J Appl Biotechnol Rep. 2022 June;9(2):576-581

Journal of
Applied Biotechnology Reports

doi 10.30491/JABR.2020.249397.1289

Original Article

Influence of Extracellular Vesicles from *Lactobacillus rhamnosus* GG on the Cell Adhesion and *mmp2* and *mmp9* Genes Expression in Colorectal Cancer Cells

Amir Mohammad Parsa [1], Hamideh Mahmoodzadeh Hosseini [1*], Seyed Ali Mirhosseini [1*]

[1] Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

Corresponding Authors: Hamideh Mahmoodzadeh Hosseini, PhD, Associate Professor; Seyed Ali Mirhosseini, PhD, Assistant Professor- Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Vanak Sq. Molasadra St., Tehran, Iran. Tel: +98-2182482592, E-mail: hosseini361@yahoo.com; ali.mirh@gmail.com

Received September 21, 2020; **Accepted** December 5, 2020; **Online Published** June 18, 2022

Abstract

Introduction: *Lactobacillus rhamnosus* GG is a probiotic bacterium with anti-cancer and anti-microbial characteristics. In addition, Extracellular Vesicles (EVs) from *Lactobacillus rhamnosus* GG showed apoptotic impacts on colorectal cancer cells. In this study, we aimed to isolate EVs from *Lactobacillus rhamnosus* GG (EVL) and identify its effect on cell adhesion and expression of *mmp2* and *mmp9* genes.

Materials and Methods: HT29 cells were exposed to 16, 32, 64, 128, 256, 512, 1024, and 2048 µg/ml EVL, and the cell viability, cell adhesion, and *mRNA* gene expression were investigated using the MTT assay, cell adhesion assay, and real-time PCR.

Results: We found that 128, 256, 512, 1024, and 2048 µg/ml of EVL led to attenuated cell viability of HT29 cells (p<0.05). Moreover, the expression of both *mmp2* and *mmp9* genes and cell adhesion significantly reduced at 1024 and 2048 µg/ml EVL compared to untreated cells (p<0.05).

Conclusions: According to the findings of the present study, it is suggested that EVL could be effective in cancer cell cytotoxicity.

Keywords: Probiotic, *Lactobacillus rhamnosus* GG, Colorectal Cancer, Cell Adhesion, Extracellular Vesicle

Citation: Parsa AM, Mahmoodzadeh Hosseini H, Mirhosseini SA. Influence of Extracellular Vesicles from *Lactobacillus rhamnosus* GG on the Cell Adhesion and *mmp2* and *mmp9* Genes Expression in Colorectal Cancer Cells. J Appl Biotechnol Rep. 2022;9(2):576-81. doi:10.30491/JABR.2020.249397.1289

Introduction

Cancer is known as a complicated disease which is a collection of more than 100 diseases. In spite of many efforts to improve cancer treatment, many common cancer treatment strategies are inefficient due to their toxicity and side effects.[1] Colorectal Cancer (CRC) is the third most common cancer in men and the second in women, with 1.65 million new cases and almost 835,000 deaths in 2015.[2] It is believed that genetic and lifestyle factors are the main causes of CRC. Mutations in genes that control cell growth and division lead to cancer. After cells detach from the tumor and invade surrounding tissues, it is likely that the tumor forms secondary tumors at other locations in a process called metastasis. The best method to treat CRC is to prevent and fight metastases. The spread of this type of cancer is a serious problem.[3] Matrix Metalloproteinases (MMPs) are a category of proteins that play a key role in cancer progression and are involved in different events such as metastases, cancer invasion, and cell growth. MMP2 and MMP9 are the two main members of this family.[4] Recently, researchers have applied microbial metabolites, probiotics, and toxins in cancer therapy.[5]

The gastrointestinal tract hosts a complex and diverse ecology of microorganisms.[6] These microbes are important to the health and destabilization of this microorganism ecosystem leading to several gastrointestinal disorders. Probiotics are live microorganisms being beneficial to the host in terms of health features if administered in sufficient amounts. It is believed that they improve the symptoms of several gastrointestinal diseases.[7]

Probiotics may play a key role in preventing cancer and treating infectious diseases, primarily by modifying the host's innate and acquired immune system.[8] However, it was suggested that probiotic bacteria are potential anti-cancer agents.[9] The anti-cancer properties of probiotic microorganisms have been verified, but the exact mechanisms are unknown.[10] In addition to the interaction of probiotics with the body systems, metabolites and bioactive molecules and structures secreted by these microorganisms such as parasporin-2Aa1, ε-poly-L-lysine, and nisin were introduced as anti-cancer agents.[11,12] Recent studies have shown the role of probiotics in Extracellular Vesicles (EVs) secretion by inducing IL-10 pathway probably leading to regulatory T cells (Tregs), differentiation, and proliferation.[13]

Lactobacillus rhamnosus GG is also a nonpathogenic probiotic bacterium being almost resistant to acid and bile. This probiotic produces an antimicrobial agent that could help to decrease inflammation and treat infectious diseases.[14]

Copyright © 2022 The Author(s). This is an open-access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

EVs are nanometer-scale membrane-contained vesicles released in an evolutionally conserved manner by several cells,[15] through easing the transfer of proteins, nucleic acids, and other molecules between cells.[16] The bacterial EVs might also include antibiotics, toxins or various virulence factors significantly involved in pathogenesis, host cell invasion, eliminating competitors, drug resistance and dodging the immune system. In pathogenic bacteria, EVs play a key role in bacterial infection. These vesicles are also involved in cell-to-cell communication and transporting genetic information being like eukaryotic EVs in this regard. Since EVs composition varies among numerous bacteria, the biosynthetic pathway of these vesicles is unclear.[17,18]

It has been shown that EVs act as a vital intercellular delivery system transferring vast types of signals between various recipient cells. In fact, EVs influence immune system responses and might transmit disease biomarkers for therapeutic coverage. EVs are acknowledged as shuttles transferring several components to the target cells influencing the signals whose transmittance into the recipient cells is essential.[19]

Previous reports have revealed that EVs could affect other cells near and/or far from the origin of secreting cells. Cancerous cells are the particular cells that both release and receive the discrepant EVs, such as exosomes with diverse characteristics. Thus, applying EVs to therapeutic purposes against tumor progression has been favored by the researchers. Investigations on EVs revealed that they carry proteins, mRNA and miRNA molecules and play a role in promoting or inhibiting cancers; furthermore, they are associated with apoptosis characteristics within various kinds of cells. The EVs are significantly involved in various kinds of vital processes such as inhibiting, promoting, and regulating gene expression, as well as differentiation and proliferation in the cells that receive them. Among EVs, those containing microRNA molecules are also involved more significantly in connections with gene expression via linking to mRNA molecules and manipulating the translation process of proteins. Note that the secreted EVs will be fused with the recipient cell membrane, and the molecules such as RNA molecules will be released as metabolites within the body fluids. These features helped EVs to be effective activating and regulating structures.[20]

In the present study, *lactobacillus rhamnosus* GG was selected as a non-pathogenic and benefit probiotic strain and the EVs were isolated from culture medium after 48 h growth. After confirming the morphology, the effects of EVs were evaluated on the cell adhesion and the expression of *mmp2* and *mmp9* genes in colorectal cancer cells.

Materials and Methods

Isolation of EVs from Lactobacillus rhamnosus GG

Lactobacillus rhamnosus GG (PTCC1637 strains) was purchased from the Iranian Research Organization for Science and Technology (Tehran, Iran). The bacteria were put to culture in Man Rogosa Sharp (MRS) broth (Inoclon, Tehran, IRAN) anaerobically at 37 °C for 48 h. Then, to isolate the EVL, culture medium was centrifuged for 30 min at 8000 g and the supernatant without bacterial cells and debris was transferred to the fresh tube. Next, a 0.22 mm filter (GVS filter technology, UK) was applied to filter the collected supernatant. To concentrate the supernatant, Centricon Plus-70 (Millipore, MA, USA) was utilized. Ultimately, an ultracentrifuge TL-100 rotor was used for ultracentrifugation at 100000 g for 60 min. The purified pellet was suspended in Phosphate Buffer Saline (PBS) and kept at 80 °C for further analysis. To measure the EVL concentration, the protein content was measured at 230 nm employing a Nanodrop spectrophotometer (Thermo-Scientific, US).[21]

Transmission Electron Microscopy Analysis

To study the EVL in terms of size and morphology, Transmission Electron Microscopy (TEM) analysis was performed. In brief, 5 µg EVL was fixed with 2.5% glutaraldehyde and then transferred to a firmware/carbon-coated grid (Iran University of Medical Sciences, Tehran, Iran). After 20 min incubation at room temperature, the grid was stained by negative stain (uranyl oxalate/methyl cellulose/uranyl acetate (Merck, Darmstadt, Germany). Finally, the images were prepared by TEM (LEO906, Germany, at 80 kV).[22]

MTT Test

To identify the cytotoxic effect of EVL, the MTT assay was done. HT29 cell line, as a colorectal cancer cell line, was bought from the cell bank of Pasture Institute (Tehran, Iran). RPMI1640 containing 10% Fetal Bovine Serum (FBS), 1% 100 mg/ml streptomycin, and 100 U/ml penicillin as a complete medium was used for harvesting HT29 cells at 37 °C and 5% CO_2. All the reagents and materials for cell culture were prepared from Inoclon, Tehran, Iran. To investigate the effect of EVL on the viability of HT29 cells, 200 µl complete medium containing 8000 cells was added to each well of the 96-well plate and incubated overnight at 37 °C and 5% CO_2. Next, the cells were treated with 16, 32, 64, 128, 256, 512, 1024 and 2048 µg/ml for 24 h, separately. Then, the culture medium of each well was replaced by 100 µl RPMI1640 without FBS containing 20 µl MTT dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, USA) (5µg/ml) and incubated for 4 h at 37 °C and 5% CO_2. Finally, the culture medium of each well was removed and formazan crystal was solved in 100 µl dimethyl sulfoxide (Sigma-Aldrich, USA) and the optical density was recorded at 570 nm using ELISA reader (BioRad, USA). Culture medium without EVs was analyzed as a negative control. Each test was carried out three times.

Adhesion Assay

To investigate the impact of EVL on the cell adhesion of HT29, the cell adhesion assay was performed according to the method introduced by Pereira et al. Briefly, 5×10^4 of HT29 cells were treated with 256, 512, 1024 and 2048 µg/ml EVL for an hour and were then transferred to separate wells of 96 well plate and incubated at 37 °C and 5% CO_2 for another 3 h. Then, the detached cells were removed via washing them twice with PBS. After fixing the attached cells with cold methanol (for 5 min), 1% Toluidine blue in 1% sodium tetraurate was added to the cells and incubated for 5 min. The stained cells were washed with PBS and dissolved in SDS 1% at 37 °C for 20 min. Finally, the optical density of each well was recorded at 540 nm using ELISA reader (BioRad, USA). Culture medium without EVs was analyzed as a negative control. Each test was conducted three times.[23]

Gene Expression Analysis

To evaluate the EVL impact on the expression of *mmp2* and *mmp9* genes, real-time PCR was applied. Briefly, 1×10^6 HT29 cells were treated with 256, 512, 1024 and 2048 µg/ml EVs for 24 h. Then, Trypsin was applied to detach the cells and the RNX-Plus kit (Cinnagen, Iran) was applied following the manufacturer's instructions to extract mRNA. The agarose gel electrophoresis and spectrophotometer were applied to determine the quality and quantity of the purified mRNA, respectively. Afterwards, cDNA synthesis kit (Takara, Japan) comprising two universal primers, random hexamer, and oligo dT primers, and M-MLV reverse transcriptase were applied following the manufacturer's instructions to amplify 1µg of each purified mRNA for synthesizing cDNA. Next, the real-time PCR method was applied to measure the relative rate of *mmp2* and *mmp9* genes expression. Table 1 shows the specific primers for *mmp2* and *mmp9* genes. *β-actin* gene was tested as a housekeeping gene. In brief, 1.5µl of each cDNA was added to the reaction mixture containing 2X cybergreen solutions and 10 pmol of specific primer with the final volume of 20 µl. The thermal program was executed by Corbett Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany) for 3 min for initial denaturation step at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at annealing temperature and 30 s at 72 °C. The relative expression of each gene was calculated using Rest 2009 based on $2^{-\Delta\Delta CT}$ (Qiagen, USA).

Table 1. The sequence of primers

Gene names	Forward (5′-3′)	Reverse (5′-3′)	Product length (bp)
mmp2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC	112
mmp9	AGACCTGGGCAGATTCCAAAC	CGGCAAGTCTTCCGAGTAGT	300
β- actin	TCATGAAGATCCTCACCGAG	TTGCCAATGGTGATGACCTG	118

Statistical Analysis

To measure the discrepancy between the test and control groups, a one-way ANOVA test was conducted by SPSS (Version 11) software (SPSS, Chicago, IL, USA). *p*-values less than 0.05 were set as statistically significant

Results

TEM Findings

Figure 1 depicts the image from TEM. EVLs are the white round shapes at the range of 50 nm-1500 nm in the dark background.

Cell Viability Outcome

To investigate the effect of EVL on the proliferation of HT29 cells, MTT assay was done. The serial concentrations of EVL from 16 to 2048 µg/ml were tested (Figure 2). Based on the statistical analysis, 64, 128, 256, 512, 1024 and 2048 µg/ml of EVL led to attenuated cell viability of HT29 cells 67.41, 66.3, 65.5, 63.1, 58.5 and 52.2%, respectively ($p<0.05$). The calculated IC_{50} was 2090.3µg/ml based on the data from MTT assay, and we selected four effective concentrations under IC_{50}, including 256, 512, 1024 and 2048 µg/ml, for further investigations.

Cell Adhesion Analysis

As shown in Figure 3, 512, 1024, and 2048 µg/ml of EVL significantly decreased the cell attachment of HT29 cells after 24 h (60.6, 57.6 and 36.6% in the respective order) in comparison with the negative control (100%) ($p<0.05$).

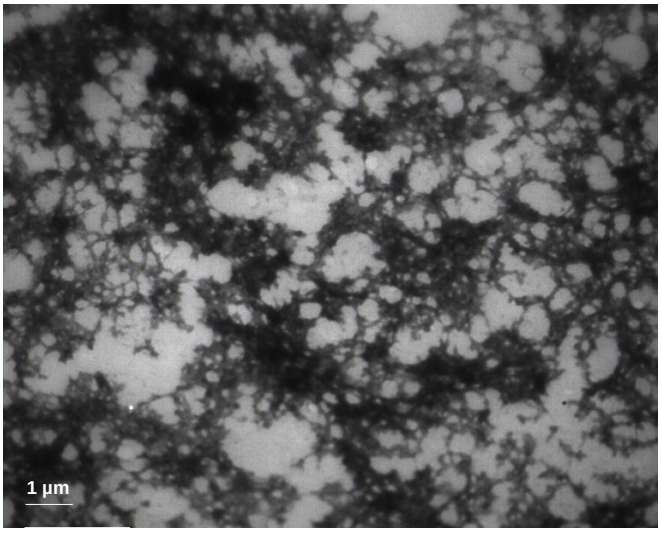

Figure 1. Image from TEM Stained with the Uranyl Acetate.

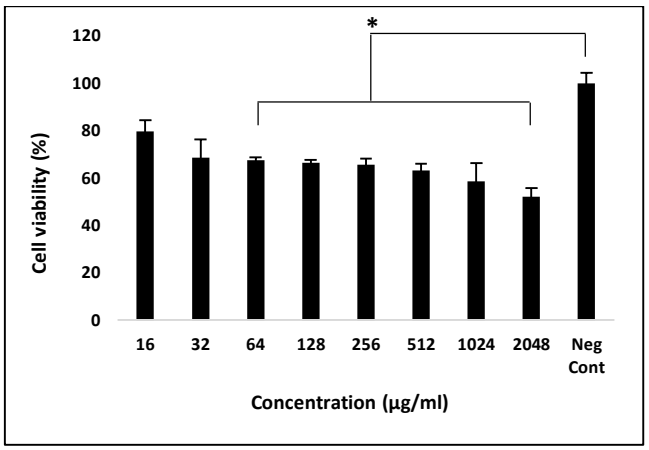

Figure 2. The Percentage of HT29 Cell Viability After Exposing to Different Concentrations of EVL. 64, 128, 256, 512, 1024 and 2048 µg/ml of EVL Reduced the Percentage of Cell Viability (67.41, 66.3, 65.5, 63.1, 58.5 and 52.2%, respectively). Star (*) indicates the statistically significant difference results compared to negative control with *p*-values less than 0.05.

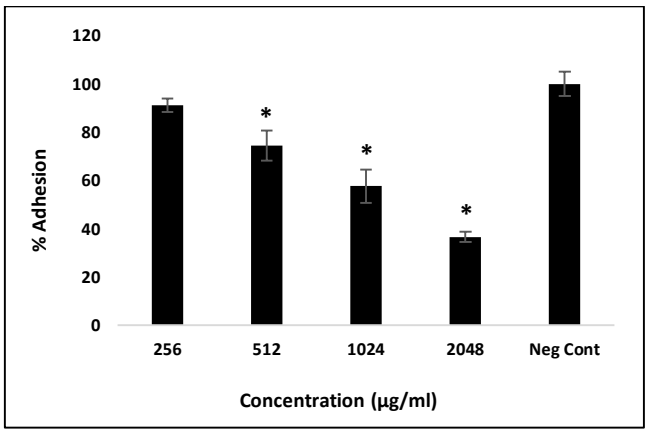

Figure 3. The HT29 Cell Adhesion After Exposing to Different Concentrations of EVL. Decrease cell attachment was significantly seen at 512, 1024, and 2048 µg/ml after 24 h (60.6, 57.6 and 36.6%, respectively). Stars (*) indicate the statistically significant difference results compared to negative control with *p*-values less than 0.05.

mmp2 and mmp9 Gene Expression

Real-time PCR was used to determine the effects of EVL on mRNA expression of two genes belonging to MMPs, *mmp2*, and *mmp9* genes. As illustrated in Figure 4a, the relative mRNA levels of *mmp2* were 1.3, 1.092, 0.84 and 0.741 fold after treating with 256, 512, 1024 and 2048µg/ml EVL, respectively. In addition, the relative expression levels of *mmp9* were 1.3, 0.99, 0.742 and 0.653 fold after treating with 256, 512, 1024 and 2048 µg/ml EVs, respectively (Figure 4b). Our findings showed that the expression of both *mmp2* and *mmp9* genes was significantly reduced at 1024 and 2048µg/ml EVs compared to the untreated cells ($p<0.05$).

Discussion

The production and release of EVs are common events among all live cells contributing to the transfer of bioactive molecules

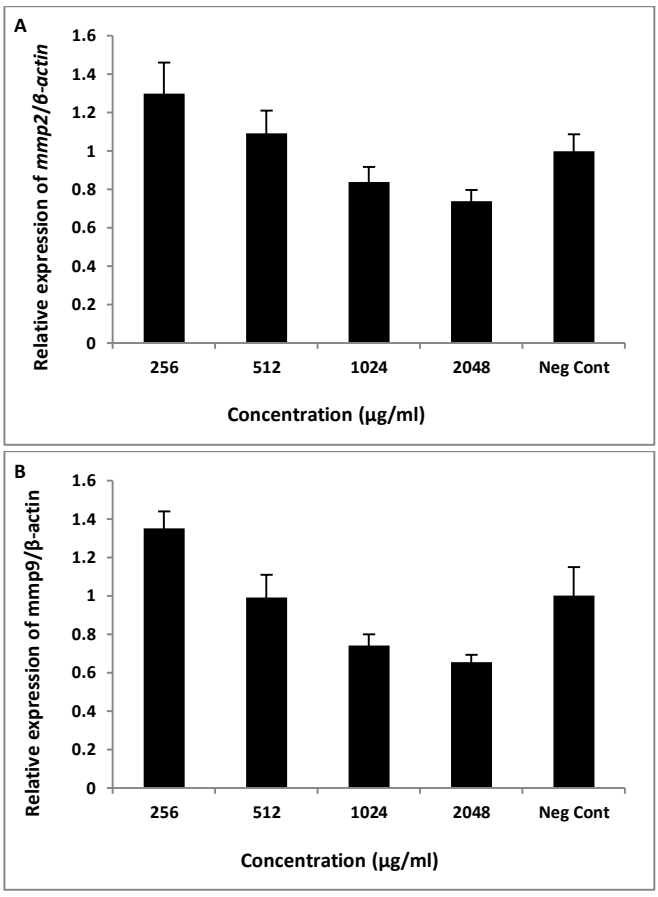

Figure 4. Relative Expression of *mmp2* gene **(A)** and *mmp9* gene **(B)** in HT29 Cells at 256, 512, 1024 and 2048 µg/ml EVL. The expression of *mmp2* and *mmp9* genes was significantly attenuated at 1024 and 2048µg/ml. Stars (*) indicate the *p*-values less than 0.05.

and macromolecule, signals and messages between the cells in a different distance from the producing cell. However, the contents of EVs are different and dependent on the type of cells and their cell cycle step. In the prokaryotes, both gram-positive and gram-negative bacteria are capable of producing EVs that play a key role in the communication between bacterial cells and bacteria population and host cells. Similar to mammalian cells, bacteria also produce and release different types of EVs in different situations with the pathologic and physiologic properties. EVs are involved in quorum sensing, biofilm formation, bacterial proliferation and growth, infection spreading and antibiotic resistance, etc.[21,24] Furthermore, it is reported that EVs from probiotic, such as *Lactobacillus rhamnosus*,[25] *Lactobacillus sakei*,[26] *Escherichia coli* Nissle 1917,[27] *Bifidobacterium longum*,[28] and *Bifidobacterium bifidum*[29] have immunomodulatory effects on cytokine profile, decreased pro-inflammatory enzyme, increased mucosal tolerance and T-reg response and mast cell apoptosis in vitro and in vivo studies. *Lactobacillus rhamnosus* GG is the gram-positive probiotic and numerous studies have been conducted concerning its anti-cancer properties. In studies conducted by Zelaya et al., Konieczna et al., and Al-Nedawi et al., it was observed that *Lactobacillus*

rhamnosus and its microvesicles resulted in an immune-modulatory effect through a TLR2-dependent regulation of DC and T cell counts.[25,30,31] Moreover, our previous finding showed the anti-cancer characteristics of EVs were isolated from *Lactobacillus rhamnosus* on the HepG2 cell line. In that study, EVLs were isolated from supernatant of *Lactobacillus rhamnosus* GG by centricon plus 70 and observed their cytotoxic impacts at 100 µg/ml of on HepG2 cancer cells. In addition, EVLs attenuated the expression of *bcl*-2 and increased the expression of *bax* gene and apoptotic index.[21] Following our previous study, in the current study, we studied the effects of EVLs on the cancer cell adhesion and the expression of *mmp2* and *mmp9*, the genes involved in cancer progression, invasion, and cancer metastases. Escamilla et al., reported that cell-free supernatant from *Lactobacillus rhamnosus* GG can down-regulate the expression of MMP9 and increase zona occludens-1 protein after treating the HCT-116 cells as the metastatic colorectal cancer cells. They suggested that the bioactive components released by *Lactobacillus rhamnosus* GG in the supernatant could prevent cancer cell invasion.[32] In another study, Maghsood et al. investigated the anti-inflammatory characteristics of the cell-free supernatant from *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* GG on the differentiated THP-1 cells. They observed that cell-free supernatant could down-regulate the expression of *mmp9* and cell surface CD147 and up-regulate TIMP-1 with no effect on the activity and expression of *mmp2* and *TIMP-2*.[33] To the best of the authors' knowledge, this study is the first observation indicating that EVs from *Lactobacillus rhamnosus* GG can affect the cell adhesion and *mmp2* and *mmp9* expression. MMP2 and MMP9 are types of gelatinase having proteolytic activity to destruct ECM proteins.[4] Here, we found that cell treatment with EVLs significantly attenuated the mRNA expression of *mmp2* and *mmp9* genes. In addition, the cell adhesions decreased by increasing the EVLs concentration. The reason for this observation could be due to the increased cell death at higher concentrations and attenuated cell number during the time of treating. Moreover, numerous proteins, factors, and molecular signaling are involved in cell detachment and metastasis, which have not been studied here.

Conclusion

In conclusion, EVLs exert cytotoxic effects on the colorectal cancer cell line and they are capable of decreasing expression of *mmp2* and *mmp9* genes. However, to confirm this outcome, further investigation on other mechanisms is required.

Authors' Contributions

Experimental works were done by AMP. The concept, and study design, data statistical analysis and manuscript preparation were carried out by HMH and SAM.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Acknowledgment

The authors would like to thank to all colleagues in Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences for their kind help.

References

1. Luqmani YA. Mechanisms of drug resistance in cancer chemotherapy. Med Princ Pract. 2005;14(Suppl. 1):35-48. doi:10.1159/000086183
2. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Akinyemiju TF, Al Lami FH, Alam T, Alizadeh-Navaei R, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2016: a systematic analysis for the global burden of disease study. JAMA Oncol. 2018;4(11):1553-68. doi:10.1001/jamaoncol.2018.2706
3. Aarons CB, Bajenova O, Andrews C, Heydrick S, Bushell KN, Reed KL, et al. Carcinoembryonic antigen-stimulated THP-1 macrophages activate endothelial cells and increase cell–cell adhesion of colorectal cancer cells. Clin Exp Metastasis. 2007;24(3):201-9. doi:10.1007/s10585-007-9069-7
4. Yao Z, Yuan T, Wang H, Yao S, Zhao Y, Liu Y, et al. MMP-2 together with MMP-9 overexpression correlated with lymph node metastasis and poor prognosis in early gastric carcinoma. Tumor Biol. 2017;39(6):1-8. doi:10.1177/1010428317700411
5. Ejtehadifar M, Halabian R, Fooladi AA, Ghazavi A, Mosayebi G. Anti-cancer effects of Staphylococcal Enterotoxin type B on U266 cells co-cultured with Mesenchymal Stem Cells. Microb Pathog. 2017;113:438-44. doi:10.1016/j.micpath.2017.11.024
6. Simon GL, Gorbach SL. Intestinal microflora. Med Clin North Am. 1982;66(3):557-74. doi:10.1016/S0025-7125(16)31407-9
7. Fooladi AA, Hosseini HM, Nourani MR, Khani S, Alavian SM. Probiotic as a novel treatment strategy against liver disease. Hepat Mon. 2013;13(2):e7521. doi:10.5812/hepatmon.7521
8. Oelschlaeger TA. Mechanisms of probiotic actions–a review. Int J Med Microbiol. 2010;300(1):57-62. doi:10.1016/j.ijmm.2009.08.005
9. Commane D, Hughes R, Shortt C, Rowland I. The potential mechanisms involved in the anti-carcinogenic action of probiotics. Mutat Res-Fundam Mol Mech Mutagen. 2005;591(1-2):276-89. doi:10.1016/j.mrfmmm.2005.02.027
10. Uccello M, Malaguarnera G, Basile F, D'agata V, Malaguarnera M, Bertino G, et al. Potential role of probiotics on colorectal cancer prevention. BMC Surg. 2012;12(1):S35. doi:10.1186/1471-2482-12-S1-S35
11. Brasseur K, Auger P, Asselin E, Parent S, Cote JC, Sirois M. Parasporin-2 from a new *Bacillus thuringiensis* 4R2 strain induces caspases activation and apoptosis in human cancer cells. PLoS One. 2015;10(8):e0135106. doi:10.1371/journal.pone.0135106
12. El-Sersy NA, Abdelwahab AE, Abouelkhiir SS, Abou-Zeid DM, Sabry SA. Antibacterial and Anticancer activity of ε-poly-L-lysine (ε-PL) produced by a marine *Bacillus subtilis* sp. J Basic Microbiol. 2012;52(5):513-22. doi:10.1002/jobm.201100290
13. Hoeppli RE, Wu D, Cook L, Levings MK. The

environment of regulatory T cell biology: cytokines, metabolites, and the microbiome. Front Immunol. 2015;6:61. doi:10.3389/fimmu.2015.00061
14. Kuisma J, Mentula S, Jarvinen H, Kahri A, Saxelin M, Farkkila M. Effect of *Lactobacillus rhamnosus* GG on ileal pouch inflammation and microbial flora. Aliment Pharmacol Ther. 2003;17(4):509-15. doi:10.1046/j.1365-2036.2003.01465.x
15. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013;200(4):373-83. doi:10.1083/jcb.201211138
16. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lutvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654-9. doi:10.1038/ncb1596
17. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat Rev Microbiol. 2015;13(10):620-30. doi:10.1038/nrmicro3480
18. Alves NJ, Turner KB, Medintz IL, Walper SA. Emerging therapeutic delivery capabilities and challenges utilizing enzyme/protein packaged bacterial vesicles. Ther Deliv. 2015;6(7):873-87. doi:10.4155/tde.15.40
19. Zhang X, Yuan X, Shi H, Wu L, Qian H, Xu W. Exosomes in cancer: small particle, big player. J Hematol Oncol. 2015;8(1):83. doi:10.1186/s13045-015-0181-x
20. Masyuk AI, Masyuk TV, LaRusso NF. Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases. J Hepatol. 2013;59(3):621-5. doi:10.1016/j.jhep.2013.03.028
21. Behzadi E, Hosseini HM, Fooladi AA. The inhibitory impacts of *Lactobacillus rhamnosus* GG-derived extracellular vesicles on the growth of hepatic cancer cells. Microb Pathog. 2017;110:1-6. doi:10.1016/j. micpath.2017.06.016
22. Mahmoodzadeh Hosseini H, Imani Fooladi AA, Soleimanirad J, Nourani MR, Davaran S, Mahdavi M. Staphylococcal entorotoxin B anchored exosome induces apoptosis in negative esterogen receptor breast cancer cells. Tumor Biol. 2014;35(4):3699-707. doi:10.1007/s13277-013-1489-1
23. Pereira FV, Ferreira-Guimaraes CA, Paschoalin T, Scutti JA, Melo FM, Silva LS, et al. A natural bacterial-derived product, the metalloprotease arazyme, inhibits metastatic murine melanoma by inducing MMP-8 cross-reactive antibodies. PLoS One. 2014;9(4):e96141. doi:10.1371/journal.pone.0096141
24. Molina-Tijeras JA, Galvez J, Rodriguez-Cabezas ME. The immunomodulatory properties of extracellular vesicles derived from probiotics: a novel approach for the management of gastrointestinal diseases. Nutrients. 2019;11(5):1038. doi:10.3390/nu11051038
25. Al-Nedawi K, Mian MF, Hossain N, Karimi K, Mao YK, Forsythe P, et al. Gut commensal microvesicles reproduce parent bacterial signals to host immune and enteric nervous systems. FASEB J. 2015;29(2):684-95. doi:10.1096/fj.14-259721
26. Yamasaki-Yashiki S, Miyoshi Y, Nakayama T, Kunisawa J, Katakura Y. IgA-enhancing effects of membrane vesicles derived from *Lactobacillus sakei* subsp. *sakei* NBRC15893. Biosci Microbiota Food Health. 2019;38(1):23-9. doi:10.12938/bmfh.18-015
27. Alvarez CS, Badia J, Bosch M, Gimenez R, Baldoma L. Outer membrane vesicles and soluble factors released by probiotic *Escherichia coli* Nissle 1917 and commensal ECOR63 enhance barrier function by regulating expression of tight junction proteins in intestinal epithelial cells. Front Microbiol. 2016;7:1981. doi:10.3389/fmicb.2016.01981
28. Kim JH, Jeun EJ, Hong CP, Kim SH, Jang MS, Lee EJ, et al. Extracellular vesicle–derived protein from *Bifidobacterium longum* alleviates food allergy through mast cell suppression. J Allergy Clin Immunol. 2016;137(2):507-16.e8. doi:10.1016/j.jaci.2015.08.016
29. Lypez P, Gonzalez-Rodriguez I, Sanchez B, Gueimonde M, Margolles A, Suarez A. Treg-inducing membrane vesicles from *Bifidobacterium bifidum* LMG13195 as potential adjuvants in immunotherapy. Vaccine. 2012;30(5):825-9. doi:10.1016/j.vaccine.2011.11.115
30. Zelaya H, Villena J, Lopez AG, Alvarez S, Aguero G. Modulation of the inflammation–coagulation interaction during pneumococcal pneumonia by immunobiotic *Lactobacillus rhamnosus* CRL1505: Role of Toll-like receptor 2. Microbiol Immunol. 2014;58(7):416-26. doi:10.1111/1348-0421.12163
31. Konieczna P, Schiavi E, Ziegler M, Groeger D, Healy S, Grant R, et al. Human dendritic cell DC-SIGN and TLR-2 mediate complementary immune regulatory activities in response to *Lactobacillus rhamnosus* JB-1. PLoS One. 2015;10(3):e0120261. doi:10.1371/journal.pone.0120261
32. Escamilla J, Lane MA, Maitin V. Cell-free supernatants from probiotic *Lactobacillus casei* and *Lactobacillus rhamnosus* GG decrease colon cancer cell invasion in vitro. Nutr Cancer. 2012;64(6):871-8. doi:10.1080/016 35581.2012.700758
33. Maghsood F, Mirshafiey A, Farahani MM, Modarressi MH, Jafari P, Motevaseli E. Dual effects of cell free supernatants from *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* GG in regulation of MMP-9 by up-regulating TIMP-1 and down-regulating CD147 in PMA-differentiated THP-1 cells. Cell J. 2018;19(4):559-68. doi:10.22074/cellj.2018.4447