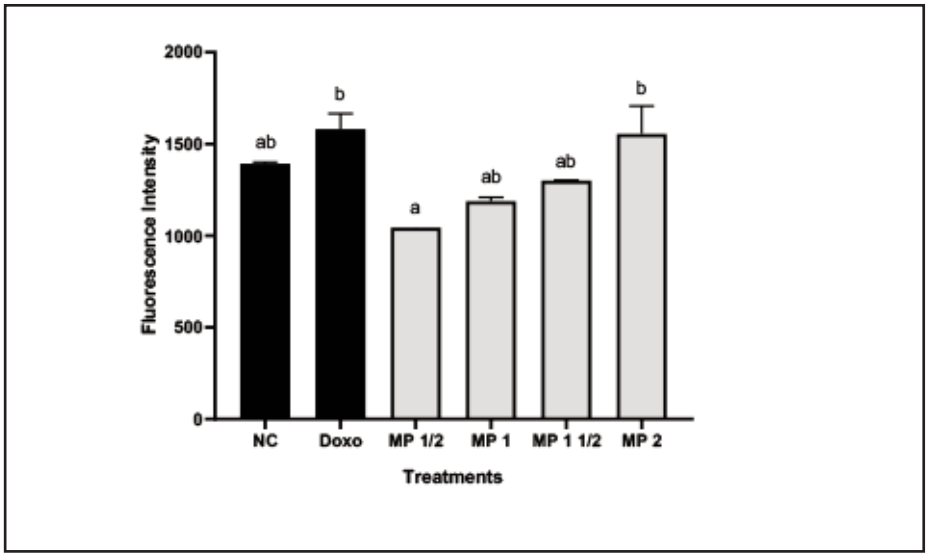

Fig. 4: Caspase-3 protein expression in multi-strain probiotic

(NC: Negative Control; Doxo: Doxorubicin; MP1/2: Multi-strain Probiotic ½ x IC_{50}; MP1: Multi-strain Probiotic 1 x IC_{50}; MP1,5: Multi-strain Probiotic 1,5 x IC_{50}; MP2: Multi-strain Probiotic 2 x IC_{50})
*The result was shown as mean ± SEM. Tukey's HSD post hoc test is used to produce statistical differences. The various letters (a,ab,b) demonstrate a significant difference between various treatments toward A549 lung cancer cell line.

IC_{50}. The results of observations of Caspase-3 and Caspase-9 protein expression were analysed using SPSS Statistics version 20.0. After a normality test was performed, normal data were analysed with analysis of variance followed by Tukey's HSD post hoc test, with a significance threshold of $p \leq 0.05$.

RESULTS

Cytotoxic Assay

Toxicity testing was carried out using the Resazurine method, which aims to determine the toxic effects of probiotics and measure the concentration of these probiotics on A549 lung cancer cells. The concentration tested based on the literature is 50, 100, 250, 500, 750, 1.000, 5.000, 10.000, 15.000, 30.000, 45.000, 60.000, 85.000, 100.000, 125.000, 150.000 ppm. The use of PrestoBlue reagent in this method will result in a colour change from the blue resazurin compound to the pink resazurin compound due to the reduction by enzymes in

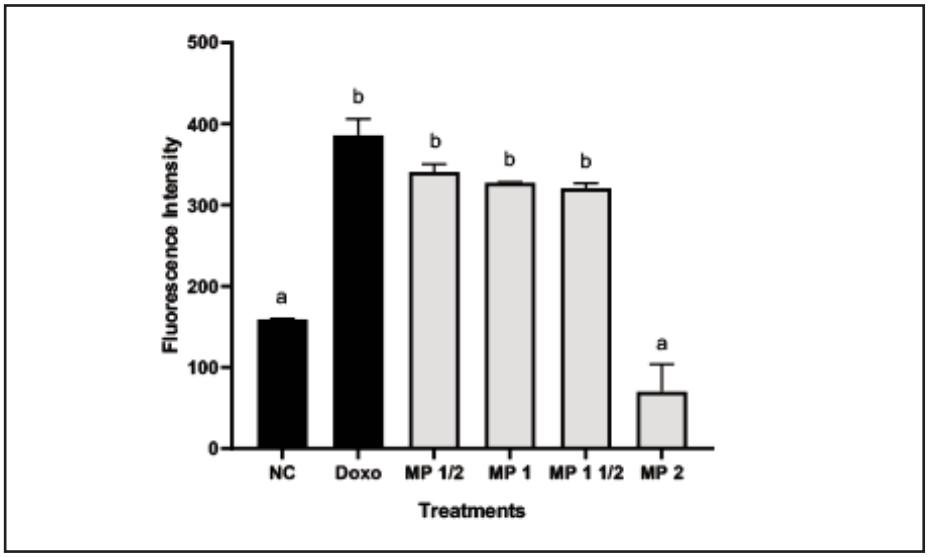

Fig. 5: Caspase-9 protein expression in multi-strain probiotic

(NC: Negative Control; Doxo: Doxorubicin; MP1/2: Multi-strain Probiotic ½ x IC50; MP1: Multi-strain Probiotic 1 x IC50; MP1,5: Multi-strain Probiotic 1,5 x IC50; MP2: Multi-strain Probiotic 2 x IC50)
*The result was shown as mean ± SEM. Tukey's HSD post hoc test is used to produce statistical differences. The various letters (a,b) demonstrate a significant difference between various treatments toward A549 lung cancer cell line.

the mitochondria. Then the absorbance will be measured at a wavelength of 570 and 600nm with the Multimode Microplate Reader Tecan Infinite m200 Pro. Figure 1 shows the cytotoxic test curve of multi-strain probiotics on A549 lung cancer cells.

In Cell Western Assay
The Caspase-3 fluorescence results of the multi-strain probiotic can be seen in Figure 2. The red colour is the result of a wavelength of 700 nm, which aims to determine the number of cells, while the green colour indicates the expression of Caspase-3 protein (primary antibody) from a wavelength reading of 800 nm.

The fluorescence results of Caspase-9 from the multi-strain probiotic can be seen in Figure 3. The red colour is the fluorescence result from a wavelength of 700 nm, which aims to determine the number of cells, while the green colour indicates the expression of Caspase-9 protein (primary antibody) from a wavelength of 800 nm. Figure 4 shows the graph of Caspase-3 expression in multi-strain probiotics. Meanwhile, Figure 5 shows the graph of Caspase-9 expression in multi-strain probiotics.

DISCUSSION

Multi-strain probiotics are a type of probiotic that contains more than one strain of bacteria originating from the same species, or from different species, such as containing fungi species (*Saccharomyces* species).[24] The use of multi-strain probiotics can show better inhibitory effects on enteropathogens and increase the benefits of the preparation due to the combined effects of different strains in one preparation compared to single strains.[25] Therefore, testing was conducted using probiotic finished products containing multi-strain probiotics, such as *L. bulgaricus, L. rhamnosus, L. fermentum, L. casei, L. plantarum*, and *Rhodopseudomonas palustris*. Initial testing was conducted by conducting a toxicity test using the Resazurin method to determine the magnitude of Inhibitory Concentrations 50% (IC_{50}) against A549 lung cancer cells.

Probiotic toxicity is obtained due to the activity of postbiotic metabolites contained in probiotics. The term postbiotic refers to micro- and macromolecular complexes, such as inactivated microbial cells, cell fractions such as muropeptides, teichoic acids, endo and exopolysaccharides, and proteins on the surface layer, or cell metabolites such as SCFA, organic acids, bacteriocins, and enzymes formed due to the fermentation process that occurs in probiotics.[26] Therefore, initial testing of the toxicity of the multi-strain probiotics used may reflect the activity of postbiotic metabolites such as SCFAs present in the multi-strain product.

Several studies that have previously obtained IC_{50} values are studies using Lactobacillus lactis KC24 obtained from kimchi isolation in SK-MES-1 cells with a concentration of 106 CFU/well capable of inhibiting proliferation by 86.53%, and *Lactococcus lactis* NK34 in SK-MES-1 cells with a concentration of 106 CFU/well capable of inhibiting proliferation by 96%.[27,28] Based on the cytotoxicity test result curves in Figure 1, the IC_{50} value of the multi-strain probiotic on A549 lung cancer cells is 58.952 ppm. The higher the IC_{50} value, the higher the concentration required to inhibit cancer cells by 50% is also higher. Referring to the classification of the cytotoxic strength of compounds, compounds with IC_{50} <100ppm are categorized as potentially cytotoxic, compounds with IC_{50} between 100-1000ppm are categorized as moderately cytotoxic, and compounds with IC_{50}

>1000ppm are categorized as non-toxic, so the multi-strain probiotic product tested is included in the non-toxic category.[27]

The IC_{50} results of multi-strain probiotic products in testing on A549 lung cancer cells can be influenced by several factors. Interactions between probiotic strains contained in multi-strain products can affect each other's effectiveness.[28] Differences in strains can provide different mechanisms of action and provide a greater scope of effects.[29] Unfortunately, this can also cause antagonistic intra-strain inhibition of different probiotic strains.[30] Research using multi-strain probiotics containing *L. plantarum, L. rhamnosus, L. acidophilus, Enterococcus faecium* showed that *L. plantarum* was the strongest strain in inhibiting *L. rhamnosus*, and *Vice versa*, while *L. plantarum* and *L. rhamnosus* were the strongest strains in inhibiting *L. acidophilus* and *Enterococcus faecium* in the product.[31] Other studies were conducted by analyzing single and multi-strain probiotics using 14 different strains to determine the inhibitory effects between strains using the cross-streak assay method and agar spot test.[30] The strains used were *L. acidophilus, L. delbrueckii subsp bulgaricus, L. casei, L. plantarum, L. rhamnosus, L. salivarius ssp Salivarius, L. fermentum, L. helveticus, Lactococcus lactis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum,* and *Streptococcus thermophilus*. The results obtained were from the *Lactobacillus* group, *L. casei* could inhibit *L. plantarum* weakly, inhibit *L. rhamnosus* moderately, and inhibit *L. fermentum* weakly. However, when compared with other groups, *Lactobacillus* is a probiotic with the strongest strain to inhibit other bacterial groups ($p<0.001$).[30]

In the probiotic product, there are five types of *Lactobacillus* strains mixed with *Rhodopseudomonas palustris* where, when compared to previous studies, *L. plantarum* will have the most dominant effect to inhibit other strains. It can be concluded that the type of strain used in a multi-strain probiotic product greatly determines whether the product is able to increase the effectiveness of each other between strains or can actually reduce the activity of other strains, as in previous studies.[32] The interaction between strains contained in this multi-strain product will affect postbiotic metabolites such as SCFA, which are expected to increase when strains are combined in multi-strain products.

In addition to strain-specific activity, the effectiveness of SCFAs as bioactive metabolites is influenced by several factors, including osmotic pressure, environmental pH, nutrient availability, and energy sources required for SCFA synthesis. In this study, the multi-strain probiotic formulation had a pH of 3.10, while the optimal pH range for SCFA production is 5.5–6.0. An environment that is too acidic ($pH<5.5$) can inhibit acidogenic activity. As a result, SCFA production is likely to be disrupted, so the effectiveness of probiotics in inducing apoptosis may not be optimal. Under physiological conditions, normal cells maintain a pH range of 7.35-7.45, while cancer cells exhibit a more acidic pH between 6.3 and 7.0 due to altered metabolic activity. Exposure of multi-strain probiotics to A549 lung cancer cells with low pH may further acidify the intracellular environment, potentially disrupting SCFA-mediated apoptotic signals and allowing cancer cells to survive.

SCFA itself is an intermediate compound synthesized in the anaerobic digestion process from substrates such as polysaccharides, proteins, and lipids. These substrates will be hydrolyzed first before entering several pathways for the formation of SCFA.[33] Pyruvate will be produced through the glycolysis pathway, and propionate will be produced through the succinate or propanediol pathway. Butyrate will be produced through the activity of the butyrate kinase enzyme. Acetate will be produced from the activity of the acetate CoA transferase enzyme.[34,35] The anaerobic digestion process from hydrolysis to various fermentation pathways to obtain these SCFAs cannot be observed in this study, because the exposure of multi-strain probiotics directly to A549 lung cancer cells occurs without any digestion process.

Further testing was conducted to observe the expression of Caspase-3 and Caspase-9 proteins as apoptosis markers after exposure to a multi-strain probiotic in A549 lung cancer cells. Apoptosis is a form of non-inflammatory programmed cell death mediated by Caspase activation that can occur through intrinsic or extrinsic pathways.[36] The intrinsic pathway is a pathway that is activated due to mitochondrial damage, where cytochrome c will be released into the cytoplasm along with Apaf-1, which will then form an apoptosome, where this apoptosome will activate Caspase-9. Caspase-9, which is an initiator Caspase, will then activate Caspase-3 as an executor Caspase in the apoptosis process.[37]

In this study, tests were conducted to see Caspase expression at concentration ranges of 0.5x IC_{50}, 1xIC_{50}, 1.5xIC_{50}, and 2xIC_{50}. The Caspase-3 expression of the multi-strain probiotic showed a significant difference ($p\leq0.05$). The multi-strain probiotic at a concentration of 2xIC_{50} showed higher Caspase-3 expression compared to the other group. The multi-strain probiotic at a concentration of 0,5xIC_{50} gave the highest Caspase-9 expression. The expression of Caspase-3 and Caspase-9 is influenced by the activity of SCFAs exposed by the administration of a multi-strain probiotic. In previous studies, research on SCFA type sodium butyrate with a concentration of 5mM was able to reduce proliferation and migration of A549 lung cancer cells and could increase the expression of miR-3955.[38] As for propionate, it was found that at a concentration of 10mM of sodium propionate it was able to reduce proliferation and increase cell cycle arrest and could increase apoptosis in H1299 and H1703 human non-small cell lung carcinoma cells.[39]

In lung cancer, SCFA type butyrate can accumulate as a histone deacetylase (HDAC) inhibitor in the nucleus, where HDAC is an enzyme that plays a role in tumour cell expression.[37] Accumulation of HDAC inhibitor from butyrate is then able to increase inhibition of lung cancer cell expression and increase the apoptosis process by increasing proapoptotic proteins such as BAX and BCL-2 homologous antagonist/killer (BAK) or through a pathway of decreasing the expression of anti-apoptotic proteins such as Bcl-2.[40] In addition to butyrate, SCFA type propionate can activate cell apoptosis and the cell cycle by decreasing Survivin expression and increasing p21 expression. Survivin is an antiapoptotic protein whose expression increases in several types of cancer,

one of which is lung cancer. With a decrease in Survivin expression, there will be induction of BCL-2–associated death promoter (BAD) and BAX expression, where there will be induction of cells entering the G2/M arrest phase.[39]

It has been explained previously that due to the absence of a digestion process because A549 lung cancer cells were exposed to probiotics directly, it can result in the anaerobic digestion process may not be optimal to obtain SCFA, which can affect the amount of SCFA obtained in the research samples used. In this study, quantitative measurements were also not carried out on the amount of SCFA, such as butyrate or pyruvate concentrations, which are suspected of affecting A549 lung cancer cells.

In previous studies, the use of cell-free supernatant in lung cancer research was carried out on the testing of *Faecalibacterium prausnitzii* cell-free supernatant form on A549 lung cancer cells by measuring anti-inflammatory cytokines such as Interleukin (IL)-10, Tumour Growth Factor (TGF)-β2, and IL-1Ra.[40] The results of this study showed that multi-strain probiotics were able to dysregulate the expression of several cytokines. This is as seen in the results of Caspase-3 and Caspase-9 expression in this study, where Caspase-3 and Caspase-9 can still be expressed. Despite the suboptimal formation of SCFAs and the non-toxic nature of multi-strain probiotics, as demonstrated by cytotoxicity assay, this study demonstrated that the probiotic formulation effectively stimulated apoptosis in A549 lung cancer cells. Specifically, Caspase-3 expression was significantly increased at a concentration of 2×IC_{50}, while Caspase-9 expression peaked at 0.5×IC_{50}.

CONCLUSION

A multi-strain probiotic is included in the non-toxic category, with IC50 values in cytotoxic tests conducted on A549 lung cancer cells of 58.952ppm. The use of a multi-strain probiotic at a concentration of 2xIC_{50} can stimulate the expression of Caspase-3. The use of multi-strain probiotics at concentrations of 0.5xIC_{50} can stimulate the expression of Caspase-9 in A549 lung cancer. Thus, multi-strain probiotics can activate major apoptotic pathways without causing cytotoxic effects, indicating their potential as a safe and effective adjuvant therapy for the treatment of lung cancer.

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AUTHOR CONTRIBUTIONS

MEG contributed to the conception and design of the study, definition of intellectual content, data analysis, manuscript editing, and manuscript review. RL contributed to liter ature research, conducted experimental studies, performed data acquisition, and participated in manuscript preparation and review. GRG was responsible for literature research, experimental studies, data acquisition, statistical analysis, manuscript preparation, and review. IGYD conducted experimental studies and statistical analysis, and contributed to manuscript editing and review. AK was involved in the conception and design of the study, definition of intellectual content, data analysis, manuscript editing, and manuscript review.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest regarding the data presented in this manuscript.

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