A co-culture study to determine the supportive role of probiotics on immune system against cancer cells

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ABSTRACT

In this study, two different in vitro co-culture systems were established in order to determine the supportive role of probiotic Lactobacillus acidophilus LA-5 on macrophages when used in combination with doxorubicin in both human- and mouse-origin cancerous cell lines. First, a co-culture study was undertaken using RAW 264.7 mouse macrophage cells that activated by LA-5 against 4T1 mouse triple negative breast adenocarcinoma cells. After the initial study, a second co-culture was performed using THP-1 human monocyte-like cells activated by LA-5 against CaCo-2 human colorectal adenocarcinoma cells. The supplementary potential of LA-5 on the macrophages was evaluated by investigating the viabilities of 4T1 and CaCo-2 cells via an MTT test. The data obtained in the study indicated that the viability of cancerous cells 4T1 and CaCo-2 decreased to ≈40% and ≈30% in the groups that used LPS-activated macrophages stimulated with LA-5 and the combination of LA-5 and doxorubicin (1/2 IC50), respectively. In conclusion, the use of LA-5 could be encouraged as a dietary supplementary for cancer patients due to its superior effects on immune stimulation.

Keywords: Probiotics, Immune system, Lactobacillus acidophilus LA-5, Cancer cells

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Introduction

In recent years, there has been an increasing interest in the studies focusing on probiotics and their potential effects on health (Duary et al., 2014). Probiotics are defined as live microorganisms that are beneficial for human health when administered in adequate amounts (Mack, 2005). The intestines contain trillions of bacteria with more than a thousand species living in a symbiotic relationship with the host and likewise with probiotic microorganisms (Shida and Nomoto, 2013). Nowadays, probiotics have a wide range of use and probiotics, especially Lactobacillus and Bifidobacterium species can be found in various products in the market, such as kefir, yoghurt, milk, cosmetics, oral health care products or in the pharmacies as capsules, sachets and in some other pharmaceutical forms (Saxelin, 2008; Sanders et al., 2018). Various factors including dietary disorders, stress, ageing, infections, and unnecessary use of antibiotics could result in imbalance in gut microbiota which leads to dysfunction in the gut (Penders et al., 2007; Damakos and Kolios, 2008; Turnbaugh, 2008; Shida and Nomoto, 2013). It is known that this dysfunction or a defect in gut microbiota can result in serious health problems, such as inflammatory bowel disease (IBD), Crohn’s disease (CD), ulcerative colitis (UC). Individuals suffering from these diseases are given probiotics as a supplementary product as a treatment to restore the gut microbiota (Brown and Valiere, 2004; Sheil et al., 2007; Mallappa et al., 2012; Duary et al., 2014).

It is well known that gut microbiota and probiotics are great stimulators of immune response. Probiotics are involved in stimulation of immune and inflammatory responses, playing a vital role in activating both innate and adaptive immune responses (Maslowski and Mackay, 2011; Mallappa et al., 2012). Studies have demonstrated that a probiotic species, Lactobacillus, is a prominent immune stimulator and plays a vital role especially in dendritic cell and macrophage activation (Kaji et al., 2018). Macrophages are immune cells that have abilities in direct killing foreign microorganisms and infected cells. Furthermore, they produce immune stimulatory cytokines, which makes them very good contributors to the immune defense mechanism against various diseases including cancer (Ivec et al., 2007). Probiotic microorganisms are also noble inducers of cytokine responses, promoting the activation of immune responses directed against pathogens (Kaji et al., 2018). In an in vitro study, the effects of Lactobacillus and Bifidobacterium probiotics on macrophages were investigated against vesicular stomatitis virus (Ivec et al., 2007). The researchers found that macrophages induced by probiotics increased nitric oxide synthesis and inflammatory cytokine production that resulted in the inhibition of viral replication (Ivec et al., 2007). Other studies demonstrated the capabilities of probiotics on increasing the phagocytic activity of macrophages (Galdeano et al., 2007; Fagfouri et al., 2015). Lactobacillus surface protein (Slp-A) is known for its activities in immune stimulation. It is a potent activator of both dendritic cells and macrophages. In a study undertaken by Konstantinov et al. (2008), Slp-A was emphasized as a crucial surface protein responsible for activating dendritic cells and their pro-inflammatory cytokines release such as interleukin-12 (IL-12), tumor necrosis factor-alpha (TNF-α) and Interleukin 1 beta (IL1β) (Galdeano et al., 2007; Konstantinov et al., 2008). Slp-A from the Lactobacillus membrane is also a potent activator of TLR-2 receptor of macrophages that further stimulates pro-inflammatory factors (TNF-alpha and COX-2) (Piccart- Gebhart et al., 2005; Taverniti et al., 2013).

Cancer is a leading worldwide cause of death and although it is regarded as one of the most dangerous diseases, no effective treatment has yet been found. Current treatment strategies are not sufficient for the treatment and prophylaxis of cancer; thus, alternative approaches are needed to overcome cancer (Piccart- Gebhart et al., 2005). Probiotics are known for their positive effect on general health; however, they can also be given as supplementary products along with conventional therapies in cancer treatment (Chen et al., 2007; Zamberi et al., 2016). A well balanced diet in terms of protecting microflora would be beneficial in cancer prevention and treatment (Zitvogel et al., 2017). Some probiotic strains of Lactobacillus have been proven to disrupt cancer-specific mechanisms, as well as protecting against various infections (Dasari et al., 2017). A few examples of the anti-cancer properties of probiotics are the suppression of harmful bacterial growth important in carcinogen and mutagen production, protection against the oxidative damage of DNA along with the regulation of immune system (Mack, 2005). Clinical and experimental studies have demonstrated the beneficial effects of probiotics on health, such as inducing apoptotic death mechanisms and immune stimulation apart from their antioxidant activities (Dasari et al., 2017). L. acidophilus is composed of various strains with different biological, immunomodulatory activities. In a study carried out by Parvinder and Aruna (2012), the immunomodulatory effects of four different strains of L. acidophilus were tested for antibody production using a delayed type hypersensitivity test, iNOS, as well as a phagocytic activity test. According to the comparison of the various strains of L. acidophilus, it was concluded that L. acidophilus LA-5 demonstrated the most potent immunomodulatory effect among other species (Parvinder and Aruna, 2012).
This in vitro study was performed to determine the supportive effect of probiotic *L. acidophilus* LA-5 on macrophages with low-dose doxorubicin (1/2 IC$_{50}$), a chemotherapeutic agent currently used in our clinic. For this purpose, two different co-culture studies were undertaken in the selected murine and human cell lines: the first using RAW 264.7 mouse macrophage cells as the immune cells stimulated by *L. acidophilus* LA-5 against 4T1 mouse triple negative breast adenocarcinoma cells, and the second co-culture study using THP-1 human monocyte-like cells as the immune cells to be stimulated by *L. acidophilus* LA-5 against CaCo-2 human colorectal adenocarcinoma cells. The main aim of the study was to draw attention to the positive effect of *L. acidophilus* LA-5 on the immune system and emphasize that it could be a beneficial supplementary product in the diet of cancer patients undergoing chemotherapy.

**Materials and Methods**

**Cell Culture**

The RAW 264.7 (murine macrophage cells), 4T1 (murine breast cancer cells) and CaCo-2 (human colorectal adenocarcinoma) cells were purchased from ATCC (Manassas, VA, USA). The THP-1 (Human monocyte-like cells) cell line was generously given by Prof. Dr. Kemal Sami Korkmaz (Ege University, Bioengineering Department, Bornova, Izmir, Turkey). The RAW 264.7 and THP-1 cells were cultivated in Roswell Park Memorial Institute 1640 (Serox, Mannheim, Germany), supplemented with 10% fetal bovine serum (FBS) (Serox, Mannheim, Germany), 2 mM/L glutamine, 100 U/mL of penicillin and 100 mg/mL of streptomycin. The 4T1 and CaCo-2 cells were cultivated in DMEM/F12 (Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12) (Serox, Mannheim, Germany), supplemented with 10% FBS, 2 mM/L glutamine, 100 U/mL of penicillin and 100 mg/mL of streptomycin. The cells were incubated at 37°C in a 95% humidified atmosphere of 5% CO$_2$.

**Preparation of UV-inactivated *L. acidophilus* LA-5**

*L. acidophilus* LA-5 (Christian Hansen, Denmark) was cultured at 37°C for 24 h in De Man, Rogosa and Sharpe (MRS: Merek, Germany) broth. After cultivation, centrifugation (Hettich Rotofix II, Germany) at 6600 x g for 30 min was applied to collect the bacterial cells. The cells were then washed twice in sterile physiological solution (0.85% NaCl) and resuspended in DMEM/F12 to the original volume. *L. acidophilus* LA-5 cells in the plate were then killed by being exposed to 30 min long ultraviolet light (UV-C: 15W, Philips, Netherland) for two times consecutively (distance between plate and UV lamp: 3.5 cm). The cell count was confirmed before and after the UV treatment, using a conventional plate count method (Kishimoto et al., 2017).

**Determination of the IC$_{50}$ Values of Doxorubicin on 4T1 and CaCo-2 Cells**

The cytotoxicity of doxorubicin was determined using an MTT [3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide)] (Acros Organics, New Jersey, USA) assay that detects the activity of mitochondrial reductase of viable cells assay. The assay is based on cleavage of MTT that forms formazan crystals. This cleavage appears in living cells with succinate-dehydrogenase. Adding dimethyl sulfoxide to wells helps formazan crystals to be resolved (Mosmann, 1983; Nalbantsoy et al., 2016). For this purpose, 4T1 and CaCo-2 cells were cultivated for 24 h in 96-well microplates with an initial concentration of 1×10$^5$ cells/well in a 95% humidified atmosphere with 5% CO$_2$, at 37°C. The cultured cells were then treated with three different concentrations of doxorubicin (0.2, 2.0, 20 µg/mL), followed by incubation for 48 h at 37°C. The optical density of the dissolved material was measured at $\lambda=570$ nm (reference filter, $\lambda=620$ nm) with a UV-visible spectrophotometer (Thermo Multiskan Spectrum). The viability (%) was determined by the following formula:

$$\%\text{Viable cells:} \left[ \frac{\text{(absorbance of treated cells)} - \text{(absorbance of blank)}}{\text{(absorbance of control)} - \text{(absorbance of blank)}} \right] \times 100$$

**Co-culture Studies**

Two different systems were employed according to the co-culture experiments. In the first study, mouse cell lines were tested. RAW 264.7 mouse macrophage cells were used as immune cells and activated by probiotic *L. acidophilus* LA-5 against 4T1 mouse triple negative breast adenocarcinoma cells (Table 1). In the second co-culture study, human cell lines were studied. THP-1 human monocyte-like cells were used as immune cells and activated by probiotic *L. acidophilus* LA-5 against CaCo-2 human colorectal adenocarcinoma cells (Table 1).
Table 1. Groups showing the components of 12 different combinations tested in both co-culture studies.

| Group | Co-Culture Study 1 | Co-Culture Study 2 |
|-------|--------------------|--------------------|
| 1     | Only 4T1           | Only CaCo-2        |
| 2     | 4T1 + RAW 264.7    | CaCo-2 + THP-1     |
| 3     | 4T1 + RAW 264.7 (LPS) | CaCo-2 + THP-1 (LPS) |
| 4     | 4T1 + Probiotic - *Lactobacillus acidophilus* LA 5 | CaCo-2 + Probiotic - *Lactobacillus acidophilus* LA 5 |
| 5     | 4T1 + RAW 264.7 (LPS) + Probiotic - *Lactobacillus acidophilus* LA 5 | CaCo-2 + THP-1 (LPS) + Probiotic - *Lactobacillus acidophilus* LA 5 |
| 6     | 4T1 + RAW 264.7 (LPS) + Probiotic - *Lactobacillus acidophilus* LA 5 | CaCo-2 + THP-1 (LPS) + Probiotic - *Lactobacillus acidophilus* LA 5 |
| 7     | 4T1 + LPS          | CaCo-2 + LPS       |
| 8     | 4T1 + Doxorubicin (1/2 IC<sub>50</sub>) | CaCo-2 + Doxorubicin (1/2 IC<sub>50</sub>) |
| 9     | 4T1 + Doxorubicin (1/2 IC<sub>50</sub>) + RAW 264.7 | CaCo-2 + Doxorubicin (1/2 IC<sub>50</sub>) + THP-1 |
| 10    | 4T1 + Doxorubicin (1/2 IC<sub>50</sub>) + RAW 264.7 (LPS) | CaCo-2 + Doxorubicin (1/2 IC<sub>50</sub>) + THP-1 (LPS) |
| 11    | 4T1 + Doxorubicin (1/2 IC<sub>50</sub>) + Probiotic - *Lactobacillus acidophilus* LA 5 | CaCo-2 + Doxorubicin (1/2 IC<sub>50</sub>) + Probiotic - *Lactobacillus acidophilus* LA 5 |
| 12    | 4T1 + Doxorubicin (1/2 IC<sub>50</sub>) + RAW 264.7 (LPS) + Probiotic - *Lactobacillus acidophilus* LA 5 | CaCo-2 + Doxorubicin (1/2 IC<sub>50</sub>) + THP-1 (LPS) + Probiotic - *Lactobacillus acidophilus* LA 5 |

**4T1 & RAW 264.7 Cells**

The 4T1 cells were seeded to 96-well microplates at a density of 10⁵ cells/mL and incubated for 24 h. The RAW 264.7 cells were activated with lipopolysaccharide (LPS) (10 ng/mL) (Sigma Aldrich, St. Louis, Missouri, USA) for 24 h before being used in the co-culture experiments. LPS activates macrophages to produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL) -1, IL-6, IL-8 and IL-12 and macrophages secrete a wide variety of other biological response agents, in response to LPS, including free radicals such as platelet activating factor, prostaglandins, enzymes and nitric oxide (Fujihara et. al., 2003). The UV-inactivated probiotic *L. acidophilus* LA-5 at the concentration of 10⁶ cells/mL was added to the co-culture of 4T1. Following 24 h incubation of the RAW 264.7 cells with LPS, the cells were added to the final concentration of 10⁵ cells/well over the 4T1 cells. In this study, various groups were also investigated along with doxorubicin in order to determine the supportive potential effect of *L. acidophilus* LA-5 on macrophages in terms of cancer treatment.

**CaCo-2 & THP-1 Cells**

The CaCo-2 cells were seeded onto 96-well plates at a density of 10⁵ cells/ml and incubated for 24 h. The THP-1 monocytes were incubated with 15 ng phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, St. Louis, Missouri, USA) for 24 h to be differentiated into human macrophage-like cells. After the differentiation process, the THP-1 cells were treated with LPS (10 ng/mL) for another 24 h for full activation. Following incubation, the THP-1 cells were added into the CaCo-2 cells at a concentration of 10⁵ cells/well. The UV-inactivated probiotic *L. acidophilus* LA-5 at the concentration of 10⁶ cells/mL was added into the co-culture of the CaCo-2 and THP-1 cells.
**In Vitro Cytotoxicity Assay**

The viability of cancer cells in co-culture systems were determined by following the general procedure based on cell viability. According to this procedure, a modified colorimetric MTT was used to determine the IC₅₀ value of doxorubicin used in the experiment (Mosmann, 1983; Nalbantsoy et al., 2016). For this purpose, the CaCo-2 and 4T1 cells were cultivated for 24 h in 96-well microplates with an initial concentration of 1 x 10⁵ cells/mL. Following 24 h incubation, the RAW 264.7 (1 x 10⁵ cells/mL) and THP-1 cells (1 x 10⁵ cells/mL) and with a low dose of doxorubicin (1/2 IC₅₀) were added along with *L. acidophilus* LA-5 to the cultures. The low dose of doxorubicin was preferred for accurate evaluation. The co-cultures were further cultivated for 48 h and viability measurements were carried out two times at 24 h intervals (24th and 48th h). In each culture, the percentage of surviving cells was determined after incubation with macrophages and *L. acidophilus* LA-5. The viability (%) was determined by the following formula:

\[
\text{%Viable cells: } \frac{(\text{absorbance of treated cells} - \text{absorbance of blank})}{(\text{absorbance of control} - \text{absorbance of blank})} \times 100
\]

**Statistical Analysis**

The study was organized in triplicates and presented as mean ± standard error of mean (SEM) of samples. Graph Pad Prism 5.0 software (San Diego, USA) was used to calculate the IC₅₀ values and analyze variance (standard deviation calculation). The data were statistically analyzed using one-way ANOVA, followed by Tukey’s multiple comparison test. The significance level was set to 0.05 for one-way ANOVA, and 0.0001 for Tukey’s multiple comparison test.

**Results and Discussion**

The study was conducted to determine the supplementary role of probiotic strain *L. acidophilus* LA-5 in cancer treatment, when taken together with a chemotherapeutic agent, doxorubicin. For this purpose, an MTT assay was performed to calculate the minimal dose of doxorubicin to kill 50% of the cells. After determining the doxorubicin concentration, co-culture experiments were undertaken. Firstly, a mouse cell line based co-culture system was constructed using RAW 264.7 macrophage cells and 4T1 breast cancer cells. After completing this stage, another co-culture study with human cell lines was conducted using PMA stimulated THP-1 (macrophage like cells) and CaCo-2 (human colon adenocarcinoma) cells.

**Determination of the IC₅₀ Values of Doxorubicin on 4T1 and CaCo-2 Cells**

For this purpose, three different doses of doxorubicin were tested and the IC₅₀ values were determined as 4.15 µg/mL for 4T1 cells and 7.213 µg/mL for CaCo-2 cells. The study was planned to use a 1/2 dose of doxorubicin. For this purpose, half these concentrations were used for the co-culture experiments (2.07 µg/mL for 4T1 cells, 3.60 µg/mL for CaCo-2 cells) (Figure 1).

![Figure 1. Percentage viability graph of 4T1 and CaCo-2 cells after treatment with three different concentrations of doxorubicin (20, 2.0, 0.2 µg/mL) for 48 h exposure. The cell viability was determined by an MTT assay. The unexposed cells were taken as 100% viability. *p<0.01, ****p<0.0001](image-url)
The Effect of RAW 264.7 Cells Upon Stimulation with Probiotic L. acidophilus LA-5 on the Viability of the 4T1 Cell Line

The results indicated that the effect of the probiotic and doxorubicin showed significant inhibition on the 4T1 cell viability in 48 h when compared to 24 h \( (p<0.0001) \) (Figure 2). Groups 8-12 administered with doxorubicin demonstrated a remarkable effect when compared to the groups without chemotherapy. The results indicated the supplementary effect of probiotic L. acidophilus LA-5 on RAW 264.7. The most significant inhibition of cancerous cell viability was observed in Group 12 (≈40%) in 48 h, superior to other treatment and control groups \( (p<0.0001) \).

The Effect of THP-1 Cells Upon Stimulation with Probiotic L. acidophilus LA-5 on the Viability of the CaCo-2 Cell Line

The data obtained showed that the general trend between the groups in the study carried out on the murine cell lines correlated with the results from the co-culture study of human-origin cell lines. Generally, similar to the first study, 48 h treatment demonstrated more significant results than 24 h treatment in terms of colon cancer cell viability. The groups with doxorubicin (Groups 8-12) exhibited significant inhibition of cell viability when compared to those without doxorubicin \( (p<0.0001) \), Figure 3. The effect of probiotic L. acidophilus LA-5 on RAW 264.7 demonstrated a remarkable effect by decreasing of the viability of cancerous cells \( (p<0.0001) \). Against human colorectal adenocarcinoma, Group 12 was the most affected with a significant decrease in the viability to ≈30% \( (p<0.0001) \) (Figure 3). Group 12 was followed by Group 9, in which the viability of 4T1 cells were significantly inhibited when compared to the control group \( (p<0.0001) \) (≈35% viability). In the 24 h incubation time, there was also a very effective response in Group 9, decreasing the cell viability to ≈50% (Figure 3).

**Figure 2.** Percentage viability graph of the 4T1 breast cancer cell line after co-culture treatment using RAW 264.7 murine macrophage cells stimulated with L. acidophilus LA-5 for 24 and 48 h. The cell viability was determined by an MTT assay. The control cells were untreated 4T1 breast cancer cells and taken as 100% viability. **\( p<0.01 \), ****\( p<0.0001 \)**
The popularity of probiotic goods and supplements in the market has increased due to the superior beneficial effects of these inactivated microorganisms on general health (Saxelin, 2008). They are not only strengthening our general health, but are also potential immune stimulants as they keep our immune system awake to any harmful conditions (Galdeano et al., 2007). Cancer is one of the leading causes of death in the world. Although many studies have been conducted on this field of research, no effective treatment has yet to be found against this disease. Surgery, chemotherapy and radiotherapy are common therapeutic strategies currently being used in the treatment of cancer (Siegel et al., 2012); however, it is known that alternative approaches and supplementary products are needed for cancer treatment strategies. These alternative approaches can be direct or indirect (supportive). Immunotherapy is currently regarded as the next best alternative treatment approach for cancer (Sathyanarayanan and Neelapu, 2015).

It is basically the manipulation of immune system that augments the patients’ immunity against tumor cells (Couzin-Frankel, 2013). Probiotics are important immune stimulants and have shown to possess significant effects on immune cell activation, giving them a great value in terms of supplementary potential when administered together with a therapeutic agent (Foo et al., 2011).

In this study, the immune stimulatory potential of probiotic L. acidophilus LA-5 was investigated together with a low dose of doxorubicin (1/2 IC₅₀), which is a chemotherapeutic agent currently used in the treatment of various types of cancer. The study also aimed to investigate the potential effect of the probiotic L. acidophilus LA-5 on macrophage activation against two different cancer types (mouse breast and human colon carcinomas). The supportive effects of probiotics together with chemotherapeutics were also performed in a study by Masuno et al. (1991). In that study, Lactobacillus casei LC9018 was tested clinically with doxorubicin in order to treat malignant pleural effusion secondary to lung cancer patients. In the group treated with Lactobacillus casei LC9018, a significant increase in performance status (PS)

Figure 3. Percentage viability graph of the CaCo-2 human colon adenocarcinoma cell line after co-culture treatment using PMA-treated THP-1 human macrophage-like cells for 24 and 48 h. The cell viability was determined by an MTT assay. The control cells were untreated CaCo-2 human colon adenocarcinoma cells and taken as 100% viability.

*p<0.1, **p<0.01, ****p<0.0001
with fewer symptoms apart from a longer survival rate were detected in comparison to the control group. The results obtained from that study revealed the potential benefits of using probiotics in cancer treatment. It is known that probiotic bacteria first interact with macrophages and dendritic cells upon ingestion, and modulate both the innate immune system, especially phagocytosis, and the adaptive immune response (Galdeano et al., 2007; Faghfoori et al., 2015). The surface protein Slp-A is known for its role in the activation of the TLR2 signaling pathway and pro-inflammatory response in macrophages (Galdeano et al., 2007; Konstantinov et al., 2008). The *L. acidophilus* LA-5 strain was tested in a study to investigate its effect on immune cells and was found to induce RAW 264.7 cells for the production of IL1-α and TNF-α. These results promoted the macrophage stimulatory potential of LA-5 strain in producing immunological factors (Masuno et al., 1991). The effects of *L. acidophilus* LA-5 on macrophages were also investigated on the murine macrophage-like cell line J774 and reported to enhance the phagocytic activity of macrophages (Hatcher and Lambrecht, 1993). Macrophages show anti-tumor activities by increasing phagocytosis and induction of *in vitro* macrophage proliferation (Foo et al., 2011).

In this study, murine macrophages (RAW 264.7) were stimulated with both LPS and probiotic *L. acidophilus* LA-5. The groups stimulated with both probiotic *L. acidophilus* LA-5 and LPS (Group 12: 4T1 + doxorubicin (1/2 IC_{50}) + RAW 264.7 (LPS) + Probiotic – *L. acidophilus* LA-5) showed significant cytotoxic activity against the 4T1 murine breast cancer cells and decreased the viability of cancer cells to approximately 80% in 24 h incubation (*p* < 0.0001) (Figure 2). The group 6: 4T1 + RAW 264.7 (LPS) + Probiotic – *L. acidophilus* LA-5, did not show significant inhibition, (Figure 2). In the combination therapy group (Group 12; 4T1 + doxorubicin (1/2 IC_{50}) + RAW 264.7 (LPS) + Probiotic – *L. acidophilus* LA-5), the role of *L. acidophilus* LA-5 on LPS-activated macrophages with doxorubicin were investigated in terms of cancer cell viability *in vitro*. Regarding the groups that included doxorubicin as a main therapeutic agent, it is clear that the percentage viability of 4T1 cancer decreased significantly (50%) when compared to the control group (*p* < 0.0001) (Group 1; only 4T1) upon 48 h incubation exerting the best cytotoxic effect among other groups (Figure 2). These data demonstrated that there is a need for chemotherapy as a main agent as mentioned in the study of Masuno et al. (1991). The doxorubicin alone group [Group 12; 4T1 + doxorubicin (1/2 IC_{50})] decreased the cell viability to 72% and 85% in 24 and 48 h of incubation, respectively. In the initial co-culture study, the best combination group was found to be the LPS-activated macrophage group that was also stimulated with probiotic *L. acidophilus* LA-5 along with doxorubicin (1/2 IC_{50}) (Group 12). Group 12 decreased the 4T1 cell viability significantly to 40% (*p* < 0.0001). The preliminary data obtained from this study demonstrated promising results in terms of the supplementary role of probiotics in cancer treatment when used with a drug (i.e., doxorubicin), correlating with the results of Masuno et al. (1991).

As reported by Perdigon et al. (2003), lactic acid bacteria had a great capacity in stimulating gut immune system. The authors showed that lactic acid bacteria enhanced macrophages activity to induce IFNγ and TNFα cytokines favoring both Th1 and Th2 immune responses desired for anti-cancer studies (Perdigon et al., 2003). Slp-A protein, found at the surface of *L. acidophilus* LA-5, is also known for its superior activity in initiating both adaptive and innate immune systems. Slp-A can be recognized by both macrophages and dendritic cells (Konstantinov et al., 2008; Taverniti et al., 2013). In the current research, following the initial study performed on murine cell lines, another co-culture study was implemented to determine the effect of probiotics on macrophages on human-origin cell lines.

For the second co-culture system, CaCo2 human colon adenocarcinoma and PMA-stimulated THP-1 macrophage-like cells activated by LPS were used. The second co-culture study aimed to investigate the effect of probiotic *L. acidophilus* LA-5 on colon cancer cells, which is the main place they would reach soon after being ingested. The results obtained in this co-culture study were more promising when compared to the initial co-culture study (4T1 & RAW 264.7). Figure 3 illustrates that upon the probiotic stimulation of human macrophage-like cells, the percentage viability of CaCo2 cells significantly decreased to 70% and 60% in 24 and 48-h, respectively (*p* < 0.0001) (THP-1) (Group 5; CaCo2 + THP-1 + Probiotic – *L. acidophilus* LA-5). The group 6; CaCo2 + THP-1 (LPS) + Probiotic – *L. acidophilus* LA-5, did not show significant inhibition, (Figure 3). It could be concluded that the probiotic stimulation of human macrophage-like cells (THP-1) increased the cytotoxic activities against CaCo2 cells, which might be related to the Slp-A protein found at the surface of *L. acidophilus* LA-5, as it is known for its activities in TLR2 (Konstantinov et al., 2008). Similar to the previous co-culture study that was undertaken with murine cell lines, groups with doxorubicin showed significant activity in decreasing the viability of CaCo2 cells, as expected (Groups 11; CaCo2 + doxorubicin (1/2 IC_{50}) + Probiotic – *L. acidophilus* LA-5; 12; CaCo2 + doxorubicin (1/2 IC_{50}) + THP-1 (LPS) + Probiotic – *L. acidophilus* LA-5). In Group 8, in which the CaCo2 cells were treated with a 1/2 IC_{50} dose of doxorubicin, there was a remarkable decrease in the viability of CaCo2 cells to 80%-55% levels upon 24 and 48 h exposure, respectively (*p* < 0.0001). From the data, the combination group
(Group 12; CaCo^2 + doxorubicin (1/2 IC_{50}) + THP-1 (LPS) + Probiotic – *L. acidophilus* LA-5) was found to be the most affected group as the viability of CaCo^2 cells was significantly inhibited (30%) (p<0.0001). In a study carried out by Baldwin et al. (2010), *L. acidophilus* and *L. casei* probiotics were found to be apoptosis inducers when tested on colorectal carcinoma cell line, suggesting that probiotic bacteria itself may possess an anticancer activity. It is also well known that probiotics also have significant effect on immune stimulation (Mack, 2005). Additionally, probiotics are found to exert their anticancer effect through immune stimulatory effect on T-cells, macrophages and neutral killer cells (Shida and Nomoto, 2013). In a population based study, a beverage containing *L. casei* Shirota was found to reduce the breast cancer occurrence risk (Toi et al., 2013). *L. casei* Shirota was also found to reduce the recurrence rate of bladder cancer apart from increasing the efficacy of chemotherapy in patients (Naito et al., 2008). These data obtained from preliminary research in the literature and the data obtained in this study together suggest the beneficial effect of probiotic *L. acidophilus* LA-5 as a supplementary medicine in the diet of patients under chemotherapy treatment in terms of their capacity in apoptosis induction, immune stimulation, and anticancer activities. It is also well known that upon chemotherapy treatment, a decrease in *Lactobacillus* quantity is observed, followed by increasing pathogenic species in gut flora. In the study by Mlu et al. (2013), probiotic consumption together with chemotherapy in lung cancer patients was found to be very useful in restoring gut microflora reducing gastrointestinal complaints apart from its beneficial effects on immune system (Mlu et al., 2013; Sharma et al., 2018).

The data obtained from the two different co-culture studies emphasized that *L. acidophilus* LA-5 has a great potential in stimulating macrophages against cancer cells. The use of probiotic *L. acidophilus* LA-5 in cancer patients receiving chemotherapy would be an alternative strategy to combat cancer via strengthening the immune response of patients.

**Conclusion**

Probiotics have gained a lot of interest in recent years with their beneficial roles in human health due to their anticancer and immune stimulatory activities. In this study, two different co-culture studies were performed using both murine and human cell lines. In both experiments, the supplementary potential of *L. acidophilus* LA-5 on macrophages was evaluated by investigating the cell viability of the 4T1 murine breast cancer and CaCo-2 human colon cancer cells via an MTT assay. However, future work should investigate the mechanism action of cell death.

In brief, according to these data suggest that patients undergoing chemotherapy can be encouraged to include probiotic products containing the *L. acidophilus* LA-5 strain in their diet to strengthen their immune response, as well as disrupting cancer progression mechanisms.

**Compliance with Ethical Standard**

**Conflict of interests:** The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

**Ethics committee approval:** Author declare that this study does not include any experiments with human or animal subjects.

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