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Modulatory Effects of Multi-Strain Probiotics on Inflammatory Cytokines and Apoptotic Protein Expression in MCF-7 Breast Cancer Cells *in vitro*

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ABSTRACT

Breast cancer (BC) is one of the most prevalent cancers with a high morbidity rate. Increased estrogen production in women can cause inflammation, which contributes to the development of BC. The most abundant proinflammatory mediators involved are Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6). In addition, evasion of apoptosis is a hallmark of cancer, with caspase-3 and caspase-9 playing central roles in the programmed cell death pathway. Multi-strain probiotics have the potential to modulate the immune system and have anti-cancer effects. This study aimed to evaluate the effects of multi-strain probiotics on the expression of inflammatory markers (TNF-α and IL-6) and apoptotic proteins (caspase-3 and caspase-9) in MCF-7 BC cell culture *in vitro*. Multi-strain probiotics were used, and their cytotoxicity against MCF-7 cells was determined using the resazurin assay to calculate the half-maximal inhibitory concentration (IC_{50}). The expression levels of TNF-α, IL-6, caspase-3, and caspase-9 were analysed using the In-Cell Western (ICW) method at various concentrations (0.5× IC_{50}, 1× IC_{50}, 1.5× IC_{50}, and 2× IC_{50}). The IC_{50} value of the multi-strain probiotics in MCF-7 cells was 37433 µg/mL. Treatment with multi-strain probiotics led to increased expression of TNF-α (+2204.00) and IL-6 (+1327.16), indicating a modulatory effect on inflammatory responses. Simultaneously, there was a significant upregulation of caspase-3 (+123.13) and insignificant upregulation of caspase-9 (+128.75), suggesting activation of the apoptosis pathways. Multi-strain probiotics exhibit potential as adjuvant anti-BC agents by increasing inflammatory cytokines and promoting apoptosis through increased expression of caspase-3 and caspase-9 in MCF-7 cells.

Keywords: Apoptosis, Breast cancer, Cytokines, Inflammation, MCF-7, Probiotics

Introduction

Breast cancer (BC) is the most common cancer among women globally. Based on GLOBOCAN 2022 data, there were approximately 2.31 million new BC cases (11.6 % of all cancer cases).[1] In Asia, 0.98 million new cases and 0.31 million deaths were recorded. In Indonesia, BC accounts for 68 thousand of 396 thousand total cancer cases and is the second leading cause of cancer-related deaths after lung cancer.[2] While early-stage BC has a high cure rate (70–80%), advanced stages remain difficult to treat effectively.[3] BC development is driven by extrinsic and intrinsic factors. Extrinsic factors include a high-fat diet, physical inactivity, alcohol, and radiation exposure. Intrinsic factors involve hormonal influences, aging, and genetic mutations such as Breast Cancer Gene 1/2 (BRCA1/2), Tumor Protein 53 (TP53), and Ataxia Telangiectasia Mutated (ATM).[4] In postmenopausal women, estrogen is synthesized mainly in adipose tissue.

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Elevated estrogen levels promote luminal cell proliferation and activate Nuclear Factor Kappa Beta (NF-κB), a key regulator of inflammation, which contributes to tumor progression and therapy resistance.[5] Pro-inflammatory mediators such as Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) play critical roles in BC. TNF-α can promote proliferation and immune modulation, while paradoxically also inducing apoptosis.[6] IL-6 is elevated in the tumor microenvironment and promotes macrophage polarization into tumor-associated M2 phenotypes, enhancing growth, metastasis, and angiogenesis via Janus Kinase (JAK), Signal Transducer and Activator of Transcription (STAT3), Phosphoinositide 3-Kinase (PI3K), Protein Kinase B (AKT), Rat sarcoma (RAS), and Mitogen-Activated Protein Kinase (MAPK) pathways.[7,8] Additionally, gut microbiota dysbiosis in BC patients exacerbates inflammation. Increased β-glucuronidase-producing bacteria enhance estrogen recycling, while lipopolysaccharide triggers cytokine release.[9,10] Apoptosis, or programmed cell death, is a physiological process crucial for maintaining cellular homeostasis. In cancer, this process is often disrupted, allowing uncontrolled growth. The intrinsic apoptosis pathway involves mitochondrial signaling, with caspase-9 activating caspase-3, leading to DNA fragmentation and cell death. Overexpression of anti-apoptotic proteins such as B-cell lymphoma 2 (BCL-2) in BC blocks this process. Thus, targeting caspase-3 and -9 activation is a promising strategy for BC therapy.[11] Probiotics, defined as live microorganisms providing health benefits when consumed adequately, have emerged as potential adjuvant agents in cancer therapy. Their anticancer effects include gut microbiota modulation, NF-κB suppression, reduction of BCL-2, and upregulation of BCL-2–associated X protein (BAX), caspase-3, and caspase-9.[12-13] Multi-strain probiotics offer synergistic advantages, producing diverse

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metabolites such as lactic acid, peptidoglycans, and short-chain fatty acids (SCFAs), notably acetate, propionate, and butyrate, that are known to induce apoptosis. SCFAs also modulate immunity by increasing IL-10 and suppressing BCL-2, thereby activating caspase cascades.[14,15] Several *in vitro* studies using single-strain probiotics like *Lactobacillus plantarum*, *Enterococcus lactis*, and *Lactococcus lactis* have shown the ability to induce apoptosis in Michigan Cancer Foundation-7 (MCF-7) and MD Anderson – Metastatic Breast (MDA-MB)-231 breast cancer cells through NF-κB inhibition and caspase activation. However, most focus only on IC_{50} determination, without assessing broader anti-inflammatory and apoptotic responses, particularly for multi-strain probiotics.[16-18] Moreover, few studies have simultaneously measured pro-inflammatory markers (TNF-α, IL-6) alongside apoptotic indicators (caspase-3, -9), limiting insight into their dual-action potential.

Multi-strain probiotics have better gut adhesion and colonization capacity than single strains, producing more diverse bioactive metabolites. A study involving a combination of *Lactobacillus acidophilus, L. plantarum, L. rhamnosus*, and *Enterococcus faecium* demonstrated effective colonization and enhanced SCFA production, along with increased IL-10 and IL-6 levels.[15] Despite this, research on their role in modulating inflammation and inducing apoptosis in BC is still limited. This study is the first to comprehensively evaluate the dual modulatory effects of multi-strain probiotics on pro-inflammatory cytokines (TNF-α and IL-6) and apoptotic markers (caspase-3 and caspase-9) in MCF-7 breast cancer cells *in vitro*. Therefore, this study aimed to investigate the effect of multi-strain probiotics on TNF-α, IL-6, caspase-3, and caspase-9 expression in MCF-7 cells.

Materials and Methods

Materials

The cell line used in this study is Michigan Cell Foundation (MCF)-7 breast cancer cells. The chemical and reagents used in this study include Roswell Park Memorial Institute (RPMI-1640) medium, fetal bovine serum (FBS), 2 mM L-glutamine, penicillin-streptomycin, phosphate-buffered saline (PBS), Trypsin-EDTA, primary antibodies (caspase-3, caspase-9, TNF-α and IL-6), secondary antibodies, 8% paraformaldehyde, Cell Tag 700 stain, Tween 20, Tris-buffered saline, and Triton X-100. Multi-strain probiotics containing *Lactobacillus bulgaricus, L. rhamnosus, L. fermentum, L. casei, L. plantarum,* and *Rhodopseudomonas palustris*. The equipment used includes a 96-well microplate, a CO_2 incubator, and Odyssey® XF.

MCF-7 Cell Culture

MCF-7 BC cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (penicillin-streptomycin). The cells were cultured in an incubator at 37 °C with 5 % CO_2, and the culture medium was replaced every 48 h. When cell confluence reached 80–90%, the cells were harvested by treating them with 0.25 % trypsin-EDTA for 3–5 min to detach them from the culture surface. The trypsinization process was then neutralized using complete medium, followed by centrifugation at 1,200 rpm for 5 min. The resulting cell pellet was counted using a hemocytometer for further experiments.[19,20]

Cytotoxic Assay

MCF-7 cells were seeded in a 96-well plate at a density of 1×10^4 cells per well and incubated for 24 h at 37 °C with 5 % CO_2. After 24 h of incubation, the culture medium was replaced with a medium containing multistrain probiotic at various concentrations: 1, 2, 4, 8, 16, 32, 64, 128, 1171.88, 2343.75, 4687.50, 9375, 18750, 37500, 75000, and 150000 µg/mL. The cells were then incubated for 48 h at 37 °C with 5 % CO_2. After the incubation period, PrestoBlue reagent was added to the 96-well plate, followed by an additional incubation in a CO_2 incubator for 2 hours. The absorbance was then measured using a Multimode Microplate Reader, Tecan Infinite m200 Pro, at a wavelength of 570 nm.[21]

Analysis of Protein Expression Using in-Cell Western (ICW)

MCF-7 cells were seeded in a 96-well plate at a density of 1×10^4 cells per well and incubated for 24 h at 37°C with 5 % CO_2. After incubation, the culture medium was replaced with a medium containing multistrain probiotic suspension at various concentrations (0.5× IC_{50}, 1× IC_{50}, 1.5× IC_{50}, 2× IC_{50}). The treatment was applied for 24 h at 37 °C with 5 % CO_2. Following treatment, the cells were fixed with 4 % formaldehyde for 15 minutes at room temperature to preserve protein integrity in situ. The cells were then permeabilized with 0.1 % Triton X-100 for 5 min to enhance antibody penetration. Blocking was performed using 5 % BSA for 1 hour to minimize nonspecific binding. The samples were subsequently incubated overnight at 4 °C with primary antibodies specific to TNF-α, IL-6, caspase-3, and caspase-9, followed by washing with PBS-Tween 20 to remove unbound antibodies. Fluorophore-conjugated secondary antibodies were added and incubated for 1 h at room temperature in the dark to prevent degradation of the fluorescence signal. After washing, protein expression was analysed using a 96-well microplate reader and detected with the Odyssey® XF system.[21] The experiment employed two types of controls: a positive control using cisplatin and a negative control consisting of untreated cells. The probiotic treatment was administered at four concentrations corresponding to 0.5×, 1×, 1.5×, and 2× IC_{50}.

Statistical Analysis

Data were analysed using IBM SPSS Statistics for Windows, Version 25.0. (Armonk, NY: IBM Corp). Normality was assessed using the Shapiro-Wilk test. If the data followed a normal distribution, parametric analysis was conducted using One-Way Analysis of Variance (ANOVA) with a 95% confidence level ($\alpha = 5\%$). If the data were not normally distributed, a non-parametric test was performed using the independent k-sample t-test with a post-hoc analysis based on the Kruskal-Wallis method. Cytotoxicity data were analysed using linear regression in GraphPad Prism version 8.0.1. (GraphPad Software, San Diego, CA, USA) to determine the 50% inhibitory concentration (IC_{50}) value.

Results and Discussion

Cytotoxicity Assay

The cytotoxicity assay revealed that the IC_{50} value of the multistrain probiotics was 37433 µg/mL (Figure 1), which, according to Fitri et al.[22] classifies the preparation as non-toxic (IC_{50} >1000 µg/mL). This is consistent with other studies, such as those by Chuah et al.[23] and Abdoli et al.[24], which also reported relatively high IC_{50} values for probiotic preparations against MCF-7 cells. In contrast, Tan et al.[25] reported a much lower IC_{50} value of 10.83 µg/mL using *L. plantarum* metabolites, categorized as potentially cytotoxic.[25] These variations highlight the influence of factors such as strain composition, culture medium, metabolite concentration, pH levels, and the presence of SCFAs.

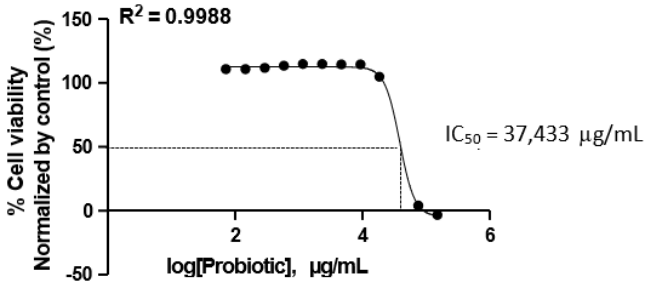

Figure 1: Cytotoxicity Assay Curves of Multistrain Probiotics on MCF-7 Cells

One possible explanation for this finding is the composition and concentration of SCFAs, such as butyrate, in the metabolite fraction. Butyrate is a well-established histone deacetylase inhibitor (HDACi) that promotes apoptosis in cancer cells by modulating p53, BCL-2, and BAX expression, ultimately activating caspase-3.[26] Semaan et al.[27] found that 1 mM butyrate reduced MCF-7 cell proliferation by 57 % within 24 h and arrested the cell cycle in the G1 phase. However, *in*

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vitro experiments often fail to replicate optimal SCFA production due to the absence of metabolic fermentation pathways present in the gastrointestinal tract, which may explain the suboptimal effects observed in this study.

Expression of TNF-α and IL-6 in MCF-7 Cells Treated with Multistrain Probiotics

The fluorescence readings at wavelengths of 700 nm and 800 nm are shown in Figure 2. The 700 nm wavelength produces a red signal, indicating the number of cells in each well, while the 800 nm wavelength produces a green signal, representing TNF-α and IL-6 expression. The results indicate a significant increase in TNF-α and IL-6 expression compared with cisplatin treatment following multistrain probiotic treatment (Figure 3). Probiotic administration plays a role in increasing the expression of TNF-α and IL-6 in the early stages of breast cancer exposure. During this phase, probiotics play an immunostimulatory and immunomodulatory role, initiating the release of inflammatory mediators at the onset of inflammation to activate the innate immune system.

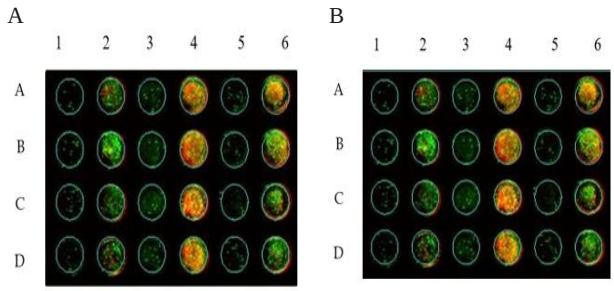

Figure 2: Fluorescence Images of TNF-α (A) and IL-6 (B) Expression in MCF-7 Cells Treated with Multistrain Probiotics
*A1-D1: Positive control background; A2-D2: Positive control (Cisplatin); A3-D3: Negative control background; A4-D4: Negative control; A5-D5: Probiotic Background at 0.5, 1, 1.5, and 2× IC_{50}; A6-D6: Probiotic at 0.5, 1, 1.5, and 2× IC_{50}.

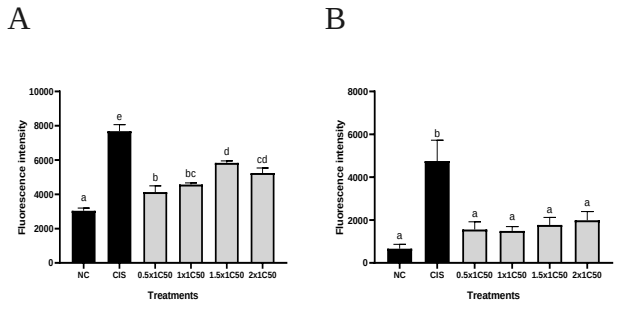

Figure 3: TNF-α (A) and IL-6 (B) Protein Expression of Multistrain Probiotics in MCF-7 Cells
(NC: Negative Control; Cis: Cisplatin) *The result was shown as mean ± SEM. Tukey's HSD post hoc test is used to produce statistical differences. The various letters (a,b,bc,cd,d,e) in TNF-α expression demonstrate a significant difference between various treatments toward MCF-7 cells. The letters (a,b) in IL-6 expression demonstrate the treatment has no significant difference toward MCF-7 cells.

TNF-α and IL-6 are proinflammatory cytokines released by monocytes and macrophages, components of the innate immune system that prevent pathogen invasion by releasing cytotoxic molecules. Macrophages can recognize bacteria, which in this study were presented as probiotics. Bacteria express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which are recognized by macrophages. Furthermore, macrophages also recognize microbe-associated molecular patterns (MAMPs) found on the surface of probiotic bacteria. TLR activation induces various responses, including cytokines, and can initiate the NF-κB pathway, resulting in increased expression of TNF-α and IL-6. It is known that the increase in IL-6 expression by macrophages peaks in the first 24 hours after exposure to probiotics such as *Lactobacillus* sp. strains, which are also known to increase the activation and translocation of NF-κB in macrophages tested with the addition of *Lactobacillus* sp.[28]

Expression of Caspase-3 and Caspase-9 in MCF-7 Cells Treated with Multistrain Probiotics

The fluorescence images at wavelengths of 700 and 800 nm from MCF-7 cells treated with multi-strain probiotics are shown in Figure 4. The 700 nm wavelength produces a red signal, indicating the number of cells in each well, while the 800 nm wavelength produces a green signal, representing caspase-3 and caspase-9 expression. The results indicate a significant increase in caspase-3, but not a significant increase in caspase-9 expression, following treatment with either multistrain probiotic, as evidenced by the identical notations on each bar (Figure 5). This insignificant increase in Caspase-9 may be due to insufficient SCFA concentration, short exposure duration, or oxygen level variations in the culture environment. Although butyrate-induced apoptosis primarily involves caspase-9 activation, certain conditions may lead to caspase-3 activation through alternative pathways.

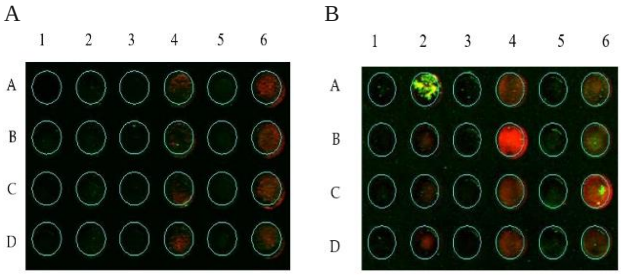

Figure 4: Fluorescence Images of Caspase-3 (A) and Caspase-9 (B) Expression in MCF-7 Cells Treated with Multistrain Probiotics
*A1-D1: Positive control background; A2-D2: Positive control (Cisplatin); A3-D3: Negative control background; A4-D4: Negative control; A5-D5: Probiotic Background at 0.5, 1, 1.5, and 2× IC_{50}; A6-D6: Probiotic at 0.5, 1, 1.5, and 2× IC_{50}.

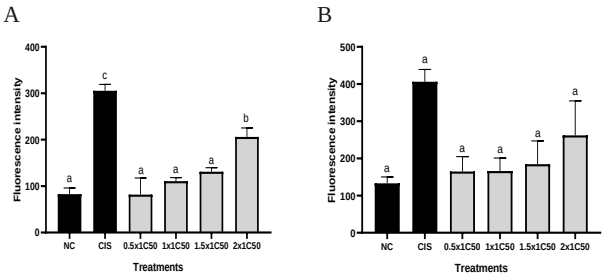

Figure 5: Caspase-3 (A) and Caspase-9 (B) Protein Expression of Multistrain Probiotics in MCF-7 Cells
(NC: Negative Control; Cis: Cisplatin) *The result was shown as mean ± SEM. Tukey's HSD post hoc test is used to produce statistical differences. The various letters (a,b,c) in caspase-3 expression demonstrate a significant difference between various treatments toward MCF-7 cells. The letters (a) in caspase-9 expression demonstrate that the treatment has no significant difference in MCF-7 cells.

Despite these observations, the specific metabolites responsible for the effects remain unidentified. It is assumed that SCFAs are present, but the types and concentrations were not analyzed. This represents a major limitation of the current study. Identification of SCFA profiles would provide valuable insight into the precise bioactive mechanisms of the probiotic preparation. Future studies should include SCFA profiling and comparison with pure SCFA controls to determine their contributions. Another limitation is the absence of pH measurement following probiotic addition. Since cancer cells are sensitive to pH changes, and

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pH alterations can independently induce apoptosis or necrosis, it is unclear whether the observed cytotoxicity was solely due to probiotic bioactivity or influenced by pH shifts. The optimal pH range for MCF-7 cells is 6.7–7.0, while extreme acidic or alkaline environments (e.g., pH 6.0 or 9.0) can significantly affect viability. Therefore, future studies should monitor and control pH levels to ensure accurate interpretation of probiotic effects.

Interestingly, while multi-strain probiotics are often presumed to act synergistically, antagonistic interactions between strains may reduce their overall effectiveness. For example, Lactobacillus species can produce acids and bacteriocins that suppress the growth of other strains. Chapman et al.[29,30] reported that such inter-strain antagonism could compromise the intended benefits of multi-strain formulations. This raises the possibility that, despite their theoretical advantages, multi-strain products may require optimization to prevent inhibitory interactions and maximize therapeutic potential.

Conclusion

This study showed that multi-strain probiotics did not show cytotoxic effects against MCF-7 breast cancer cells due to their low IC_{50} category. The treatments significantly increased the expression of TNF-α and IL-6 compared with cisplatin and significantly upregulated the expression of the apoptotic marker caspase-3; however, the increase in caspase-9 expression was not statistically significant, indicating their potential to induce cancer cell apoptosis. Further studies are necessary to identify the specific bioactive metabolites responsible for these effects, assess their efficacy in *in vivo* cancer models, and clarify the underlying molecular mechanisms of apoptosis.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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