

COMPARISON OF THE ANTI-CARCINOGENIC EFFECTS OF SOME PROBIOTIC BACTERIA AND THEIR POSTBIOTICS ON COLORECTAL CANCER CELLS

 Serap Niğdelioğlu Dolanbay^{1*},  Belma Aslım¹

¹ Gazi University, Faculty of Science, Department of Biology, Ankara, Turkey

*Corresponding Author:

E-mail: serapdolanbay@gazi.edu.tr

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ABSTRACT. The third most widespread cancer and the second leading reason for cancer-associated death is colorectal cancer (CRC). Natural agents such as probiotics and postbiotics, that offer anti-carcinogenic effects for CRC prevention, have become an important focus in recent years. Therefore, the aim of this study is to compare the anti-proliferative effects related to anti-genotoxic and immunomodulatory effects of viable probiotics with their exopolysaccharides (EPSs), which is one of their postbiotics. For this purpose, the strains' ability to inhibit the proliferation of HT-29 cells were determined with the WST-1 assay kit, their genotoxic and anti-genotoxic effects with the Comet assay and their immunomodulatory effects with IL-8 and IL-10 ELISA kits. According to our results, both viable probiotics and lyophilized EPSs (L-EPSs) were effective in all studies, but the best anti-proliferative (51% cell death), anti-genotoxic (48% inhibition) and immunomodulatory (for IL-8: 46% suppression and for IL-10: 74% increase) (* $p < 0.05$) effect was obtained from viable probiotics (*Levilactobacillus brevis* LB63). Additionally, in the present study found that these effects of L-EPSs were close to viable probiotics. Therefore, it has been shown that postbiotics can be used as alternatively to viable probiotics, because of the properties such as reliable and no side effects of their, thus it may be a useful alternative for cancer. According to these results, new agents such as probiotic-based postbiotics will be introduced to the pharmaceutical and food industry as well as probiotic bacteria that are protective and/or therapeutic against cancer.

Keywords: *Anti-proliferative, anti-genotoxicity, immunomodulatory, exopolysaccharides, lactic acid bacteria.*

INTRODUCTION

Cancer is a disease category defined by the unregulated multiplication of agile and invasive cells [1]. The third most common cancer is the colorectal cancer (CRC) being the second leading reason for cancer-associated deaths; it affects mainly the colon and rectum in men and women [2]. CRC can be treated with depending on the progressive stage, mainly by surgery, radiotherapy, immunotherapy or chemotherapy, but alternative interventions are needed because of systemic toxicity, chemotherapy resistance and cancer repetition [3]. Therefore, the development of highly efficient and hypotoxic anti-cancer agents containing natural products is critical [4]. In this context, scientists have focused on probiotics in the alternative treatment of CRC [5]. In addition, great

importance is attached to bioactive ingredients, such as postbiotics for adjuvant treatment in CRC patients by researchers [6].

Probiotics of the genera *Lactobacillus* and *Bifidobacterium* are the most constantly used and several of their strains exhibit important *in vitro* and *in vivo* properties [5]. Lactic acid bacteria (LAB) are part of the normal microbial community and play an important role in sustaining the stability and variety of the gut microbiome [7]. These bacteria are well-known for their health-promoting properties such as anti-cancer activity, and these can be explained as: modulation of the intestinal microflora, mutagen or carcinogen inactivation, DNA protection against oxidation, cell differentiation and apoptosis modulation, inhibition of the tyrosine kinase signaling pathway, immunomodulatory effects, and lowering of intestinal pH [8, 9, 10]. LAB are known to be effective on CRC, especially with their mutagen or carcinogen inactivation and immunomodulatory effects [11]. Because, DNA repair genes, growth factors and their receptor genes, tumor suppressor genes, proto-oncogenes, cell cycle checkpoint genes, and genes linked to apoptosis are genes that are mutated at various phases of CRC advancement [12]. Therefore, it is important that probiotics bind to mutagenic agents, inhibiting DNA damage by leading to biotransformation of these agents and subsequent detoxification [13, 14, 15, 16]. On the other hand, the role of the immune system in controlling the tumor progression and promotion is crucial [17]. The effects of LAB on the immune system are generally as follows: the activation of the innate immune system cells like the phagocytes and natural killer cells and the inhibition of excessive immune reactions [18, 19]. The first effect features a preventive impact against infections and cancers, while the second effect has an inhibitory effect against allergies, autoimmune diseases and inflammatory bowel diseases (IBDs) [18]. The varying ability of probiotic strains to induce cytokine production and T cell differentiation was demonstrated [20, 21]. Some strains may cause an increase in levels of the anti-inflammatory cytokine interleukin (IL)-10 [22], while other LAB strains may lead to the inhibition of the synthesis of IL-8 that was induced by the tumor necrosis factor- α (TNF- α) [23, 24]. In addition, it is likely that their anti-genotoxic and immunomodulatory activities are due to postbiotics such as polysaccharides, peptidoglycans and secretory glycoproteins [10, 13, 25].

Postbiotics, which are functional bioactive constituents that are produced in a matrix during fermentation and can be used to support health, and may contain many different components, including microbial cell fractions, exopolysaccharides (EPSs), metabolites, cell lysates, short-chain fatty acids, functional proteins, teichoic acid, muropeptides derived from peptidoglycan, and pili-type structures [26]. It has biological activities such as postbiotics, immunomodulating, anti-oxidant, anti-inflammatory, anti-microbial, hypocholesterolemic, anti-hypertensive, anti-obesogenic and anti-proliferative [27]. In addition to these effects, postbiotics may be used as potential anti-cancer agents due to their reliable profile, established chemical structures, longer shelf life, stability in digestive system environments, non-toxicity, and resistance to hydrolysis [6]. EPSs, one of the well-defined postbiotics [28], are categorized as either homo- or heteropolysaccharides; that is to say, whether they are made up of single or multiple building blocks of monosaccharides [29, 30, 31]. EPSs are known to have health-promoting properties such as anti-cancer, anti-mutagenic, immunomodulatory, anti-ulcer, anti-oxidant, anti-thrombotic, neuroprotective, hypoglycemic, and cholesterol-lowering properties [32, 33, 34, 35].

While probiotic bacteria gathered interest with their ability anti-cancer there is no comprehensive study published to compare the basic mechanisms of probiotic bacteria

and their postbiotics (L-EPSs). In this perspective, the objective of the study was a) to determine the EPSs production capacities of *Levilactobacillus brevis* LB63, *Lactiplantibacillus plantarum* GD2, and *Lacticaseibacillus rhamnosus* E9, b) to investigate the potential anti-proliferative effect of the viable probiotics and their L-EPSs on HT-29 cells, c) to determine potential genotoxic and anti-genotoxic activities of each viable probiotics and their L-EPSs with Comet assay in human lymphocytes d) to investigate the ability of the viable probiotics and their L-EPSs to suppression IL-8 release in HT-29 cells induced with TNF- α and the effect of the viable probiotics and their L-EPSs on the secretion of IL-10 by HT-29 cells. Thus, new agents such as probiotic-based postbiotics will be introduced to the pharmaceutical and food industry, as well as probiotic bacteria that are protective and/or therapeutic against cancer.

MATERIALS AND METHODS

LAB used in the study

In the present study, we used three different types of LAB (*Lactobacillus brevis* LB63, *Lactobacillus plantarum* GD2, and *Lactobacillus rhamnosus* E9) from the stock collection of the Biotechnology Laboratory at Gazi University, Faculty of Science, Department of Biology (Ankara, Turkey) that isolated from breast-fed human feces during a previous study. However, the genus names of the strains used in our study were updated in consideration the work of Zheng et al. [36]. Accordingly, the genus name of the *Lactobacillus brevis* LB63 strain was updated as *Levilactobacillus*, the genus name of the *Lactobacillus plantarum* GD2 strain was *Lactiplantibacillus*, and the genus name of the *Lactobacillus rhamnosus* E9 strain was *Lacticaseibacillus*. All strains were stored in deep frozen (Sanyo, USA) at -30 °C in Man, Rogosa and Sharp Broth (MRS; Merck, Germany) supplemented with 50% (v/v) sterile glycerol (Merck, Germany) until ready for use.

Isolation and lyophilization of the crude EPSs from LAB

LAB were subcultured three times in MRS broth at 37 °C for 18-20 h. They were treated with trichloroacetic acid (TCA) (Merck, Germany). The suspension was gently stirred for 2 h at room temperature to promote polymer release. Supernatant was collected by centrifugation 2000 x g for 10 min. Then supernatant was evaporated for 6-8 h. The EPSs fraction was precipitated using cold-absolute ethanol (+4 °C, 12 h), followed by centrifugation 2000 x g for 10 min, resuspension in ultrapure water [37]. Isolated EPSs were lyophilized in a Christ Alpha 2-4 LSCplus freeze dryer (Marin Christ, Germany). L-EPSs powders were freeze-dried and stored at +4 °C.

Measurement of EPSs in the culture medium

LAB cultured in MRS broth were boiled at 100 °C for 10-15 min. When it has cooled, cultures were treated with 85% TCA solution. Supernatant was collected by centrifugation 21000 x g for 25 min. The EPSs fraction was precipitated using cold-absolute ethanol then was collected by centrifugation 21000 x g for 25 min, resuspension in ultrapure water. Total EPSs in each sample was determined using the phenol-sulfuric acid method. Extinction was measured at 490 nm, and concentrations were calculated using a calibration curve made up of glucose standards (0-200 mg/L) [38, 39].

Measurement of L-EPSs

L-EPSs were prepared at a concentration of 800 µg/mL in sterile water. Measurement of L-EPSs was estimated by phenol-sulfuric acid method [38, 39]. Yield (%) was determined as $\text{EPS (after lyophilization) - EPS (before lyophilization)} \times 100 / \text{EPS (before lyophilization)}$.

Preparation of viable probiotics

For routine analysis, LAB were subcultured twice in MRS broth at 37 °C for 18-20 h. Bacteria were harvested by centrifugation at 12.000 x g for 30 min (NF 400, Nuve, Inc., Turkey). After two washes in sterile phosphate buffered saline (PBS) pH 7.2 and the bacteria were resuspended in PBS. Then the bacteria were added at proper dilution to achieve a final concentration of $\sim 10^8$ cfu/mL in PBS. Optical density at 600 nm absorbance was used to estimate bacterial suspension (T80+UV/VIS Spectrometer, PG Instruments Ltd, England) [40, 41].

Cell culture

In this experimental study, human colorectal adenocarcinoma cell line (HT-29) were obtained from American Type Culture Collection (ATCC, HTB-38) were stored in liquid nitrogen. HT-29 cells cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, USA) supplemented with 1% penicillin-streptomycin (Sigma, Germany), 10% fetal bovine serum (FBS) (Gibco, USA), 1% L-glutamine (Sigma, Germany) and 40% MCDB (Sigma, Germany) at 37 °C in a humidified atmosphere with 5% CO₂ (Mco-18AIC, Sanyo, Japan). The cell culture medium was replaced every 2 days [42].

Determination of anti-proliferative effect of viable probiotics and L-EPSs

The method used by Hong et al. [43] in the determination of the anti-proliferative effect of cultures was carried out by making some modifications. In this study, WST-1 assay kit was used (Cayman Chemical Company, USA). Appropriate concentration of the viable probiotics ($\sim 10^8$ cfu/mL) or L-EPSs (800 µg/mL) were added to cell culture without antibiotics and then incubated 37 °C in a 5% CO₂ atmosphere for 24 to 48 h. After incubation, each well was treated with 10 µl of the WST-1 mixture. Generation of formazan was measured at 450 nm by microplate reader (Epoch, BioTek, USA). The anti-proliferative effects were measured by contrasting the sample's viability to that of the control (without the test sample). % of viable cells were calculated according the following formula = $[\text{OD (sample)}/\text{OD (control)}] \times 100\%$. OD (sample) and OD (control) [44].

Isolation of human lymphocyte

Blood samples were obtained by venipuncture from a non-smoking donor. 5 mL of anti-coagulated blood was diluted 1:1 with Dulbecco's phosphate-buffered saline (DPBS) and was diluted with lymphocyte separation medium (Biocoll separating solution; Merck, Germany) in a centrifugation tube. After centrifugation for 20 min at 800 x g at 4 °C, the ring with white blood cells was harvested. After wash in PBS at 800 x g for 20 min and the cell pellets were resuspended in 0.5 mL of PBS [45, 46].

Genotoxicity and anti-genotoxicity assessment using Comet assay

The Comet assay was performed basically following Singh et al. [47] protocol's, and with minor changes of Noroozi et al. [48] and Raipulis et al. [49]. Lymphocytes submitted to one of the following treatment: a) PBS for 90 min (negative control); b) viable probiotics ($\sim 10^8$ cfu/mL) or L-EPSs (800 $\mu\text{g/mL}$) treatment for 90 min; c) 50 μM hydrogen peroxide (H_2O_2) for 15 min (positive control); d) viable probiotics or L-EPSs plus 50 μM H_2O_2 (viable probiotics or L-EPSs treatment for 90 min, 50 μM H_2O_2 treatment for 15 min respectively). Treatment “a”, “c”, “d” were the experiments to test anti-genotoxicity, and treatments “a”, “b” were the experiments to test genotoxicity. After centrifugation for 5 min at 800 x g, the lymphocytes washed twice with sterile PBS pH 7.2. The lymphocytes were mixed with 75 μL of 0.5% low melting point agarose (LMA) at 37 °C, and added to the slides precoated with 1% normal melting point agarose (NMA) for 15-20 min at -20 °C. The slides were then immersed in lysis solution (2.5 M NaCl, 0.1 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4 °C. The slides were then placed into an electrophoresis tank containing electrophoretic buffer (10 M NaOH and 0.2 M EDTA, pH 13.0) for 20 min. An electric current of 25 V/300 mA was applied to the DNA for 20 min at 4 °C for electrophoresis. For 15 min at 4 °C, the slides were immersed in a neutralizing buffer (0.4 M Tris, pH 7.5), dried and stored at room temperature. The slides were examined immediately after staining with 50 μL ethidium bromide under fluorescence microscope (Leica DFC425C, Germany) connected to an image analysis system (Comet Analysis Software, version 4.0, π Perceptive Instruments Ltd., England). 100-300 individual cells were selected for each analysis. The DNA damage was stated as % Tail DNA, that % Tail DNA = [Tail intensity/(Head intensity + Tail intensity)] \times 100 [47, 48].

Enzyme-linked immunosorbent assay (ELISA) for IL-8 and IL-10

HT-29 cell lines were seeded in a 96 well plate (10^4 - 10^5 cells/well). After that treated with viable probiotics ($\sim 10^8$ cfu/mL) or 800 $\mu\text{g/mL}$ of L-EPSs. After 1 h incubation, TNF- α (Millipore, USA) was added at a concentration of 10 ng/mL, and the cells were returned to the incubator for 24 to 48 h. The concentration of cytokine released by the HT-29 cells were measured using human IL-8 and IL-10 (without induction with TNF- α) cytokine levels by ELISA kits (Invitrogen, USA) according to the manufacturer's instructions. The absorbance was measured using an ELISA reader at a wavelength of 450 nm [50, 51, 52, 53, 54].

Statistical analysis

All experiments have been carried out at least in triplicate and data were expressed as mean \pm standard deviation (SD). Statistical analysis was performed using the IBM SPSS version 16.0. Kendall-taub and Sperman's correlation tests were used to assess significant differences ($p < 0.05$) and correlation between means. Also, in studies one-way analysis of variance (ANOVA) was performed using the Kruskal Wallis test.

RESULTS AND DISCUSSION

EPSs production capacities of LAB

In this study, for determine the anti-cancer effect of EPSs, three different LAB producing high amounts of EPSs were selected in preliminary studies. The EPSs quantities that purified and lyophilized of strains producing high EPSs were also measured and are shown in Table 1 together with EPSs production quantities of the strains. The amount of L-EPSs were found to closely correlated with EPSs production capacities of strains in culture media. The EPSs isolated from *L. rhamnosus* E9 was found the highest amount (186.2 ± 3.2 mg/L) and after lyophilization, amount of EPSs was significantly increased (279.2 ± 4.3) ($p < 0.05$).

Table 1. The amounts of EPSs and L-EPSs isolated from LAB in culture medium

Codes of strains	Species	EPSs ¹ (mg/L)	L-EPSs ² (mg/L)	Yield (%)
LB63	<i>Levilactobacillus brevis</i>	132.2 ± 2.0^a	199.1 ± 4.2^a	51 ± 1.0^a
GD2	<i>Lactiplantibacillus plantarum</i>	146.3 ± 2.4^b	221.3 ± 5.0^b	51 ± 1.1^a
E9	<i>Lacticaseibacillus rhamnosus</i>	186.2 ± 3.2^c	279.2 ± 4.3^c	50 ± 1.1^a

¹Before lyophilization, ²After lyophilization. Different superscripts letters in the same column indicate the difference at the $p < 0.05$ level. Values are the mean \pm SD (n:3/group, three independent experiments).

Anti-proliferative effects of viable probiotics and L-EPSs

The anti-proliferative activity of the strains used in the study was determined with the WST-1 assay kit; in the practice, the effect of viable probiotics at concentrations of ~ 8 log cfu/mL were investigated. According to our results, viable probiotics were effective in this cancer cell line (Fig. 1). The results obtained from this study showed that, the best effect was obtained of LB63 strain ($51.2\pm 1.4\%$ cell death) ($*p < 0.05$). In applications with L-EPSs at a concentration of $800 \mu\text{g/mL}$, it was determined that L-EPSs were effective in HT-29 cell line, and the best effect was obtained from L-EPSs of E9 strain ($32.7\pm 2.5\%$ cell death) ($*p < 0.05$) (Fig. 1). When the results are evaluated in general, it can be said that although L-EPSs show a lower anti-proliferative effect compared to viable probiotics, both viable probiotics and L-EPSs show a high anti-proliferative effect.

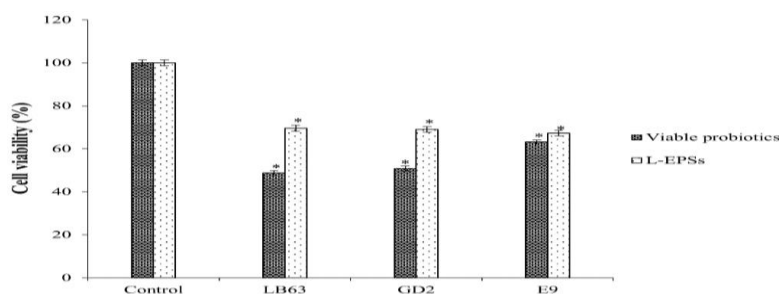


Fig. 1. Anti-proliferative effect of viable probiotics and postbiotics (L-EPSs) against HT-29 cells. Control, HT-29 cells; LB63, *L. brevis*; GD2, *L. plantarum*; E9, *L. rhamnosus*. Values are the mean \pm SD (n:5/group, three independent experiments).

$*p < 0.05$, significant differences from the control.

Genotoxic and anti-genotoxic effects of viable probiotics and L-EPSs

The anti-genotoxic effects of strains on human lymphocytes against oxidative DNA damage induced by H₂O₂ were examined by Comet assay. In addition, the possible genotoxic effect of the strains on human lymphocytes was determined in order to test their safety before use in the food industry and medicine. In the study, the tail intensity of the lymphocytes damaged upon H₂O₂ treatment was 42.6±1.9% (#*p*<0.05) (positive control), while the tail intensity of the lymphocytes treated only with viable probiotics or L-EPSs was found to be 0.1±0.0% (Table 2). These results showed that viable probiotics and L-EPSs do not cause any genotoxic effect, ie DNA damage. In Comet assay, in the positive control containing the lymphocytes treated with H₂O₂, the normal appearance of smooth edges changed to a scalloped edge appearance due to the migration of DNA fragments out of the nucleus and the lymphocytes took the form of a comet. The length of this tail correlates directly to the damage. Moreover, the fluorescent intensity at the tail parallels the extent of the damage (Fig. 2). Prior to the treatment with the H₂O₂ agent, human lymphocytes were treated with viable probiotics or L-EPSs to test the DNA-protecting effect of the strains based on the tail density data in Table 2, the best result was obtained from viable probiotics (strain LB63) (22.2±0.9%) (**p*<0.05). Viable probiotics appeared to cause a decrease the tail intensity in the damaged lymphocytes as well as the tail length. That is, the viable probiotics significantly reduced the DNA damage induced by H₂O₂. Similarly, L-EPSs have been found to significantly reduce oxidative DNA damage by reducing both tail length and tail intensity in damaged lymphocytes and the best result was obtained with L-EPSs from E9 strain (25.8±0.6%) (**p*<0.05) (Table 2). When the results are evaluated, it can be said that both viable probiotics and L-EPSs have a high anti-genotoxic effect against H₂O₂ induced DNA damage and these effects are close to each other.

Table 2. Genotoxicity and anti-genotoxicity of viable probiotics and postbiotics (L-EPSs)

Protocols	Treatment	Tail length (µm)	Tail intensity (%)	
Genotoxicity	Negative control ^a	27.9±1.8	0.0±0.0	
	Viable probiotics	LB63	29.8±1.6	0.1±0.0
		GD2	31.4±0.8	0.1±0.0
E9		27.1±0.9	0.1±0.0	
L-EPSs	LB63	28.6±1.3	0.1±0.0	
	GD2	27.3±0.7	0.1±0.0	
	E9	30.6±1.2	0.1±0.0	
Anti-genotoxicity	Negative control ^a	27.9±1.8	0.0±0.0	
	Positive control ^b	58.2±1.6 [#]	42.6±1.9 [#]	
Viable probiotics+H ₂ O ₂	LB63	29.8±1.1*	22.2±0.9*	
	GD2	31.1±1.7*	23.0±0.7*	
	E9	33.5±1.6*	24.7±1.1*	
L-EPSs+H ₂ O ₂	LB63	42.8±1.3*	31.1±1.2*	
	GD2	38.7±1.7*	29.0±1.3*	
	E9	34.6±0.7*	25.8±0.6*	

^aThe negative control contained D-PBS and lymphocytes. H₂O₂ was not treated with viable probiotics and L-EPSs. ^bThe Positive control contained D-PBS, lymphocytes and 50 µM H₂O₂. Values are the mean ± SD (n:3/group, three independent experiment). [#]*p*<0.05, compared with negative control; **p*<0.05, compared with positive control.

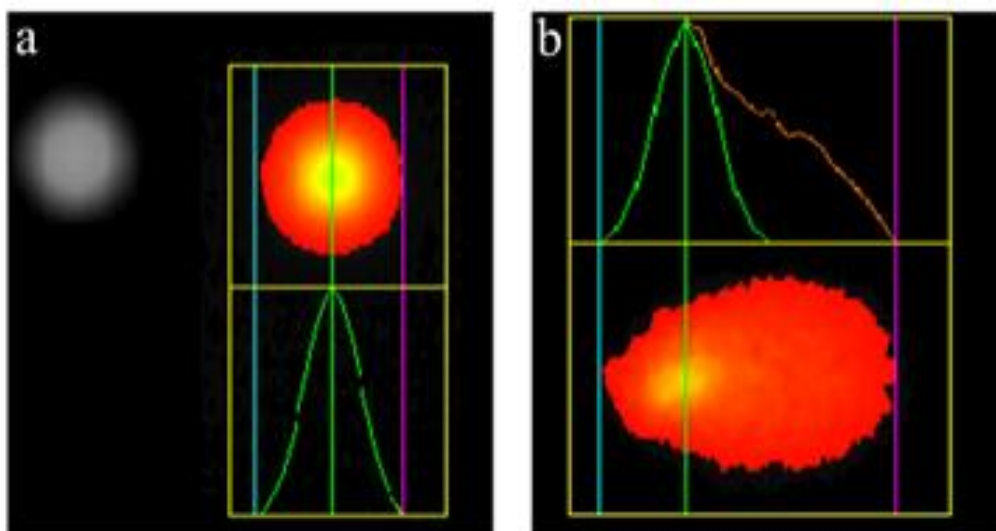


Fig. 2. Images of undamaged; negative control (a) and damaged; positive control (b) human lymphocyte cells

Immunomodulatory effects of viable probiotics and L-EPSs

The inhibitory effects of the strains on TNF- α induced IL-8 cytokine and activating effects on the anti-inflammatory cytokine IL-10 in HT-29 cells and thus their immunomodulatory effects were investigated by ELISA. IL-8 level of only HT-29 cells (negative control) was determined as 73.1 ± 8.3 - 76.1 ± 5.4 pg/mL, while the level of IL-8 of HT-29 cells induced by TNF- α (positive control) was determined as 530.6 ± 10.8 - 557.3 ± 8.1 pg/mL ($*p < 0.05$). On the other hand, it was determined that both viable probiotics and L-EPSs suppress this increase in IL-8 level. The highest suppression in IL-8 levels released from TNF- α -induced HT-29 cells by the viable probiotics (strain LB63) and L-EPSs (strain E9) were observed as 302.2 ± 6.2 and 372.1 ± 9.7 pg/mL, respectively ($^{\#}p < 0.05$). According to these results, it can be said that viable probiotics are more effective than L-EPSs in suppressing IL-8 level (Fig. 3). In applications with the IL-10 cytokine, only HT-29 cells (negative control) had an IL-10 level of 48.1 ± 2.4 - 49.3 ± 1.5 pg/mL. In the study, both viable probiotics and L-EPSs increased IL-10 levels in HT-29 cells. The highest increase in IL-10 levels released from HT-29 cells by the viable probiotics (strain LB63) and L-EPSs (strain E9) were observed as 85.4 ± 2.1 and 68.1 ± 3.2 pg/mL, respectively ($*p < 0.05$). According to these results, it can be said that viable probiotics are more effective than L-EPSs in increasing IL-10 levels (Fig. 4). When the results are evaluated, it can be said that both viable probiotics and L-EPSs have immunomodulatory effects.

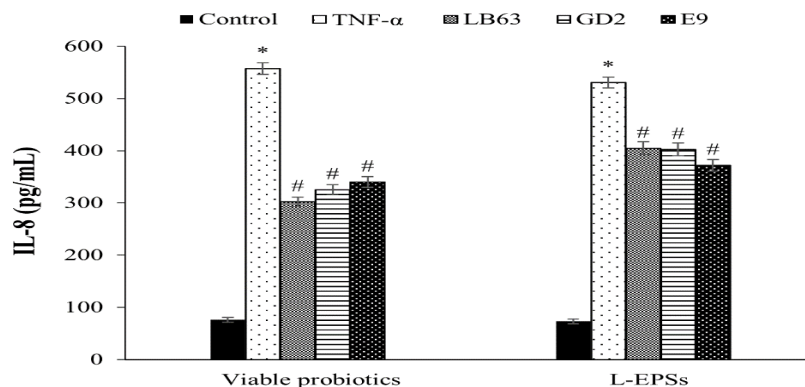


Fig. 3. Effects of viable probiotics and postbiotics (L-EPSs) on TNF- α induced IL-8 production in HT-29 cells. Control, HT-29 cells; TNF- α , TNF- α treated cells; LB63, *L. brevis*; GD2, *L. plantarum*; E9, *L. rhamnosus*. (n:5/group, three independent experiments). * p <0.05, compared with control; # p <0.05, compared with TNF- α .

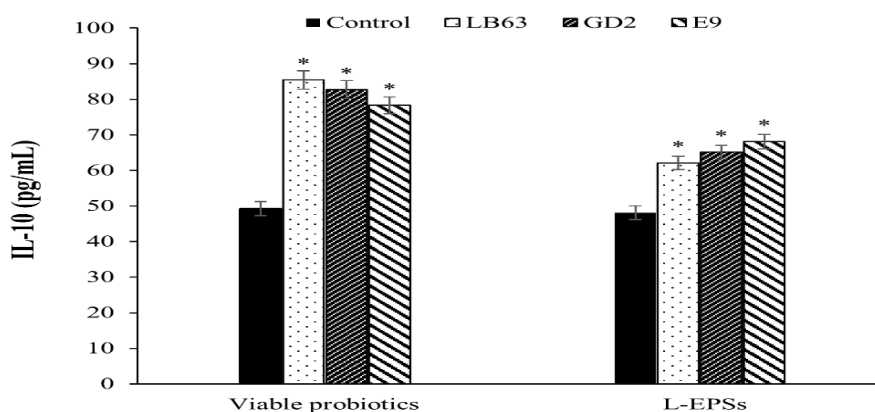


Fig. 4. Effects of viable probiotics and postbiotics (L-EPSs) on IL-10 production in HT-29 cells. Control, HT-29 cells; LB63, *L. brevis*; GD2, *L. plantarum*; E9, *L. rhamnosus*. (n:5/group, three independent experiments). * p <0.05, significant differences from the control.

Recently, human microbiota is on the agenda as a promising method of study in the protection and/or therapy of CRC [11]. In this context, scientists have focused on probiotics in the alternative treatment of CRC [5]. In addition, great importance is attached to bioactive ingredients such as postbiotics for adjuvant treatment in CRC patients by researchers [6]. Along with their various properties (acid resistance, tolerance to bile salts, substrate and niche competition etc. bacterial advantages) EPSs [55], one of the postbiotics, are known to play a direct or indirect role in probiotic effects like anti-genotoxic, anti-inflammatory, and anti-tumor effects [44, 56, 57]. Therefore, in this study, we examined the EPSs production capacity of the strains and the anti-proliferative effects related to anti-genotoxic and immunomodulatory effects of the strains producing high

EPSs. It was observed that EPSs production varied among the strains, and it was determined that the EPSs production capacity of the E9 strain was higher than the other two strains (Table 1). Dupont et al. [58] evaluated the EPSs production produced by *L. paracasei* Type V and found the amount of EPSs as 85 ± 36 mg/L in the glucose source. In another study conducted with *L. delbrueckii* ssp. *bulgaricus* strain, it was reported that the amount of EPSs produced by the bacteria in different environments ranged between 75-354 mg/L [59]. In our study, glucose was used as a carbon source and EPSs production was generally higher than in study using carbon source in the literature.

Since the probiotic bacteria used in the study are located in the intestinal system, the HT-29 cell line similar to human intestinal cells was used in anti-proliferative effects applications [60]. In our study, it was determined that viable probiotics were effective in HT-29 cell line and the most effective one among the bacteria was the LB63 strain (Fig. 1). In literature studies, viable probiotics have been reported to inhibit the development of cultured cancer cells [61]. In a study by Grimoud et al. [62], they reported that the anti-proliferative effect of some probiotic bacteria on HT-29 cells alone was more than 20%, but when combined with prebiotics, the effect reached 66.31%. Tuo et al. [63] reported that the heat-killed *L. coryniformis* subsp. *torquens* T3L and *L. rhamnosus* SB31L strains were effective in HT-29 CRC cell line by 30% and 19%, respectively. According to the findings of the literature, the live cells of the strains used in our study are more effective in preventing cancer cell proliferation compared to cells that are not combined with prebiotics and have been heat-treated. On the other hand, alternative agents that potential such as postbiotics are used to content the composition of the intestinal microbiota and overcome some clinical and other problems associated with the use of viable probiotics in recent years [50, 64, 65]. In this context, in our study, where we examined the anti-proliferative effect of L-EPSs, which is a postbiotic compound, it was determined that L-EPSs were also very effective and the most effective were L-EPSs obtained from the E9 strain (Fig. 1). Liu et al. [44] using the MTT-assay with the HT-29 cell line, reported that the EPSs obtained from the *L. casei* 01 strain exhibited 26% anti-proliferative effect after 24 h. Considering the variability in the parameters such as cell counts used in studies, employed method, as well as the EPSs concentration, the results of our study are highly significant. Therefore, in the preparation stage of various preparations or mixed substrates, we are considering the possible use of L-EPSs, particularly of the EPSs from E9 strain against potential unfavorable situations such as failure in the stabilization of viable cells.

The cancer induction, which is a multi-step process, starts with the accumulation of mutations in tumor suppressor genes and proto-oncogenes [66, 67]. Thus, using the Comet assay to determinate DNA damage in primary cells is more important than using other standard genotoxicity assays in bacteria or cultured cells to examine protective effects [67]. Therefore, in our study, the anti-genotoxic effects of both viable probiotics and L-EPSs to prevent possible genotoxic and H₂O₂-induced oxidative DNA damage were investigated by Comet assay. According to the results we obtained, it was seen that viable probiotics and L-EPSs do not cause any DNA damage. The fact that the viable probiotics and L-EPSs do not cause a DNA damage in human lymphocytes has shown that they can be used safely. In the experimental models performed, LAB were shown to significantly lower the DNA damage in gastric and colon mucosa due to chemical carcinogens [68]. Human lymphocytes were used in our study and a high DNA damage was obtained (Table 2). In addition, it was determined that lymphocyte cells treated with H₂O₂ acquired an irregular edged appearance and the shape of a comet due to the initiation

of migration of DNA fractures out of the nucleus (Fig. 2). Noroozi et al. [48] in their study with rat lymphocytes with 50 μM H_2O_2 about 48% DNA damage were obtained. Therefore, the DNA damage we have created on lymphocytes is compatible with the literature, and viable probiotics have significantly reduced this damage. In a study by Koller et al. [69], DNA damage was induced by H_2O_2 in HT-29 cells. The resulting damage was inhibited by 30% by *L. brevis* VM21 strain at a concentration of 3.10^7 bacteria/mL, and by 47% by *L. acidophilus* VM19 strain. In our study, viable probiotics were incubated with lymphocytes and the best effect was obtained from the LB63 strain (Table 2). Our results are consistent with the literature, and it can be said that viable probiotics are very effective in suppressing DNA damage. Probiotics' anti-mutagenic activity is linked to postbiotics including peptidoglycans, polysaccharides, and secretory glycoproteins, according to *in vitro* studies [6, 10]. Mutagens binding, inducing chemopreventive enzymes, suppressing carcinogen enzymes, and preventing DNA damage are some of the essential molecular pathways involved in postbiotics' anti-mutagenic properties [6, 13]. Therefore, in our study in which we investigated the antigenotoxic effect of L-EPSs, which is one of the postbiotics, it was determined that L-EPSs inhibited the DNA damage that occurred. In their study, Liu et al. [44] reported that the EPSs (50 $\mu\text{g}/\text{mL}$) obtained from the *L. casei* 01 strain inhibited the damage in intestine 407 cells induced by 4-NQO. In our study, L-EPSs of the E9 strain effectively decreased the existing DNA damage (Table 2). Considering the variability in the mutagenic agent used in studies, cells, EPSs concentration difference and incubation times, the results of our study are highly significant. When the results are evaluated, suggesting that it will have properties that regulate the gastrointestinal system (GIS) balance against inflammation-related diseases that may occur due to oxidative DNA damage in GIS due to the anti-genotoxic activities of both viable probiotics and L-EPSs.

Studies have shown that most pro-inflammatory cytokines stimulate tumor angiogenesis and thus accelerate tumor progression [70]. IL-8 is a pro-inflammatory cytokine released from normal cells as well as from tumor cells and it is responsible from the initiation of acute inflammatory reactions [40, 70]. Of the anti-inflammatory cytokines, IL-10 exerts its protective activity by inhibiting the inflammation mediators such as IL-1 β , TNF- α , IL-8, IFN- γ , IL-6 and prostaglandin metabolites, also it has anti-metastatic and anti-tumor activity [40, 71, 72]. Recently, the ability of LAB to regulate the release of pro-inflammatory and anti-inflammatory cytokines is drawing attention [23]. In various studies, it has been demonstrated that postbiotics, like probiotics, are also efficient in adjusting the immune system reaction and have appropriate immunomodulatory effect [6, 73, 74]. Therefore, we investigated the immunomodulatory effects of viable probiotics and L-EPSs in our study. According to our results, it was noted that inflammation of HT-29 cells was successfully modeled by TNF- α also viable probiotics and L-EPSs that with high anti-proliferative and anti-genotoxic effect significantly decrease inflammation (Fig. 3). In another study with HT-29 cells, Bai et al. [50], similar to our study, co-incubated the strains (8 log cfu/mL) and the cells before the TNF- α treatment for 1 h. However, unlike our study, the cells were incubated after the TNF- α treatment for 3 h. After administration, the IL-8 release, which was 639.5 ± 62.3 ng/L, decreased to 515.4 ± 55.4 ng/L with *L. bulgaricus* LB10 strain. Accordingly, the results obtained in our study are better than the findings reported by Bai et al. [50]. In our study, both viable probiotics and L-EPSs increased IL-10 release in HT-29 cells without TNF- α induction (Fig. 4). Gao et al. [51] investigated the effect of bacterial concentration on IL-10 release in a study of HT-29 cells and *Clostridium butyricum* and found that the

best result was approximately 8 log cfu/mL bacterial concentration used in our study. While the results obtained are significant in their entirety compared to the control, they are lower than the result obtained by Gao et al. [51]. On the other hand, in another study by Vemuri et al. [54], *L. acidophilus* DDS-1 strain increased the IL-10 level in HT-29 cells (from 2.72 ± 0.51 to 25.25 ± 1.99 pg/mL) less than the viable probiotics we used in our study. This difference in IL-10 production is thought to be due to cell number and other cell-related conditions and the strain used [75]. When the results were evaluated in general, it was determined that both viable probiotics and L-EPSs show immunomodulatory effects. Therefore, we recommend the use of both agents as adjuvant therapy in inflammation-related diseases such as IBDs, atopic dermatitis, allergic diseases, hepatic encephalopathy, as well as CRC.

CONCLUSION

In this study, we compared the anti-proliferative effects related to anti-genotoxic and immunomodulatory effects of viable probiotics and postbiotics obtained from their. The live cells of the strains came to the fore with both their anti-genotoxic and immunomodulatory effects and the anti-proliferative effects associated with these effects. On the other hand, L-EPSs that postbiotic were found to have a similar effect to viable probiotics. Therefore, in the course of preparation of various preparations or mixed substrates, we anticipate that the use of L-EPSs, especially L-EPSs of the E9 strain, may be possible against possible adverse events such as failure to achieve stabilization of viable cells. The results of the current study suggested that viable probiotics and L-EPSs could be used a natural anti-cancer agents for the prevention of CRC. According to the results, the viable probiotics (especially strain LB63) and the L-EPSs (especially strain E9) may have the ability to regulate the oxidant/anti-oxidant balance in GIS with its anti-genotoxic activity. In addition, the viable probiotics and the L-EPSs can stabilize the immune system by suppressing the release of pro-inflammatory cytokines (IL-8 etc.) and increasing the release of anti-inflammatory cytokines (IL-10 etc.). This suggests that viable probiotics and L-EPSs can be used as an immunotherapy agent for both pre-disease resistance and for the treatment of the disease process. Therefore, it may be possible to gain natural resources such as probiotic bacteria and their postbiotics into the pharmaceutical and food industry as an alternative to current treatments many diseases, especially cancer.

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