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The Effect of *Lactobacillus Plantarum* and Bacterial Peptidoglycan on the Growth of Mouse Tumors *in vivo* and *in vitro*

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Abstract: Some members of the microbiota have been shown to be effective strategies for inhibiting tumor growth through stimulation of host anti-tumor immune responses. Anti-tumor immune effects were observed when *Lactobacillus plantarum* (*Lp*), a member of the gut microbiota, was used to treat colorectal cancer in mice. Moreover, constituents of bacteria, including peptidoglycan (PG), have been shown to exhibit tumoricidal effects. The aim of this study was to investigate the anti-tumor effects of *Lp* on serum levels of angiogenic and immunostimulatory cytokines in melanoma-bearing mice *in vivo*; as well as the effect of PG on the growth of mouse melanoma and breast cancer cells *in vitro*. Fifty C57BL/6 female mice were divided into two groups. Prior to tumor implantation, *Lp* was administered via oral gavage for 2 weeks to the experimental group. After receiving subcutaneous injections of B16F10 melanoma cells, *Lp* administration was continued once per week for 3 weeks to the experimental group. After the last bacterial administration, serum levels of Vascular Endothelial Growth Factor (VEGF) and Interleukin-12 (IL-12) were determined by ELISA. Additionally, mice from both groups were monitored for survival. Moreover, B16F10 melanoma and EMT6 breast cancer cells were incubated separately with two PG concentrations for 48 h and percent viability was determined. A significant decrease in the serum levels of VEGF and a significant increase in the serum levels of IL-12 were observed in the group treated with *Lp* Moreover, 20% survival rate was noted in the group treated with *Lp* In vitro, a marked decrease in the viability of mouse melanoma and breast cancer cells was observed 48 h post-incubation with PG. It appears that *Lp* possesses anti-tumor activity, by both stimulating the immune system and suppressing angiogenesis. Moreover, *Lp* appears to have a direct tumoricidal effect through PG.

Keywords: B16F10 Melanoma Cells, EMT6 Breast Cancer Cells, *Lactobacillus plantarum*, Peptidoglycan, VEGF, IL-12

Introduction

Melanoma and breast cancer are among the leading causes of cancer mortality worldwide with a rising incidence over recent years (Kazarian *et al*., 2017; Gray-Schopfer, 2007). Deaths occur despite conventional treatments and innovative techniques in surgery, chemotherapy and radiotherapy (Palathinkal *et al*., 2014). Attempting to reduce the death rate, new treatment modalities with low side effects and maximum efficacy are being investigated (Yu *et al*., 2017). One such treatment modality is immunotherapy, which is based on stimulating or priming the patient's immune system to attack malignant tumors.

Immunotherapy has been around for more than 100 years. In the late 19th century, Dr. William Coley, the Father of Immunotherapy, treated human malignancies with a bacterial preparation later known as the Coley's toxin (Nelson *et al*., 2015; McCarthy, 2006).

Nowadays, research in cancer immunotherapy focuses on several approaches, one of which is blocking Killer inhibitory Receptors (KIRs) on Natural killer (NK) cells by anti-KIR monoclonal antibodies. It has been shown that such blockade renders the NK cell

active in killing tumor cells (Ruggeri *et al*., 2016; Romagné *et al*., 2009; Muntasell, 2017). Immune checkpoint therapy by immune checkpoint blockade is another approach in cancer immunotherapy. Monoclonal antibodies that inhibit the function of immune checkpoints CTLA-4 and PD-1 increase T cell activity against tumors (Melero *et al*., 2007).

Recently, it has been reported that checkpoint therapy was curable in some but not all patients with melanoma. Later, variability in response to checkpoint therapy was related to the constituents of the microbiota (Vétizou *et al*., 2015).

Mammals harbor over 104 microbial cells, outnumbering mammalian cells 10-fold (Christian *et al*., 2015; Bultman, 2016). Nearly 1000 different microbial types colonize different surface locations of the body. Studies show that colonization of the gut by commensal bacteria is key to immune development (Nelson *et al*., 2015). Also, accumulating evidence indicates that gut microbiota is involved in assisting the immune system against cancer (Goldszmid *et al*., 2015).

Lactobacilli are Gram positive, microaerophilic, lactic acid producing bacteria, belonging to the gut and vaginal microbiota. They are important in food and fermentation industries. They are also prominent probiotics (Tannock, 2004). It has been reported that Lactobacilli can induce differentiation to Th1 immune response, which is important for tumor inhibition (Hu *et al*., 2015). Previous studies in mice showed that administration of L. acidophilus and *Lp* modulate the immune response against breast cancer and colorectal cancer, respectively (Maroof *et al*., 2012; Hu *et al*., 2015).

The largest component of Lactobacilli cell wall is PG, an essential polymer that establishes the shape and maintains the integrity of the bacterial cell. PG is a conserved structure, with molecular motifs unique to bacteria and thus is an excellent target for the immune system (Vollmer *et al*., 2008). The mammalian innate immune system expresses several receptors that recognize PG, such as CD14, Toll-like receptor 2, Nod 1 and 2 (Kawai and Akira, 2009).

The current study was carried out in an effort to investigate the anti-tumor effect of *Lp* and PG, a constituent of its cell wall, on tumor growth. For this purpose, an in vitro study was performed to determine if PG had a direct toxic effect on mouse melanoma and breast cancer cells. Moreover, an *in vivo* study was done to determine survival rates of B16F10 melanoma-bearing mice as well as their serum levels of IL-12 and VEGF treated with *Lp* compared to untreated controls.

It was anticipated that the results obtained will clarify the direct and/or immunomodulatory effects of *Lp* and its cell wall constituent on the growth of tumors.

Materials and Methods

In Vivo Studies

Lactobacillus Plantarum (*Lp*)

Lp was cultured in deMan-Rogosa_Sharpe (MRS) medium at 37°C for 48 h, under aerobic conditions. API 50 CH was performed to confirm identity. On the day of bacterial administration to mice, bacterial colonies were suspended in Phosphate Buffered Saline (PBS); "DENSIMAT" densitometer was used to prepare a bacterial suspension with a turbidity equivalent to 5 McFarland units (~109CFU/ml).

B16F10 Melanoma Cells

B16F10 metastatic melanoma cells are Syngeneic with the C57BL/6 mice. They are adherent cells and were maintained as monolayers in vitro in RPMI 1640 culture medium supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (Lonza, B-4800 Verviers, Belgium) and incubated at 37°C in a 5% CO_2 incubator.

On the day of tumor induction into mice, the melanoma cells were detached with trypsin (2.5% trypsin in 10× in HBSS without calcium or magnesium, Lonza, B-4800 Verviers, Belgium). The viable cell count was determined by trypan blue exclusion method using a Neubauer Chamber and the cells were re-suspended in RPMI-1640 medium. The amount of B16F10 melanoma cells injected subcutaneously was 106 cells per mouse suspended in 0.4 mL RPMI-1640.

Mice and Ethics Statement

The use of mice in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Medicine, American University of Beirut. Fifty C57BL/6 female mice, 6 to 8 weeks old were used and all experiments were performed in strict accordance with the guidelines of this committee.

Administration of Lp and Challenge with Tumors

Fifty C57BL/6 mice were randomly divided in two groups. Prior to tumor implantation, Lpwas administered orally via oral gavage for 14 consecutive days (concentration: 1 x 108 CFU/mouse) to the experimental group. Feces samples were cultured before and after bacterial administration. An equal volume of Phosphate Buffered Saline (PBS) (100ml) was administered via oral gavage to the control group. At day 15, B16F10 melanoma cells were implanted subcutaneously into mice in both groups. After tumor implantation, *Lp* administration was continued once per week for 3 weeks (concentration: 1×108 CFU/mice) to the experimental groups and an equal volume of PBS (100ml) was administered orally to the control group (Table 1).

Table 1. Protocol used in treating the mice

Groups Time (Weeks)	1 – Control (25 mice)	2 – Experimental (25 mice)
1	PBS daily	*Lactobacillus plantarum* daily
2	PBS daily	*Lactobacillus plantarum* daily
3	Tumor implantation	Tumor implantation
4	PBS once per week	*Lactobacillus plantarum* once per week
5	PBS once per week	*Lactobacillus plantarum* once per week
6	PBS once per week	*Lactobacillus plantarum* once per week

Route of administration: Orally by oral gavage
Amount of *Lp* administered: 0.1 ml of a suspension containing 108 CFU per mouse
Amount of PBS administered: 0.1 ml per mouse

Procurement of Specimens

At two, 4 and 6 h post-last administration of *Lp*, five mice from each group were euthanized then dissected and blood was collected by cardiac puncture. Blood from each group was pooled and serum was separated and used for VEGF and IL-12 quantification. The remaining 10 mice from each group were monitored to determine the survival rate.

VEGF and IL-12 Quantification

VEGF mouse ELISA kit (Abcam, ab100751, USA) and IL-12 p70 mouse ELISA kit (Abcam, ab119531, USA) were used to determine the serum levels of VEGF and IL-12, respectively. The procedures were performed according to the manufacturers' protocol.

In Vitro Evaluation of Peptidoglycan (PG) Effect

Peptidoglycan

Peptidoglycan from Bacillus subtilis, a commercially available peptidoglycan extract, was used in this study.

Culture of EMT6 Breast Cancer Cells in the Presence of PG

EMT6 metastatic breast cancer cells are adherent cells and were maintained as monolayers in vitro in Waymouth culture medium supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 13% Fetal Bovine Serum (Lonza, B-4800 Verviers, Belgium) and incubated at 37°C in a 5% CO_2 incubator. EMT6 breast cancer cells were counted and seeded in 24-well plates, (seeding density of 2×104 cells/500µl/well), then incubated for 24 h at 37°C and 5% CO_2. After incubation, 500 µL of two different PG concentrations (1 and 2.5 mg/mL) were added to the different wells. No PG was added to the control wells.

The samples were run in duplicates. Wells were then incubated at 37°C in a 5% CO_2 incubator for 48 h. After incubation, EMT6 breast cancer cells were detached with trypsin, then suspended in culture medium and a viable cell count was determined.

Culture of B16F10 Melanoma Cells in the Presence of PG

B16F10 melanoma cells were counted and seeded in 24-well plates, (seeding density of 2×104 cells/500µl/well), then incubated for 24 h at 37°C and 5% CO_2. After incubation, 500 µL of two different PG concentrations (1 and 2.5 mg/mL) were added to the different wells. No PG was added to the control wells.

The samples were run in duplicates. Wells were then incubated at 37°C in a 5% CO_2 incubator for 48 h. After incubation, melanoma cells were detached with trypsin, then suspended in culture medium and a viable cell count was determined.

Statistical Analysis

Whenever applicable, data were expressed as Mean ± SD. Mice survival was evaluated by generating Kaplan–Meier survival curves. The unpaired student T-test was implemented to assess the sample variations between groups using the Graph pad online software. Results were considered to be statistically significant at P value <0.05.

Results

In Vivo Results

API 50 CH Results from Feces Samples

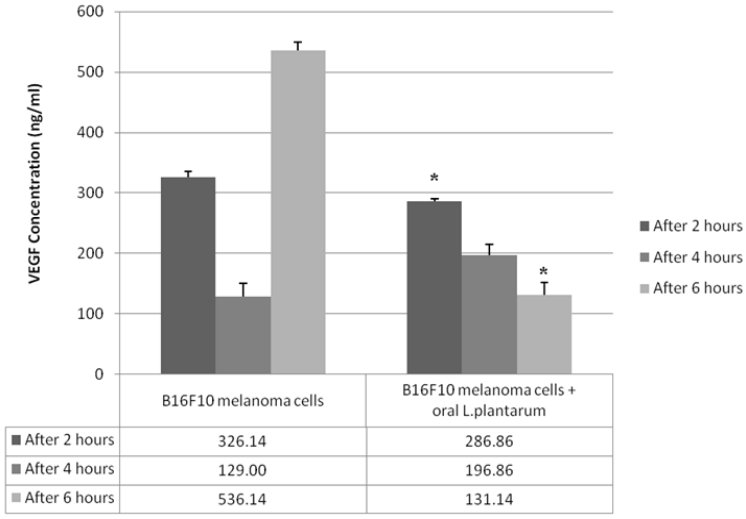
Feces samples cultured on MRS agar prior to the administration of Lpshowed complete absence of *Lp* in stools. Feces samples cultured on MRS agar after 14 days of *Lp* administration resulted in colonies very similar to the *Lp* that was cultured from stock. API 50 CH confirmed that the strain found in the feces sample is *Lp*.

VEGF Serum Levels

The serum levels of VEGF in the treated mice (experimental group) significantly decreased (p-value = 0.0306) at 2 h after the last administration of *Lp*, compared to those of the control mice, which received an equal volume of PBS. A profound decrease (p-value = 0.0018) in serum levels of VEGF was obtained in the treated mice compared to that of the control mice 6 h post bacterial administration (Fig.1).

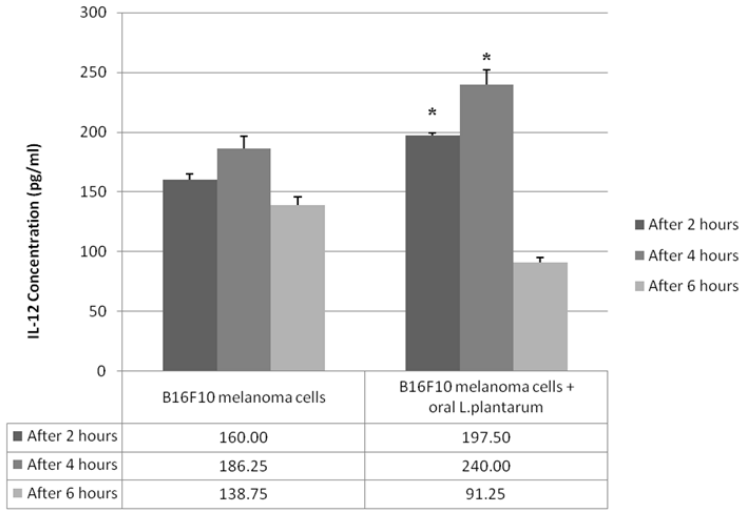
IL-12 Serum Levels

The serum levels of IL-12 in the treated mice, compared to those of the control mice, increased significantly 2 h (p-value = 0.0109) and 4 h (p-value = 0.0430) post bacterial administration. However, the serum levels of IL-12 in the treated mice, compared to those of the control mice, decreased significantly (p-value = 0.0136) 6 h post bacterial administration (Fig. 2).



	B16F10 melanoma cells	B16F10 melanoma cells + oral L.plantarum
After 2 hours	326.14	286.86
After 4 hours	129.00	196.86
After 6 hours	536.14	131.14

Fig. 1. Serum VEGF levels as detected by ELISA. VEGF levels decreased significantly in the experimental group that received *Lp* treatment orally at 2 and 6 h post treatment *: Statistically significant at p-value <0.05



	B16F10 melanoma cells	B16F10 melanoma cells + oral L.plantarum
After 2 hours	160.00	197.50
After 4 hours	186.25	240.00
After 6 hours	138.75	91.25

Fig. 2. Serum IL-12 levels as detected by ELISA. Significant increase in IL-12 levels was seen in the experimental group that received *Lp* treatmentorally at 2 and 6 h post treatment. *: Statistically significant at p-value <0.05

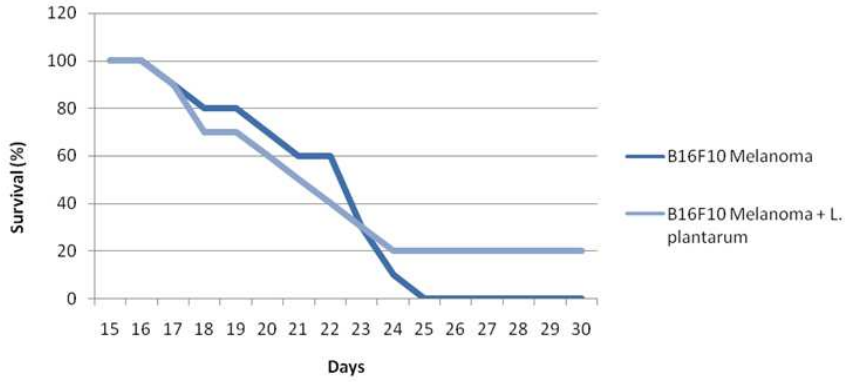


Fig. 3. Survival curve of the 2 groups of mice followed over a period of 15 days

Mice Survival

Mice of the control group, which received PBS by oral gavage, were all dead by day 25 (0% survival). By day 30, eight out of ten mice administered with *Lp* by oral gavage at a dose of 108CFU per mouse were dead (20% survival) (Fig. 3).

The survival results were further evaluated by generating the Kaplan Meier survival curves, showing the probability of survival in a given period of time (Fig. 4). The p-values were calculated to assess the statistical significance of the results obtained. P-values ≤ 0.05 were considered statistically significant. Although 20% of the experimental group survived compared to the control group the difference was not statistically significant.

In Vitro Results

Effect of PG on Breastcancer cells

Compared to the control well, a significant decrease in viable EMT6 breast cancer cell count was obtained in the wells treated with 500µl of 1 mg/ml (p-value = 0.0006) and 2.5mg/ml (p-value = 0.0001) of PG respectively (Fig. 5).

Effect of PG on Melanoma Cells

Compared to the control well, a significant decrease in viable B16F10 melanoma cell count was obtained in the wells treated with 500µl of 1 mg/ml (p-value = 0.0145) and 2.5 mg/ml (p-value = 0.00328) of PG respectively (Fig. 6).

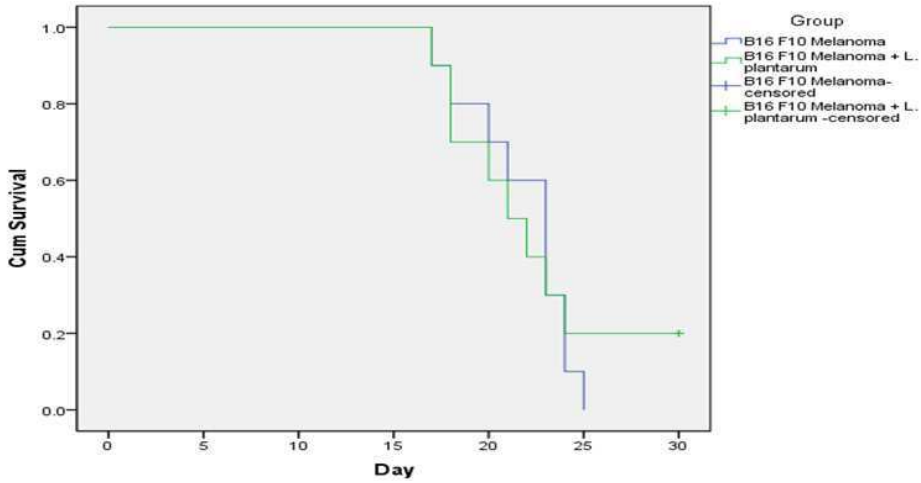


Fig. 4. Kaplan Meier survival curves for the two groups. When the experimental group was compared to the control group, it did not show any statistically significant survival rate

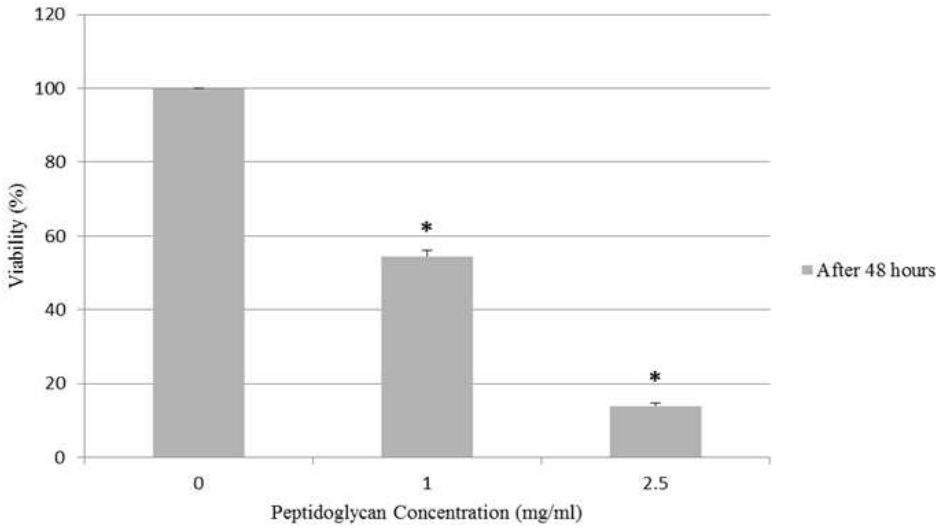


Fig. 5. Assessing the effect of PG on the growth of mouse breast cancer cells *in vitro*. The viability of EMT6 mouse breast cancer cells significantly decreased with increasing concentration of PG. *: statistically significant at p-value <0.05

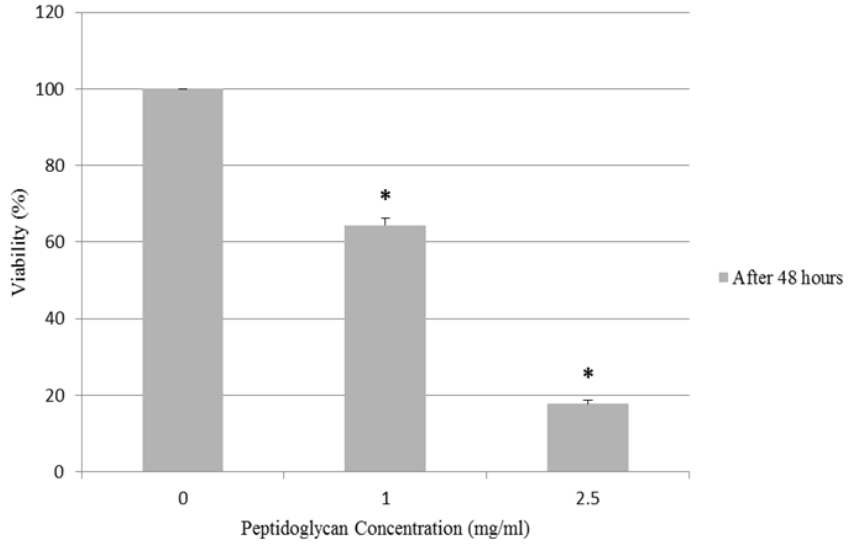


Fig. 6. Assessing the effect of PG on the growth of mouse melanoma cells *in vitro*. The viability of B16F10 mouse melanoma cells significantly decreased with increasing concentration of PG. *: statistically significant at p-value <0.05

Discussion

Reports have implicated the normal microbial flora in the development of the immune system and its anti-tumor activity. *Lp*, a normal member of the intestinal and vaginal flora, was used to treat colorectal cancer in mice. It was reported that the anti-colorectal cancer effect of *Lp* is attributed to its ability to enhance the immune response against cancer cells (Hu *et al*., 2015). Based on these results, treatment of melanoma-bearing mice with *Lp* was attempted.

In order to survive, tumors need a blood supply. For this purpose, Angiogenesis, the formation of new blood vessels, is a crucial process that is required for tumor growth and metastasis and is mainly promoted and regulated by the pro-angiogenic cytokine VEGF (Niu and Chen, 2010). In contrast, IL-12 promotes a Th1 response and is known to possess strong anti-tumor and anti-metastatic properties (Zeh III *et al*., 1993; Valerie *et al*., 1993). Decreased levels of VEGF and increased levels of IL-12 at different time intervals in tumor-bearing mice treated with Lp, as compared to untreated controls, supports the anti-tumor effect of *Lp*. This anti-tumor effect is consistent with the findings reported by previous studies; Hu *et al*. (2015) evaluated tumor size and demonstrated that *Lp* significantly inhibited CT26 growth, while Murosaki *et al*. (2000) reported that the anti-tumor effects of heat-killed *Lp* is through restoration of impaired interleukin-12 production in tumor-bearing mice. It can be speculated that receptors expressed by several IL-12-producing cells (such as macrophages, neutrophils and dendritic cells), are engaged by *Lp* resulting in signal transmission, activation of transcription factors and production of cytokines including IL-12.

It is worth noting that the decrease in VEGF levels at 4 h after the last administration of *Lp*, in the experimental group when compared to the control group, might be explained by what is known as the oscillating effect of a biological response upon the introduction of an exogenous agent (Rando *et al*., 2010).

Treatment of melanoma-bearing mice with *Lp* resulted in an increased survival (20% survival rate), which is concurrent with the ELISA results obtained. This result did not reach statistical significance, yet a prolongation of survival was observed. It is thought that manipulation of dosages used might result in a statistically significant survival outcome. It is worth mentioning that combination treatment with check point therapy might further improve prolongation of survival or even result in complete cure. This idea stems from the report that the presence of Bacteroides fragilis as member of the microbiota resulted in improved cure rates in patients getting check point therapy (Vétizou *et al*., 2015).

Peptidoglycan, a major constituent of the cell wall of Gram positive bacteria, exhibited a direct toxic effect on mouse melanoma and mouse breast cancer cells. These results were consistent with those obtained from previous studies conducted by Fichera and Giese (1994) who demonstrated that PG derived from Lactobacillus casei has a direct inhibitory activity on tumor cells and Kim *et al*. (2002) who showed that PG of ten Lactic Acid bacteria had significant anti-proliferative activities against several cancer cell lines *in vitro* .It should be noted that PG extracted from *Lp* was not commercially available. Schleifer and Kandler (1972) reported that PG from Bacillus subtilis (Bs) and *Lp* share similar characteristics, therefore PG from Bs was used in this study.

Conclusion

In conclusion, it appears that *Lp* has multiple anti-tumor effects. It suppresses angiogenesis as indicated by a decrease in VEGF levels; it boosts adaptive immunity by generating Th1 lymphocytes and has a direct toxic effect on tumor cells. Studies using combination therapy with check point therapy are future plans.

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Authors' Contributions

Arpa Aintablian and Dalal F. Jaber: Co-investigators, contributed to the conception and design of this study, acquisition of laboratory data, data analysis and/or interpretation, drafting and /or critical revision of the manuscript and approved this final version.

Mary Ann Jallad: Participated in the conception of this study, drafting and/or critical revision of the manuscript and approved this final version.

Alexander M. Abdelnoor: Principle Investigator. Introduced the conception and design of this study. He drafted and made a critical revision of the manuscript and approved this final version.

Conflict of Interest

The authors declare that they have no conflict of interests.

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