



Research Article

Prophylactic Role of *Lactobacillus paracasei* Exopolysaccharides on Colon Cancer Cells through Apoptosis Not Ferroptosis

Yalda Rahbar Saadat^{1,2}, Bahram Pourghassem Gargari^{1,3}, Arman Shahabi⁴, Yousef Nami⁵, Ahmad Yari Khosroushahi^{6,7*}

¹Nutrition Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

²Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.

³Department of Biochemistry and Diet Therapy, Faculty of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran.

⁴Molecular Medicine Department, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran.

⁵Branch for North-West and West Region, Agricultural Biotechnology Research Institute of Iran, Tabriz, Iran.

⁶Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

⁷Department of Medical Nanotechnology, Faculty of Advanced Medical Science, Tabriz University of Medical Sciences, Tabriz, Iran.

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Abstract

Background: Nowadays despite conventional methods in colon cancer treatment, targeting vital molecular pathways and induction of various forms of cell death by safe probiotic components like exopolysaccharides (EPSs) are of great importance and are considered as potential therapeutic agents. This study aimed to investigate the inhibitory effect of the EPS of *L. paracasei* on different colon cancer cell lines (SW-480, HT-29, and HCT-116).

Methods: For this purpose, several cellular and molecular experiments including MTS assay, DAPI staining, Annexin V/PI assay, quantitative real-time PCR (qPCR) and some important ferroptosis-related assays were performed.

Results: Based on the findings, *L. paracasei* EPS can induce apoptosis confirmed by all apoptosis-related assays and could not act through ferroptosis pathways. *L. paracasei* EPS could hinder the Akt1, mTOR, and Jak-1 mRNAs, and induces apoptosis through down-regulation of the anti-apoptotic gene (Bcl-2), up-regulation of pro-apoptotic genes (BAX, caspase-3, 8).

Conclusion: The exploited EPS of an indigenous probiotic strain with anticancer potential with low/insignificant cytotoxicity to normal cells is proposed for future applications in molecular-targeted therapy of colon cancer treatment. Furthermore, *in vivo* and clinical trials should be performed to evaluate the applicability of this component besides conventional methods to increase the survival rate of colon cancer patients.

Introduction

Colon cancer, as a complex and multifactorial disease, is one of the most frequent gastrointestinal tract (GIT) malignancies¹ (the second most common cancer in women/third in men).^{2,3} It has the highest cancer-related death among patients affected.^{4,5} Various factors are involved in its incidence, as its onset is often without any obvious clinical manifestations,⁶ hence, these patients are detected at advanced stages.¹ The accumulation of mutations in oncogenes and tumor suppressor genes, as well as, lifestyle, dietary factors (high fat, low carbohydrate, red meat consumption, less fruit, and vegetable consumption and alcoholic beverages), environment and carcinogenic chemicals are among factors involved in colon cancer incidence.^{6,7} Surgery, chemotherapy, and radiotherapy are among conventional methods for colon cancer

treatment,^{1,4,5,8} which in most cases have low success rates due to adverse side effects⁸ and metastasis of chemotherapy-resistant cells.⁹ Therefore, there is an urgent need for the development of novel and safe anticancer agents⁵ based on mechanisms underlying colon cancer cell growth, metastasis and invasion.³

Probiotic microorganisms are defined as 'live microorganisms when administered in adequate amounts, confer a health benefit on the host'. Most probiotics are lactic acid bacteria (LAB), a group of gram-positive bacteria, and among them, *Lactobacillus* is one of the most common species.^{2,10} They have recently acquired considerable importance because of their innumerable health beneficial properties.^{2,11,12} Many concerns about consuming live probiotics especially in immune-compromised and

*Corresponding Author: Ahmad Yari Khosroushahi, Email: yarikhosroushahia@tbzmed.ac.ir; ayarikh@gmail.com

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immature immunity during childhood, provoked researchers to investigate the health benefits of probiotic derived components.¹³ One of these important components is polysaccharides/glycans as natural biopolymers of carbohydrates. These so-called EPSs provide numerous health benefits including immunomodulatory properties, anticancer, antioxidant activity as well as blood glucose/cholesterol-lowering properties, and antihypertensive activity. Studies revealed that EPSs from probiotics like LAB exerts less adverse side effects and may act as a source of the new anticancer agent.¹³

A deeper understanding of the consequences during colon cancer development and progression will facilitate the development of novel therapeutic strategies with lower or no side effects. The phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway plays a critical role in proliferation, resistance to apoptosis, angiogenesis, and metastasis during colon cancer development.^{5,13-15} Components of the Akt signaling pathway promotes cell survival, proliferation, invasion and chemo-resistance.¹⁶ mTOR signaling controls cell survival and regulation of metabolism.¹⁷ Another vital signaling axis is the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, which is involved in numerous physiological processes including cell growth, differentiation, and migration of tumor cells, especially in colon cancer.^{18,19} Further, substantial evidence implicates overexpression of Akt, mTOR, JAK1 in colorectal cancers.¹⁴ Recent studies suggest that blocking components of PI3K/Akt/mTOR signaling pathways, are interesting targets for colon cancer therapy.^{5,12,13,15-17}

Ever-growing evidence suggested that apoptosis-which encompasses extrinsic and intrinsic pathways-acts as a vital regulator in tumor regression.^{20,21} Consequently, any defect in these pathways may result in tumor development and resistance to chemotherapeutics. Therefore, provoking apoptotic pathway seems to be a valuable target in cancer therapy.²¹ The biological importance of apoptosis is highlighted in cancer research. However, apoptosis is not the only form of programmed cell death (PCD).²² Induction of PCD is an important goal of anticancer drugs.²³ Although, resistance to apoptosis occurs in most cases, investigating non-apoptotic forms of cell death is a promising field of research in the elimination of cancer cells.²³ The term Ferroptosis was coined in 2012 by Stockwell and is a non-apoptotic form of regulated cell death which is distinct from apoptosis, necroptosis and autophagic cell death. Ferroptosis is characterized by extensive lipid peroxidation and reactive oxygen species (ROS) production, which can be suppressed by iron chelators or lipophilic antioxidants.^{22,24} The induction of ferroptosis and understanding the molecular mechanisms involved may provide new approaches in cancer treatment.²³⁻²⁵ In this regard, we carried out this research to determine the underlying mechanisms by which exopolysaccharide of *L. paracasei* can modulate aforementioned signaling

pathways on colon cancer cell lines.

Material and Methods

Reagents

Agar-agar, absolute ethanol, chloroform, DMSO and Triton X-100 were purchased from Merck Millipore, (Darmstadt, Germany). Roswell Park Memorial Institute medium (RPMI-1640), fetal bovine serum (FBS), Pen/Strep and Trypsin-EDTA were acquired from Invitrogen-Gibco (Paisley, UK). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Sigma Aldrich (St. Louis, MO, USA). CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) was bought from Promega (Madison, WI, USA). Annexin V apoptosis detection kit was obtained from Biolegend (San Diego, CA). Cell culture flasks, plates, and disposable sterile pipettes were purchased from Sorfa (Zhejiang, China (Mainland)). De Man, Rogosa and Sharpe broth (MRS) (Biolife Italiana, Milano, Italy), RNX plus (Cinnagen, Iran), Prime Script RT reagent kit (Takara Bio Inc., Tokyo, Japan), SYBR green master mix high ROX from Amplicon (Stenhuggervej, Odense, Denmark), 2',7'-dichlorofluorescein diacetate from Cayman Chemical (Ann Arbor, MI, USA), C11-BODIPY 581/591 from Thermo Fisher Scientific Inc., (Waltham, MA, USA), iron and glutathione assay kits from Abnova (Taipei, Taiwan) were used in this study.

Growth condition and EPS isolation

A stock culture of the *Lactobacillus paracasei* strain isolated from dairy products was stored at -80 °C in sterile glycerol plus media.²⁶ The strain was thawed and pre-grown in MRS broth (containing 10 g/L peptone, 8 g/L meat extract, 4 g/L yeast extract, 20 g/L dextrose, 1 g/L polysorbate 80, 2 g/L triammonium citrate, 5 g/L sodium acetate trihydrate, 0.1 g/L magnesium sulphate, 0.05 g/L manganese sulphate, 2 g/L dipotassium phosphate) incubated overnight at 37 °C. To obtain sufficient glycosidic components, this procedure was repeated in 2000 mL Erlenmeyer flasks. In brief, after 24 h of incubation at 37 °C, the culture of *L. paracasei* was centrifuged at 8000 × g for 15 min at 4°C to remove the bacterial cells, then the supernatant was collected. Then trichloroacetic acid (TCA) was added to the supernatant to a final concentration of 4% (w/v), mixed with agitation overnight at 4 °C, and precipitated proteins were separated by centrifugation (12,000 × g, 4 °C, 15 min). Next, 4 volumes of ethanol were added to the supernatant to precipitate EPS. After incubation for 12 h, EPS was gathered by centrifugation (12,000 × g, 15 min). The precipitates were dissolved in distilled water, then crude EPS was obtained by lyophilization and EPS was used for the next experiments.²⁷

Cell culture

Human colon cancer cell lines (SW-480, HT-29, HCT-116) and human embryonic kidney normal cell line (KDR/293) were purchased from Pasteur Institute, National Cell Bank of Iran, Tehran, Iran. All cell lines were cultured in RPMI-

1640 medium supplemented with 10% FBS and Pen/Strep at 37 °C and 5% CO₂. After culture reached 80% confluency cells were detached with trypsin and seeded in cell culture plates. The culture media was changed every 2–3 days.

Cytotoxicity assay

For determination of cell viability in cancerous and normal cell lines, MTS assay was performed. The cytotoxicity of *L. paracasei* EPS was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, according to the manufacturer procedures. All cell lines were seeded in 96-well plates at the density of 1.2×10^4 cells per well. After overnight incubation, cells were treated with different concentrations of *L. paracasei* EPS (in the range of 5–40 µg/mL) as well as 7 µL/well 5FU (50 mg/mL) as a positive control for 24 and 48 h. After the designated culture period, 20 µL (final concentrations of 333 µg/mL MTS and 25 µM PMS) of the combined MTS/PMS solution was added into each well containing 100 µL culture medium and were incubated similarly to the growth condition for 4 h. Afterward, 25 µL of 10% SDS were added into each well to stop the reaction. Then the absorbance was measured at 490 nm using ELISA plate reader (ELx 800; Biotek, Winooski, VT, USA). The relative growth inhibitory effect of the *L. paracasei* EPS was calculated by comparing the intensity levels of the treated cells to that of the untreated control cells.

DAPI staining

DAPI staining was performed to evince morphological changes of DNA. Here, the cells were seeded at the density of 3×10^5 cells per well on sterile coverslips in 6-well plates. After overnight incubation, the treated cells with *L. paracasei* EPS and 5FU were incubated at 37 °C for 24 h

then, the cells were fixed with 4% formaldehyde and were permeated with 0.1% Triton X-100 for 5 min. Twice washed cells with PBS were finally stained with 250 ng/mL of DAPI for 3 min at room temperature. The stained cells were evaluated by an inverted fluorescent microscope (Olympus BX64, Japan) equipped with a U-MWU2 fluorescence filter.

Flowcytometry

For evaluating the extent of apoptotic and necrotic cell death, annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit was used. For this purpose, the cells were seeded into 6-well plates (seeding density: 5×10^5 cells per well) and treated as above. Then, the cells were detached by trypsin-EDTA and washed by PBS. According to the annexin V-FITC/PI instructions the cells were re-suspended in 100 µL 1x binding buffer and 5 µL of FITC-conjugated annexin V was added to cell suspensions. Afterward, 10 µL of PI (propidium iodide) staining solution was added to the cells and incubated at room temperature under the dark condition for 15 min. The cells were analyzed using FACS Calibur flowcytometry (Becton Dickinson, San Jose, CA). Data were analyzed using Cell Quest Pro software (BD Biosciences) and total events of 10,000 cells at a rate of 900 cells/s were acquired.

RNA extraction, cDNA synthesis, and Quantitative real-time PCR

Total RNA from 5-FU and *L. paracasei* EPS treated and untreated control cells were extracted by using RNX plus reagent. The RNA pellet was dissolved in DEPC treated water, then, the yield and purity were determined using Nanodrop (Epoch™ Microplate Spectrophotometer, BioTek Instruments, USA). One microgram of isolated RNA was used for the synthesis of cDNA using Prime Script RT Reagent kit according to the manufacturer's instructions.

Table 1. List of primers used for detecting specific RNAs using Real-time PCR.

Genes	Sequence	Annealing
GAPDH	F: 5'-AAGCTCATTTCCTGGTATGACAACG-3' R: 5'-TCTTCCTCTTGCTCTTGCTGG-3'	60 °C
Caspase-3	F: 5'-GGTTCATCCAGTCGCTTTGT-3' R: 5'-AATTCTGTTGCCACCTTTTCG-3'	60 °C
Caspase-8	F: 5'-ACATGGACTGCTTCATCTGC-3' R: 5'-AAGGGCACTTCAAACCAAGTG-3'	58 °C
Bcl-2	F: 5'-GGTGGGGTCATGTGTGTGG-3' R: 5'-CGGTTCCAGGTAAGTCACTCAGTCATCC-3'	63.5 °C
Bax	F: 5'-AACATGGAGCTGCAGAGGAT-3' R: 5'-CAGTTGAAGTTGCCGTCAGA-3'	59 °C
AKT-1	F: 5'-GCAGCACGTGTACGAGAAGA-3' R: 5'-AGTGTGTGGGAAATCTGGCG-3'	57 °C
JAK-1	F: 5'-AACCTTCTCACCAGGATGCG-3' R: 5'-CTTCAGGTCATGCGTGGACA-3'	57 °C
mTOR	F: 5'-TTCCGTTCCATCTCCTTGTC-3' R: 5'-AAGGCCTCATTGACATCTGG-3'	60 °C
Nrf-2	F: 5'-ATGCAACAGGACATTGAGCA-3' R: 5'-TTGGCTTCTGGACTTGAAC-3'	60 °C
CoQ10	F: 5'-TTGGATTTCCACCTGTGTTG-3' R: 5'-CGCCAAATAGTCTCCAAATGA-3'	60 °C

F: forward primer, R: reverse primer

For performing Real-time PCR, specific primers (listed in Table 1)²⁸ were designed using the OLIGO v. 7.56 software (Molecular Biology Insights, Inc, USA). All reactions were carried out in triplicate for each sample using SYBR green master mix (high ROX) on an ABI-step I plus (Applied Biosystems, CA, USA) detection system. Real-time PCR was performed using the following profiles: 1 cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 sec, 60 °C for 35 sec and 72 °C for 10 sec. Then the melting curves were acquired by stepwise increases in the temperature from 55 °C to 95 °C to ensure the specific amplification. The $2^{-\Delta\Delta CT}$ method²⁹ was used for interpretation of results and the threshold cycle values were normalized to the housekeeping gene (GAPDH).

Ferroptosis assays

Iron level

The iron assay kit was used to monitor the intracellular iron levels in treated and untreated control cells.²⁴ Cancer and normal cell lines were seeded in 96-well plates (1.2×10^4 cells per well). After overnight incubation, cells were treated with *L. paracasei* EPS and FIN56 (5 μ M) as a positive control. According to the manufacturer's instructions, iron assay buffer and iron reducer were added to each well. After 30 min of incubation at room temperature, 100 μ L of the iron probe were added into wells and incubated for further 60 min under the dark condition at room temperature. Then, the iron level was measured at 593 nm by Nanodrop (EpochTM Microplate Spectrophotometer, BioTek Instruments, USA).

ROS level

Total ROS and lipid peroxidation was measured by 2,7-dichlorofluorescein diacetate (DCF-DA) and C11-BODIPY probe, respectively.²⁴ All treated/untreated cell lines seeded in 6-well plates (3×10^5 cells per well) were harvested by trypsin-EDTA to obtain cell suspension. 2,7-dichlorofluorescein diacetate and C11-BODIPY (581/591) were added to cell suspension at a final

concentration of 5 μ M, and incubated for 30 min at 37 °C and analyzed using a FACS Calibur flowcytometry (Becton Dickinson, San Jose, CA).³⁰

Glutathione assay

Glutathione assay was performed to measure reduced glutathione. The sulfhydryl group of GSH reacts with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). After overnight incubation of cell lines seeded on 96-well plates (1.2×10^4 cells per well), cells were treated with *L. paracasei* EPS and FIN56 (5 μ M) as a positive control. According to the manufactures instructions, the supernatant of cell lysates was added to the reagents of glutathione assay kit. After 25 min incubation at 25°C, the glutathione concentration was measured at 412 nm by Nanodrop (EpochTM Microplate Spectrophotometer, BioTek Instruments, USA).

Statistical analysis

All data were expressed as mean values \pm standard deviation (SD). Each experiment was repeated at least three times. Statistical analyses were performed using Graphpad Prism version 5 (Graphpad Software, San Diego, CA) using one-way ANOVA test considering $p \leq 0.05$ as the significance level.

Results

Cytotoxicity assay

The effect of the *L. paracasei* EPS on the viability of colon cancer cell lines (SW-480, HT-29, HCT-116) and normal cell line (KDR/293) was investigated by MTS assay. The IC_{50} value was determined to be about 15 μ g/mL at 24 h (Figure 1). As seen in Figure 1, cell viability was significantly reduced after exposure of cancerous cell lines to *L. paracasei* EPS. The MTS assay results in the significant cell viability reduction for 24 h exposure time of *L. paracasei* EPS on KDR/293 normal cells.

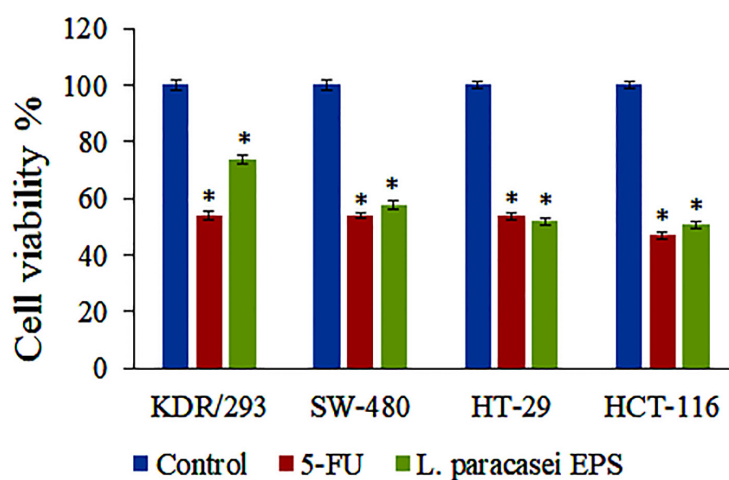


Figure 1. MTS assay of the *L. paracasei* EPS on human colon cancer cell lines and normal KDR/293 cell line after 24 h treatment, 5-FU was used as a positive control. All experiments were performed in triplicate and data were presented as mean \pm SD. Stars show $p \leq 0.05$ indicating the significance.

DAPI staining

For investigating the morphological changes as a result of induction of apoptosis on treated/un-treated cell lines, DAPI staining was applied. As shown in Figure 2, *L. paracasei* EPS treatment (15 µg/mL at 24 h) caused a significant fragmentation in the nucleus of the treated cells compared to untreated control cells, further normal

cell line showed no changes. The *L. paracasei* EPS-treated cells demonstrated significant fragmentation in the chromatin within the nucleus of colon cancer cells but their morphology did not change in untreated control and normal cell line (Figure 2). In cancerous cell lines, the nucleus seems to be separated and the formation of apoptotic bodies indicate the occurrence of apoptosis in these cells.

Flowcytometry

To distinguish early/late apoptosis and necrotic cell death, all cell lines were stained with Annexin V-FITC/PI and evaluated using flowcytometry (Figure 3). The translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the cell surface is the main characteristic of apoptotic cells, which could be detected by Annexin V staining. Cells which are in the late stage of apoptosis and necrosis, are permeable to PI due to loss of cell membrane integrity. Compared to the normal cell line, the cancerous cells treated with 15 µg/mL *L. paracasei*

EPS, demonstrated significant apoptotic cell death (early/late apoptosis) after 24 h incubation. In the treated SW-480 cells 41.16%, HT-29 41.86% and HCT-116 44.74%, were in early/late apoptosis and necrosis stages of cell death while *L. paracasei* EPS caused around 12% of apoptotic/necrotic cell death in KDR/293 normal cell line.

Based on our findings, *L. paracasei* EPS can prohibit the proliferation of cancer cell lines similar to 5-FU (as positive control which has been the first-choice chemotherapy drug for colorectal cancer for many years) (as reported in our recent research²⁸) with less cytotoxicity to the normal cell line.

Quantitative Real-time PCR

Figure 4 exhibits the expression levels of pro/anti-apoptotic genes (BAX, Caspase 3, Caspase 8 and Bcl-2), some important regulatory genes involved in the cell proliferation, apoptosis, metastasis and inflammation (AKT1, JAK1, and mTOR), and key elements of ferroptosis pathway (Nrf-2 and CoQ10) in human colon cancer and normal cell lines after treatment with the *L. paracasei* EPS, 5-FU and FIN56 (as positive controls), compared to untreated control groups. The expression of pro-apoptotic genes (BAX, caspase-3, caspase-8) was increased and the anti-apoptotic gene (Bcl-2) was significantly decreased in *L. paracasei* EPS-treated cancer cells compared to untreated control cells. The upregulation in the mentioned

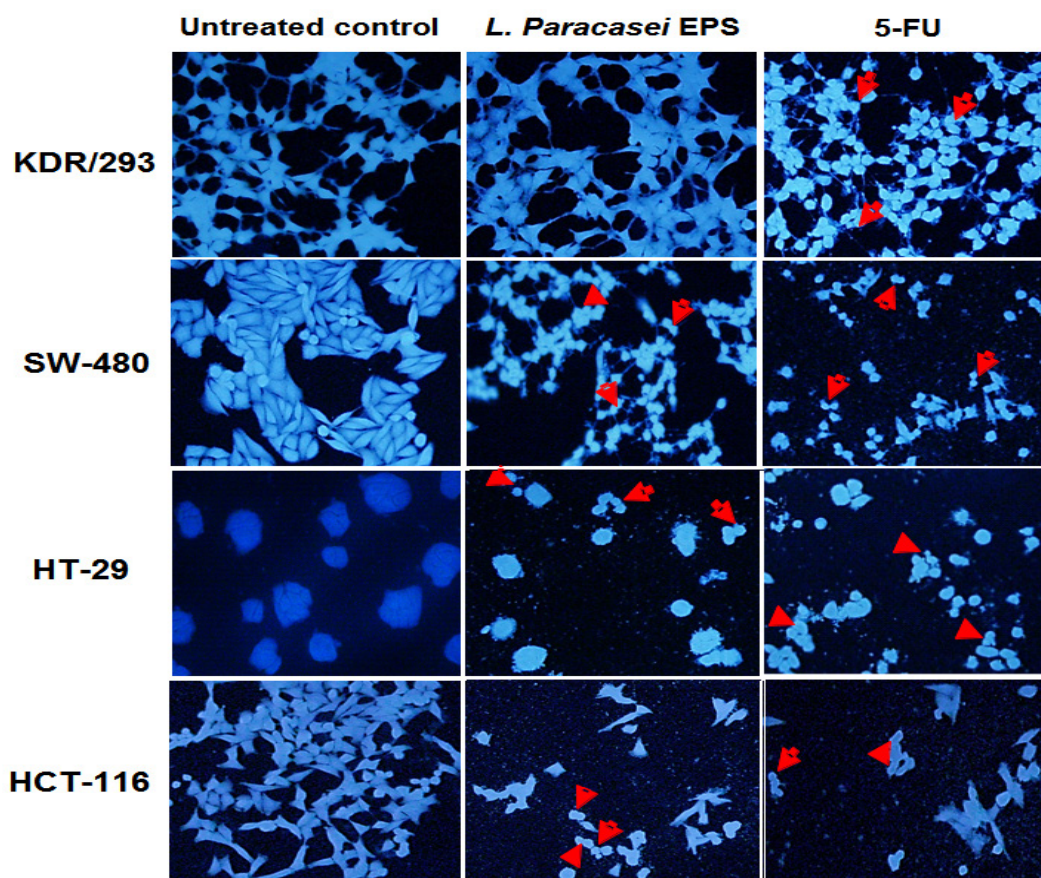


Figure 2. FITC- labeled Annexin V flowcytometry detection of apoptosis in *L. paracasei* EPS (15 µg/mL) and 5-FU (50 mg/mL) on treated and untreated control colon cancer and normal cell lines.

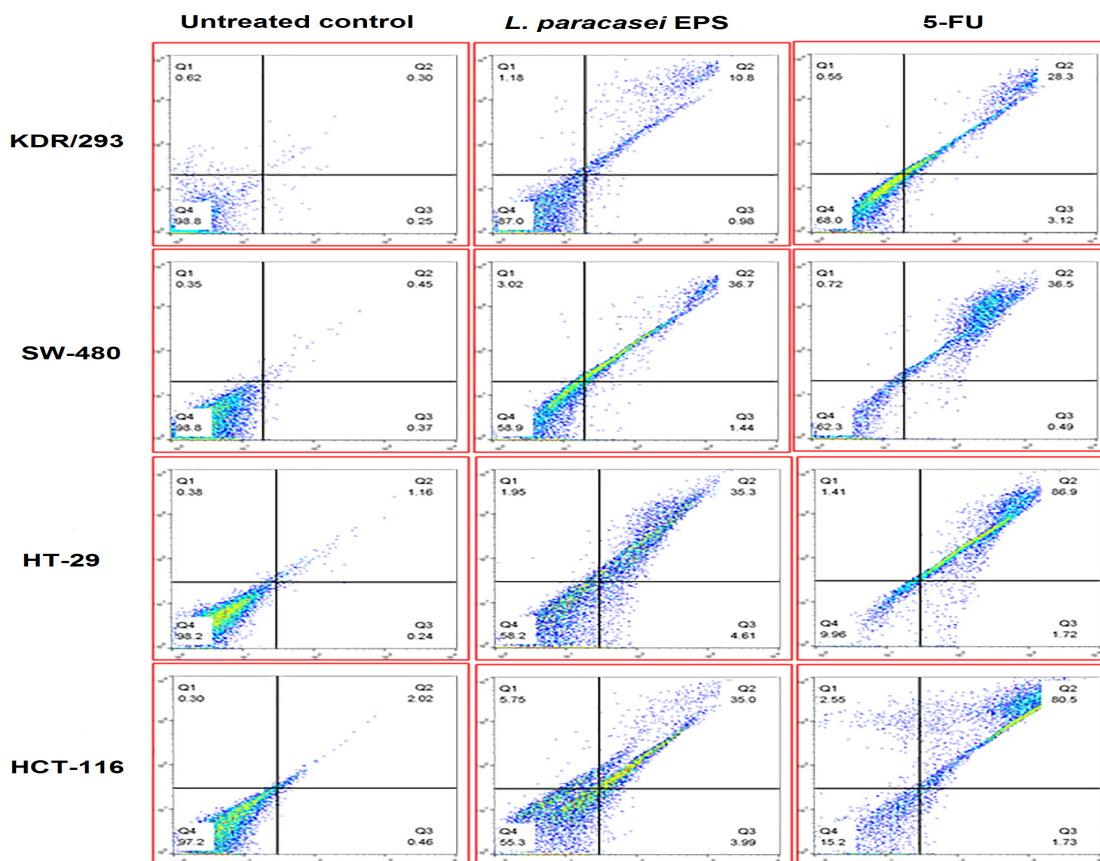


Figure 3. FITC- labeled Annexin V flowcytometry detection of apoptosis in *L. paracasei* EPS (15 µg/mL) and 5-FU (50 mg/mL) on treated and untreated control colon cancer and normal cell lines.

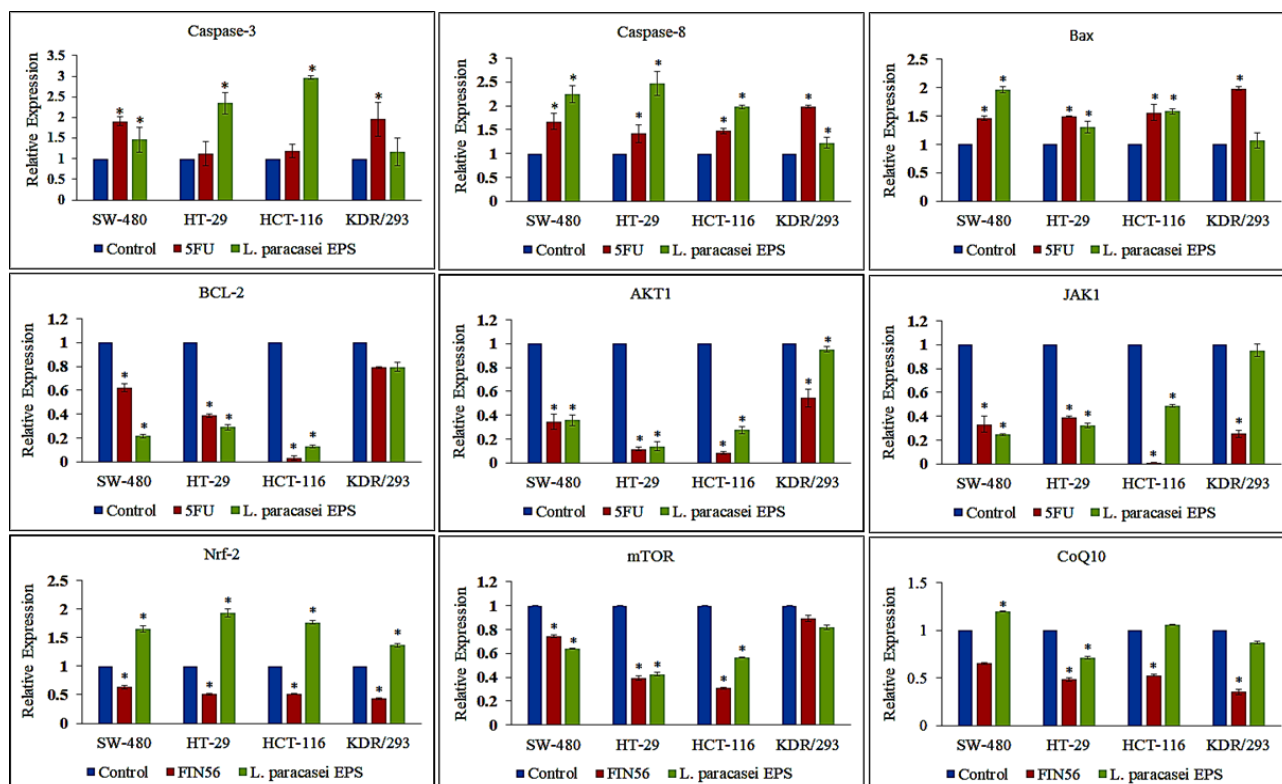


Figure 4. Effects of *L. paracasei* EPS (15 µg/mL), 5-FU (50 mg/mL) and FIN56 (5 µM) (positive control group) on human colon cancer and normal cell lines signaling pathways. Target genes were normalized with GAPDH as a reference gene. All experiments were performed in triplicate. Data expressed as fold change.

genes by *L. paracasei* EPS in all treated groups was similar to the anticancer agent (5-FU) indicating the same inducing pathways of apoptosis. According to the results of the current study, non-significant alternation on apoptotic genes expression in an epithelial normal cell line were observed. Moreover, the cancerous cells treated with *L. paracasei* EPS showed a significant reduction in AKT-1, JAK-1, and mTOR genes expression levels in all cancerous cell lines compared to untreated control cells (Figure 4) while the aforementioned genes expression levels in normal cells were non-significant. For the molecular mechanism of ferroptosis determination, Nrf-2 and CoQ10 genes expression were evaluated and as shown in figure 4, *L. paracasei* EPS could up-regulate and FIN56 down-regulate significantly the Nrf-2 gene (suppressor the formation of superoxide as well as other reactive oxygen species) expression at mRNA level. In addition, significant upregulation was not detected in CoQ10 mRNA expression levels in all *L. paracasei* EPS-treated cell lines compared to untreated control.

Total and lipid ROS levels

To evaluate total and lipid ROS, all cell lines were stained with DCF-DA and C11-BODIPY probe, respectively and assessed by flowcytometry²⁴. Treatment of cancerous and normal cells with *L. paracasei* EPS (15 µg/mL) did not exhibit an increase in fluorescence probes (Figure 5), indicating that *L. paracasei* EPS could not generate ROS and should not be considered as a ferroptosis inducer while FIN56 (5 µM) as positive control, results in increase in lipid and total ROS levels in all treated cell lines.

Glutathione and iron assays

To explore the glutathione and iron levels in all cell lines treated with *L. paracasei* EPS and FIN56, which are two important events in triggering ferroptosis, we applied glutathione and iron assay kits, respectively. Figure 6A

showed the glutathione concentration (µM) (as compared to calibrator in glutathione assay kit) in cancerous and normal cell lines. As shown in Figure 6A there is no significant depletion of glutathione level in all *L. paracasei* EPS treated-cell lines subjected to the assay. Ferrous iron (Fe²⁺) is one of the essential factors for the execution of ferroptosis. The iron chelators can prevent cells from undergoing ferroptosis. Therefore, we investigated the effect of *L. paracasei* EPS on the changes in iron levels in human colon cancer and normal cell lines. As shown in figure 6B, there is no significant alternation in iron levels in *L. paracasei* EPS-treated colon cancer cells as well as normal cell line compared to untreated control cells.

Discussion

Colon cancer is one of the common malignant digestive tract tumors. Its incidence is high in western countries as well as Iran.^{14,31} In most cases the majority of cancer cells might develop resistance to conventional therapeutic agents,⁴ therefore, targeting vital signaling pathways in colon cancer including those involved in growth signaling cascades, invasion, angiogenesis, and apoptosis is a prompt need which may exert selective cytotoxicity towards cancer cells.¹ Further, the development of new anticancer treatment modalities based on probiotics with less adverse effects is urgently needed.¹ The beneficial and antitumor effects of probiotics and their derivatives have been reported previously.^{2,6,8,9,11,13,15-17,31} However, it is noteworthy that these characteristics are completely strain-dependent.¹⁴ For instance, Zununi *et al.*¹⁷ demonstrated that the conditioned media of the probiotic *Leuconostoc mesenteroides* decreased the expression levels of AKT1 and induce mitochondrial apoptosis in HT-29 cell line. Riaz Rajoka *et al.*³² showed that three *Lactobacillus* strains exhibited anticancer activity via upregulation of BAX, Bad, Cas-3, Cas-8 and Cas-9 genes. Baldwin *et al.*³³ demonstrated a synergistic pro-apoptotic effect of the combination of

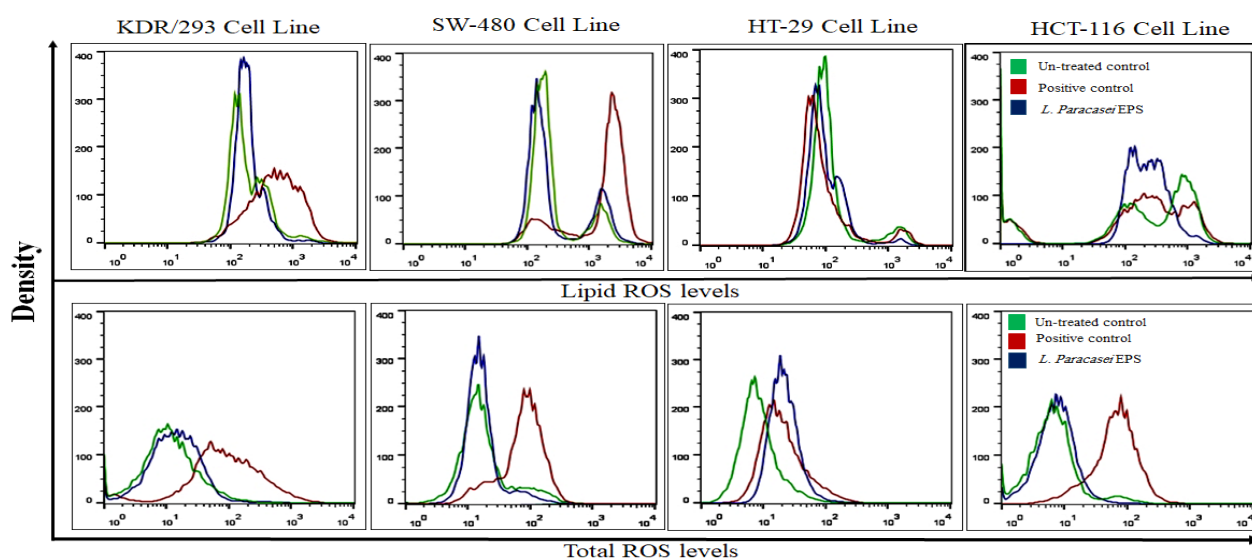


Figure 5. Effects of *L. paracasei* EPS (15 µg/mL) and FIN56 (5 µM) (positive control group) on total and lipid ROS production on human colon cancer and normal cell lines assessed by flow cytometry.

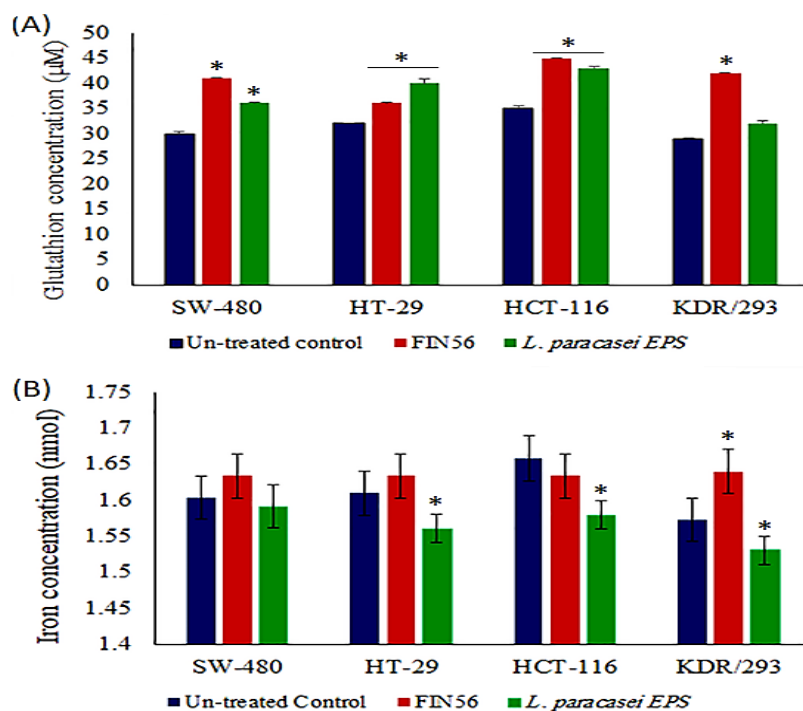


Figure 6. Effect of *L. paracasei* EPS (15 µg/mL) and FIN56 (5 µM) (positive control group) on human colon cancer and normal cell lines. (A) Glutathione concentration assay and (B) Iron concentration assay results of all treated/untreated groups.

5-FU, and *L. acidophilus* and *L. casei* probiotic strains which induce apoptosis via activation of the caspase 3 and reduction of p21 expression. Iyer *et al.*³⁴ showed that *L. reuteri* downregulated nuclear factor-κB (NF-κB)-dependent gene products and promoted apoptosis by enhancing MAPK activities in myeloid leukemia-derived cells. However, consuming live probiotics may cause concerns from the FAO and WHO, especially in immunocompromised patients.^{13,14,35,36} Thus, recently exploiting probiotic bacteria's components has been interestingly increased.^{4,6,9,14,16} EPSs – one of these components produced by lactic acid bacteria – provides numerous health benefits like antioxidant, cholesterol-lowering properties, anti-ulcer, anti-tumor, and immunomodulating activities. Researches revealed that these glycosidic components generally are less cytotoxic toward normal cells which makes them a promising candidate in cancer therapy.^{13,37,38} In this regard, we carried out experimental assays for evaluating the possible modulatory role of *L. paracasei* EPS on the molecular targets involved in cancer cell growth, apoptosis, survival, and inflammation signaling pathways. Previous studies have shown that AKT, mTOR, JAK are among important molecules, which can modulate colon cancer cell proliferation, metabolism, metastasis, and survival.^{5,17,39} Additionally studies reported the over-expression of the aforementioned genes in colon cancer. In line with their findings, our data showed that *L. paracasei* EPS is able to significantly reduce the expression levels of AKT-1, JAK-1 and mTOR mRNAs. Further, induction of apoptosis in human colon cancer cell lines was correlated with our findings of real-time PCR that showed a significant reduction in Bcl-2 and increase in

BAX mRNA expression levels in all cancerous cell lines same or even higher than 5-FU as positive control which indicates the effect of *L. paracasei* EPS on mitochondrial apoptosis pathway. Further, increased expression levels of both caspase-3 and caspase-8 mRNAs were detected in *L. paracasei* EPS treatment (Figure 4), which is confirmed by DAPI staining (Figure 2), Annexin V/PI (Figure 3) assays. Overall, cellular and molecular assays highlight the impact of *L. paracasei* EPS in apoptotic cell death in colon cancer cells. Regarding our data, the *L. paracasei* EPS could exert its apoptotic activity via Akt/ Jak1 and mTOR signaling pathway in the colon cancer cell lines. Inconsistent with our findings Di *et al.*⁴⁰ showed that the *L. casei* EPS exerts antitumor activity upon HT-29 cell line by up-regulating Bad, BAX, Cas-3 and Cas-8 genes. Sasikumar *et al.*⁴¹ revealed that *L. plantarum* BR2 EPS exerts antioxidant, antidiabetic and cholesterol-lowering properties, further they showed that the EPS was non-toxic to normal cells. Moreover, Zhou *et al.*²⁷ demonstrated the anticancer properties of *L. plantarum* NCU116 EPS (EPS116) on CT26 cells may be TLR-2 dependent also it increases the expression levels of pro-apoptotic genes. Ever-growing evidence demonstrated the vital role of induction of apoptosis in cancer research. Although resistance to apoptosis has been found in many types of cancers which in turn hinders anticancer therapeutics efficacy. Consequently, exploiting alternative regulated cell death mechanisms is of great importance in various diseases treatment especially cancer.⁴² Ferroptosis described as a form of regulated cell death which is morphologically, biochemically and genetically distinct from other forms of regulated cell death and occurs as a result of high lipid peroxidation,

production of iron-dependent reactive oxygen species (ROS).^{30,43,44} Ferroptosis cells exhibit morphological changes in mitochondrial structure without any changes in nucleus structural integrity.²⁴ However, typical features of apoptosis (mitochondrial cytochrome c release, caspase activation, and chromatin fragmentation) are not observed in ferroptosis cells⁴³. Main regulators of ferroptosis are iron metabolism, lipid peroxidation signaling and activation of the mitogen-activated protein kinase (MAPK) pathway.²⁴ Understanding the molecular mechanisms of ferroptosis in cancer treatment will open new avenues in cancer research. A vast body of evidence suggested the Nrf2 signaling activation, upregulates the genes involved in ROS detoxification and antioxidant activities of the host. At low levels of ROS, Keap1 suppresses the activity of Nrf2. When the ROS amount increases, the alternation of conformation of Keap1 results in activation of Nrf2 which in turn activates transcription of genes encoding antioxidant enzymes and detoxifying proteins. *In vitro* and *in vivo* studies regarding probiotics demonstrated that they could protect against oxidative stress through regulating the Nrf2-Keap1-ARE pathway.⁴⁵⁻⁴⁷ Thus, it seems Nrf-2 is a negative regulator of ferroptosis. The results of qPCR showed that *L. paracasei* EPS could up-regulate the Nrf-2 gene expression by which it could exert antioxidant activity. CoQ10 is an endogenous antioxidant in the mevalonate pathway, however, there is limited evidence on how it may protect cells from ferroptosis. Our results showed a reduction of CoQ10 mRNA levels in FIN56 treated cells further there is no statistically significant elevation in CoQ10 gene expression in *L. paracasei* EPS treated cells. As evidence suggests, depletion of glutathione is inconsistent with the fact that the treatment induces ROS production, causing cell death. As it's shown in Figure 6, there is no significant reduction in glutathione and iron concentration in *L. paracasei* EPS treated cells. Taken together, these data indicated that the *L. paracasei* EPS could not provoke ferroptosis cell death. To sum up, employing adjunctive therapies like probiotic therapy is one of advancing treatment modalities. The protective and anticancer effects of probiotics have been reported extensively by *in vitro/ in vivo* studies. Thus, specific inhibitors of the aforementioned signaling molecules may be explored as potential therapeutic targets. To the best of our knowledge, there is little information on the anticancer effects and molecular mechanisms involved in the interaction of EPS and cancer cells. Taken together, the results of this study demonstrated that *L. paracasei* EPS has a promising future ahead in the field of targeted therapy in colon cancer.

Conclusion

Debilitating effects of cancer, especially colon cancer with the highest incidence rate and mortality which remains an uphill battle, prompted us to investigate safe treatment modalities based on probiotic-derived components. Based on our findings, the glycosidic components of the *L. paracasei* showed promising effects on key molecular targets

which play an important role in colon cancer initiation, progression, metastasis, and chemo-resistance. *L. paracasei* EPS is able to mediate pro-apoptotic and anti-proliferative effects in colon cancer cell lines. In this research, we have exploited the EPS of an indigenous probiotic strain with anticancer potential with low or insignificant cytotoxicity to normal cells for future applications in colon cancer treatment. Furthermore, *in vivo* and clinical trials should be performed to evaluate the applicability of this component besides conventional methods to increase the survival rate of colon cancer patients.

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Author Contributions

AYK and BPG: Contributed to the study conception and designed the experiments. YRS: Performed experiments, analyzed the data and drafted the manuscript. AS and YN: Contributed significantly to the improvement of the manuscript. AYK: Critically revised the manuscript. All authors read and gave approval of the final manuscript.

Conflict of Interests

The authors declare that there are no conflicts of interest.

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