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ORIGINAL ARTICLE

Investigating the therapeutic potential of *Bifidobacterium breve* and *Lactobacillus rhamnosus* postbiotics through apoptosis induction in colorectal HT-29 cancer cells

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ABSTRACT

Background and Objectives: Colorectal cancer (CRC) is a prevalent form of cancer worldwide. Recent studies suggest that postbiotics derived from probiotic bacteria have the potential as an adjunct therapy for CRC. This study investigates the anti-cancer effects of *Bifidobacterium breve (B. breve)* and *Lactobacillus rhamnosus (L. rhamnosus)* postbiotics on the HT-29 cell line.
Materials and Methods: Through MTT and scratch assay, we investigated the anti-proliferation and anti-migration effects of *B. breve* and *L. rhamnosus* postbiotics on HT-29 cells. Furthermore, postbiotic-mediated apoptosis was assessed by analyzing the expression of *Bax, Bcl-2,* and *caspase-3.* We also investigated the effects of *B. breve* postbiotics on the expression of three important genes involved in metastasis, including *RSPO2, NGF,* and *MMP7*. Consequently, we validated the expression of selected genes in twelve adenocarcinoma tissues.
Results: The results demonstrated the significant impact of postbiotics on HT-29 cells, highlighting their ability to induce anti-proliferation, anti-migration, and apoptosis-related effects. Notably, these effects were more pronounced using *B. breve* postbiotics than *L. rhamnosus.* Additionally, *B. breve* postbiotics could inhibit metastasis through upregulation of *RSPO2* while downregulating *NGF* and *MMP7* expression in HT-29 cells.
Conclusion: Our research suggests that postbiotic metabolites may be effective biological products for the prevention and treatment of cancer.

Keywords: Apoptosis; *Bifidobacterium breve*; Colorectal cancer; *Lactobacillus rhamnosus*; Postbiotics; Probiotics

INTRODUCTION

Colorectal cancer (CRC) ranks as the third most common cancer globally, characterized by the abnormal growth of cells in the colon or rectum (1). According to the International Agency for Research on Cancer's global statistics, CRC accounted for approximately 1.9 million new cases and 0.9 million

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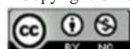
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deaths worldwide in 2020. Projections suggest that the global incidence of new CRC cases is anticipated to increase to 3.2 million by 2040. Notably, CRC incidence rates are higher in highly developed nations but are also rising in middle- and low-income countries due to the influence of Westernization (2). In recent years, the role of gut microbiota in CRC has gained significant attention as researchers have increasingly recognized the intricate relationship between the composition of the gut microbiota and the development of this prevalent form of cancer. The gut microbiota, a complex ecosystem of microorganisms residing in the gastrointestinal tract, plays a crucial role in maintaining gut health, digestion, immune function, and protection against pathogens.

Consequently an imbalance or dysbiosis in the gut microbiota has been associated with various diseases, including CRC (3, 4). In light of the potential impact of gut microbiota on CRC, researchers have explored the use of probiotics as a preventive and therapeutic strategy. Probiotics are live microorganisms that confer health benefits to the host when consumed in appropriate quantities. These beneficial bacteria aid in restoring microbial balance and promoting a healthy gut environment (5-7). More recently, a novel category of microbial-based products, known as postbiotics, has garnered substantial interest. The International Scientific Association of Probiotics and Prebiotics (ISAPP) defines postbiotics as "preparations of inanimate microorganisms and/or their components that provide health benefits to the host" (8). Postbiotics can encompass a variety of components, including metabolites, short-chain fatty acids (SCFAs), microbial cell fractions, functional proteins, extracellular polysaccharides (EPS), cell lysates, teichoic acid, peptidoglycan-derived muropeptides, and pili-type structures (9, 10). Extensive research has demonstrated the biological activities of postbiotics, which play a crucial role in promoting overall health and protecting against various diseases (11). Postbiotics offer several notable advantages over probiotics. Unlike probiotics, postbiotics can exert their biological effects by reaching various host surfaces through diverse routes, including the oral cavity, gut, skin, urogenital tract, and nasopharynx (12, 13). Moreover, they allow for a more straightforward assessment of their molecular mechanisms and their impact on disease outcomes. Nevertheless, our understanding of the significance of postbiotics in maintaining gut homeostasis is still in its nascent stages (14). Whether derived from probiotics or other microorganisms, postbiotics can be naturally synthesized or directly administered to host surfaces, enhancing human gut health. This application of postbiotics can significantly improve the prevention and treatment of gastrointestinal cancers, including CRC (8, 15, 16). Numerous strains of probiotics have been investigated for their potential effects on CRC, with species like *Bifidobacterium breve (B. breve)* and *Lactobacillus rhamnosus (L. rhamnosus)* being among the most commonly studied. These probiotics have demonstrated promise in preclinical and clinical studies by reducing inflammation, inhibiting the growth of cancer cells, and improving overall gut health. In this investigation, we compared the anti-proliferation, anti-migration, and apoptotic effects of two postbiotic extracts from *B. breve* and *L. rhamnosus.*

Consequently, the expression of crucial metastasis-related genes, *RSPO2, NGF,* and *MMP7*, within the HT-29 cellular context was evaluated. Importantly, our study extends into clinical validation through the analysis of tissue samples from adenocarcinoma patients. This comprehensive approach advances our understanding of genus-dependent postbiotics therapeutic potential and offers a unique perspective on their modulation of pivotal genes in CRC.

MATERIALS AND METHODS

This research study was conducted in compliance with ethical guidelines. The Birjand University of Medical Science ethics committee in Iran issued the approved ethics ID IR.BUMS.REC.1400.332.

Bacterial culture. The *B. breve* (ATCC 15700) and *L. rhamnosus* (ATCC 7469) strains utilized in this study were obtained from the Persian-type culture collection (Iranian Research Organization for Science and Technology, Tehran, Iran). *B. breve* and *L. rhamnosus* were cultivated in de Man, Rogosa, and Sharpe (MRS) broth enriched with cysteine medium (Merck, Germany) and standard MRS broth (Merck, Germany), respectively, with a 1% (v/v) inoculum. Both strains were incubated for 48 hours at 30°C under anaerobic conditions. Before each experiment, they underwent two subcultures, propagating for an additional 24 hours at 30°C under anaerobic conditions. Stock cultures were maintained at -80°C

in MRS broth, supplemented with 20% (v/v) sterile glycerol (Merck, Germany).

Cell culture and maintenance. The human colorectal cancer cell line HT-29 (ATCC HTB-38) and human dermal fibroblast (HDF) (ATCC PCS-201-041) used in this study were provided by the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured and maintained in a controlled environment at a temperature of 37°C in a 5% CO_2 atmosphere using DMEM medium (high glucose with GlutaMAX) (Bioidea, Iran). The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO® Invitrogen Corporation, CA, USA) and 100 IU/ml penicillin-streptomycin (Bioidea, Iran) to support cell growth and viability (17).

Preparation of postbiotics. To prepare the postbiotics, the *B. breve* and *L. rhamnosus* starter cultures were separately inoculated into 100 ml of MRS broth rich-cysteine and MRS broth, respectively. Subsequently, 1 ml of each bacterial starter culture at a concentration of 10^8 CFU/mL was inoculated into 9 mL of the respective medium. The cultures were then incubated at 37°C for 24 h without agitation, allowing both bacteria to reach the late-exponential growth phase. To obtain the postbiotics metabolite, the live *B. breve* and *L. rhamnosus* cells were separated from the culture supernatant through centrifugation at 10,000 g for 15 min at 4°C, and the resulting supernatant, containing postbiotics, was carefully collected. To ensure the quality and purity of the postbiotics, the supernatant was adjusted to a physiological pH range of 7.2-7.4. This adjustment helps maintain the stability and functionality of the postbiotics. Furthermore, the postbiotics were filtered through a 0.22 µm polyether sulfone membrane syringe filter (Millipore, USA) to remove any remaining cellular debris or contaminants (18).

Anti-proliferation effect of postbiotics on HT-29 and normal cells. HT-29 cells (6×10^3 cells per well, 100 µl per well) and HDF cells (1×10^4 cells per well, 100 µl per well) were seeded into 96-well microplates and incubated at 37°C in a 5% CO_2 incubator for the MTT assay. After a 24-hour incubation period, different postbiotic concentrations ranging from 0.62% to 40% (v/v) were introduced into a complete growth medium. Following respective time intervals of 24, 48, and 72 h, the cells were treated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma–Aldrich, USA) at a concentration of 5 mg/ml for four hours. A 10% dimethyl sulfoxide (DMSO) reagent (Sigma–Aldrich, USA) was added to dissolve the resulting blue formazan crystals. The absorbance of the formazan dye was measured at a wavelength of 570 nm with a reference wavelength of 630 nm. Each experiment was performed three times, with triplicate samples in each repetition. The percentage of cell viability was calculated using the following equation: (A570 nm sample - A570 nm blank) / (A570 nm control - A570 nm blank) × 100%, where A570 nm represents the maximum absorbance of the cells at 570 nm. To determine the concentration at which cell growth was inhibited by 50% (IC_{50}), a plot was generated by plotting the postbiotics concentration against the percentage of cell viability (19).

Anti-migration effect of postbiotics on HT-29 cells. The scratch test was employed to evaluate the efficacy of *B. breve* and *L. rhamnosus* postbiotics in inhibiting cell migration. HT-29 cells were seeded in 12-well plates at a density of 4×10^5 cells per well. After 24h treatment with a 25% IC_{50} concentration of each postbiotic, a straight scratch was created using a 200 µL yellow pipette tip. Cell migration was observed through microscopy at intervals from 0 to 72 h following treatment. Photographs were captured to document the boundary areas of the scratches at different time points. The ImageJ software (version 1.52) was utilized for analyzing the scratch area. Specifically, the ratio between the initial open space at 0 h and the remaining space after 72 h was calculated. This analysis facilitated a comparison of the migratory capacity of the cells treated with *B. breve* and *L. rhamnosus* postbiotics (20).

Study the expression of genes in adenocarcinoma tissues by RT-qPCR. Histopathological samples were collected from twelve patients diagnosed with adenocarcinoma cancer and their adjacent normal tissues to investigate the expression of three important genes related to metastasis in CRC tissues. The samples were obtained from the Biobank of Shahid Beheshti University of Medical Sciences. Total RNA was extracted from the tissue samples using an RNA extraction kit (Parstous, Iran). Subsequently, the RNA was reverse-transcribed into complementary DNA (cDNA) using the cDNA Synthesis Kit (Par-

stous, Iran), following the manufacturer's guidelines. Real-time quantitative PCR (RT-qPCR) was then conducted. The expression levels were normalized using GAPDH as an endogenous control, and the relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [ABI Step One™ Real-Time PCR System (Applied Biosystems, CA)].

Study the effects of postbiotics on gene expression of HT-29 cell line. The HT-29 cells (15×10^5 cells per well) were treated with 25% IC_{50} concentration of *B. breve* postbiotics for 24h. After this incubation period, total RNA was extracted from the cells using a total RNA extraction kit (Parstous, Iran). Subsequently, cDNA synthesis was done using a general cDNA Synthesis Kit (Parstous, Iran). Quantitative RT-qPCR was conducted to evaluate the expression levels of the *Bax, Bcl-2, caspase-3, RSPO2, NGF,* and *MMP7*, utilizing the QuantiTect™ SYBR Green PCR Master Mix (Amplicon, Denmark). Specific primers targeting the appropriate genomic regions were employed for amplification (Tabel 1). Gene amplification was performed, and the expression levels of the target genes were normalized to the reference gene *GAPDH.* The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis. The cytotoxicity and anti-proliferative effects were analyzed using analysis of variance (ANOVA), followed by pairwise multiple comparison procedures (Tukey test). The obtained results are presented as the mean ± standard error of the mean (SEM). The statistical analysis was conducted using Minitab Statistical Software, with a significance level set at $p\text{-value} < 0.05$. This threshold was used to determine the statistical significance of the observed differences between the experimental groups.

RESULTS

Postbiotics have anti-proliferation effects on HT-29 cells. The results of the MTT assay illustrated that both *B. breve* and *L. rhamnosus* postbiotics effectively reduced the viability of HT-29 cells in a time- and dose-dependent manner within the treatment groups (Fig. 1A for *B. breve* postbiotics and Fig. 1B for *L. rhamnosus* postbiotics). Subsequently, IC_{50} values were calculated for each group. It was noted that the IC_{50} values exhibited a significant reduction across different time intervals (24-72 h) when *B. breve* and *L. rhamnosus* postbiotics were administered. This decrease in IC_{50} values was particularly pronounced in cancer cells treated with *B. breve* postbiotics (IC_{50} values = 20.15 ± 0.16) as compared to those treated with *L. rhamnosus* postbiotics (IC_{50} values = 40 ± 0.9), indicating a more potent cytotoxic effect for *B. breve* postbiotics. However, the cytotoxic effects of postbiotics on HDF cells were less significant, with only limited cytotoxicity observed after 72 h of treatment, amounting to 40% for *B. breve* postbiotics and 26% for *L. rhamnosus* postbiotics (Fig. 1C for *B. breve* postbiotics and Fig. 1D for *L. rhamnosus* postbiotics).

Table 1. Primer sequences

Primer	Sequence	References
Bax	F: TGGAGCTGCAGAGGATGATTG	(21)
	R: GAAGTTGCCGTCAGAAAACATG	
Bcl2	F: TGCACCTGACGCCCTTCACC	(21)
	R: CACATGACCCCACCGAACTCAAAGA	
caspase-3	F: ATTCATAGTGGCACCAAATC	(22)
	R: TAAATCAAATCCGATGTTCC	
RSPO2	F: CTTGTAGCAGAAATAATCGCACATGT	
	R: TGCCTCATTGTCATCTTGCATC	
NGF	F: GAGCAAGCGGTCATCATCCCA	
	R: TCCATGGTCAGCGCCTTGA	
MMP7	F: GAATGTTAAACTCCCGCGTC	
	R: CGATCCACTGTAATATGCGGTA	
GAPDH	F: GTGATGCTGGTGCTGA	(22)
	R: GCTAAGCAGTTGGTGG	

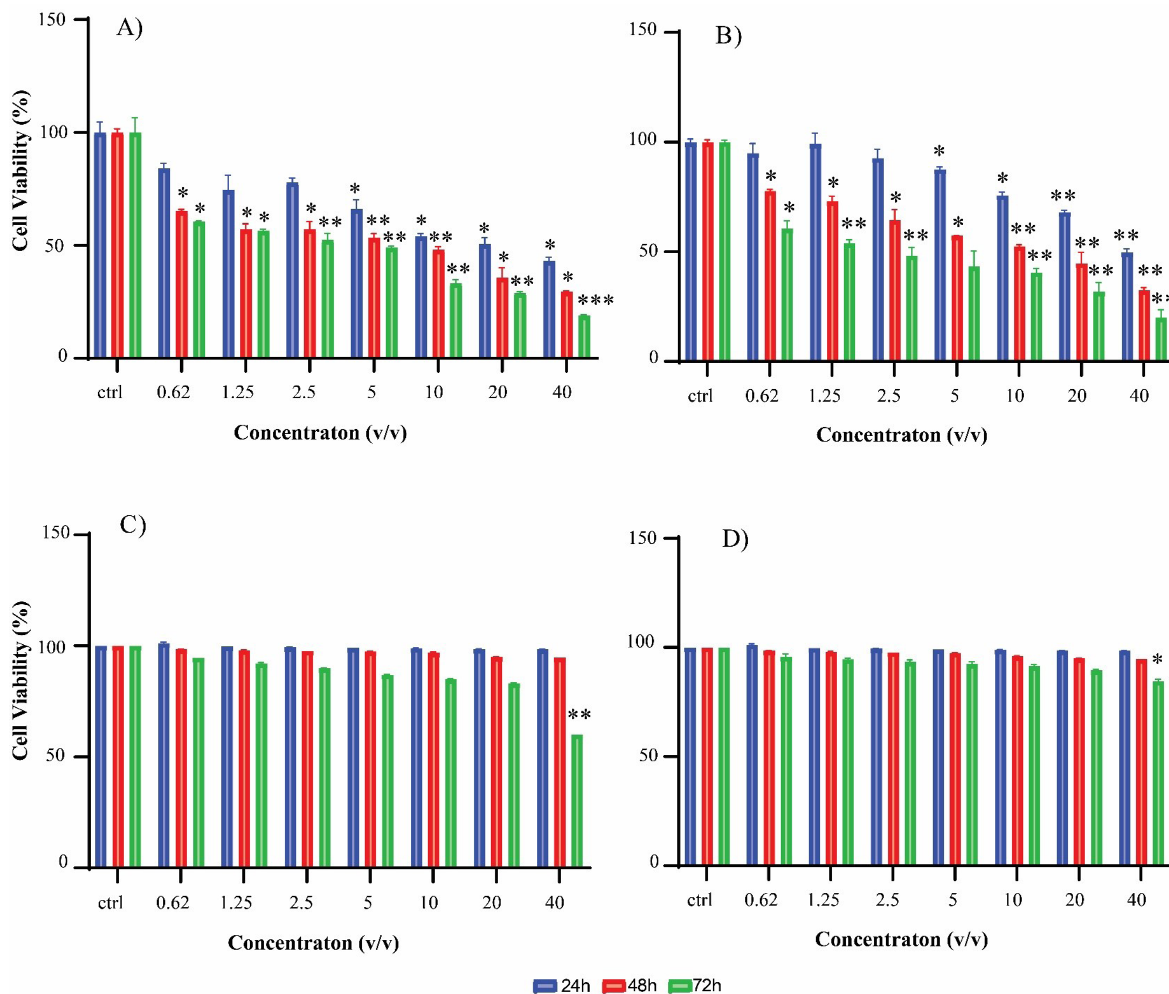

Fig. 1. MTT assay showing the effect of *B. breve* (A) and *L. rhamnosus* (B) postbiotics on reducing the viability of the HT-29 cells. HT-29 cells were treated with various postbiotic concentrations for 24h, 48h, and 72h. MTT analysis indicates that postbiotics had significant anti-proliferation effects on the viability of the HT-29 cells compared to the untreated group, with the IC_{50} value of 20.15 ± 0.16 v/v for *B. breve* in 24h and 40 ± 0.9 v/v for *L. rhamnosus* in 24h. The effects of postbiotics on HDF cells showed only limited cytotoxicity of 40% for *B. breve* (C) and 26% for *L. rhamnosus* (D) postbiotic after 72 h of treatment. ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively *, **, ***).

Postbiotics have anti-migration effects on HT-29 cells. The scratch test assessed the anti-migration effects of postbiotics on HT-29 cells. As indicated by the data presented in Fig. 2, a significant decrease in cell migration was observed within the 0-72 hour time frame in the groups treated with *B. breve* (Fig. 2B) and *L. rhamnosus* postbiotics (Fig. 2C) when compared to the control group (Fig. 2A). Additionally, no noticeable difference in the effectiveness of these two postbiotics in preventing migration was observed (Fig. 2D).

Postbiotics induce apoptosis in HT-29 cells. To explore the anti-apoptotic effects of *B. breve* and *L. rhamnosus* postbiotics, we analyzed their impact on the expression of the *Bax, Bcl-2,* and *caspase-3* genes in HT-29 cells subjected to postbiotic treatment. The results consistently revealed substantial changes in gene expression patterns. When HT-29 cancer cells were treated with *B. breve* postbiotics (Fig. 3A), we observed a significant upregulation of *Bax* (7.94-fold increase, p-value < 0.001) and *caspase-3* (4.6-fold increase, p-value < 0.01), along with a downregulation of *Bcl-2* (2.3-fold decrease, p-value < 0.05). Likewise, when the cancer cells were treated with *L. rhamnosus* postbiotics (Fig. 3B), we observed an upregulation of *Bax* (2.7-fold increase, p-value < 0.01) and *caspase-3* (3.05-fold increase, p-value < 0.01),

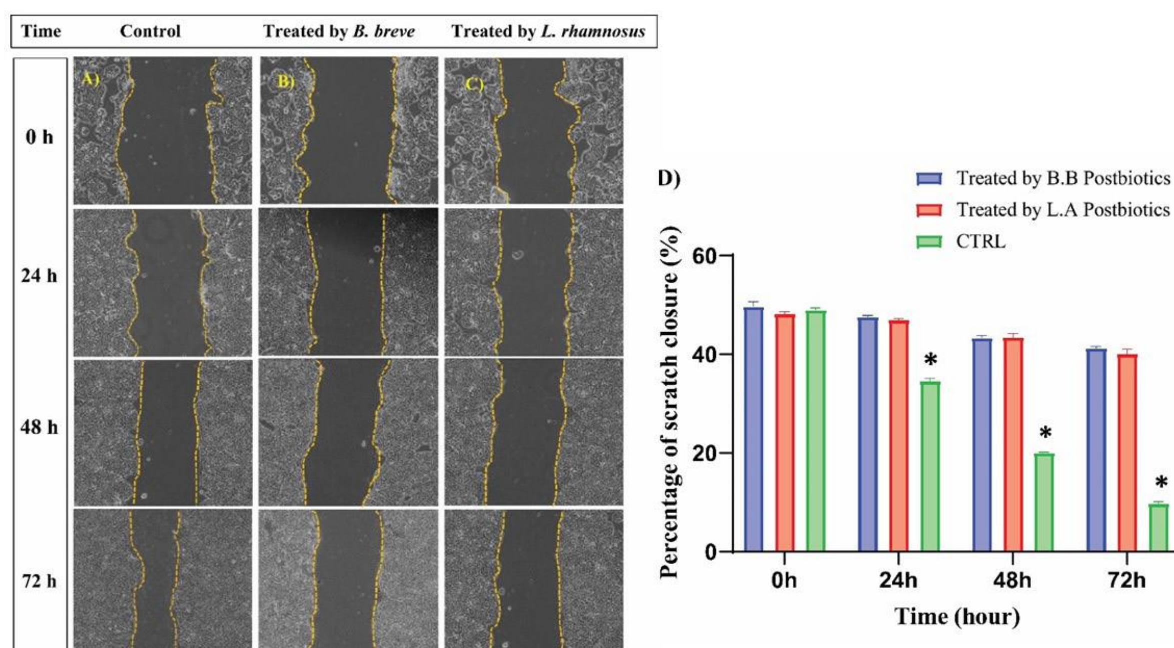

Fig. 2. Scratch test showing the control group (A), and also the effects of *B. breve* (B) and *L. rhamnosus* (C) postbiotics on reducing the migration of the HT-29 cell line. Under 80% confluence, a scratch was made on wells using a sterile yellow-colored pipette tip. Subsequently, 25% IC_{50} concentrations of postbiotics were added to the cells. Pictures were taken at 0-72 hour time frame, and the amount of migration was calculated (D) ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively *, **, ***).

and a downregulation of *Bcl-2* (1.57-fold decrease, p-value < 0.05). Remarkably, the data highlights that *B. breve* postbiotics induced a remarkable increase in the *Bax/Bcl-2* mRNA ratio by 18.2-fold in treated cancer cells, while *L. rhamnosus* postbiotics also exhibited a similar effect, elevating the ratio by 4.4-fold (Fig. 3C). This ratio increase indicates both postbiotics potential to modulate key apoptotic genes, influencing the *Bax/Bcl-2* balance in a manner conducive to anti-apoptotic effects.

Evaluating the expression of selected genes in patient tissues by RT-qPCR. The expression level of the *RSPO2, NGF,* and *MMP7* was evaluated in colorectal samples using RT-qPCR. The results showed that the expression of *RSPO2* (0.14-fold down, p-value < 0.01) in CRC samples exhibited a substantial decrease compared to the adjacent normal tissues. However, the expression of *MMP7* (2.57-fold up, p-value < 0.05) and *NGF* (5.8-fold up, p-value < 0.05) had upregulated in the CRC samples (Fig. 4).

***Bifidobacterium breve* postbiotics regulate the metastasis process in HT-29 cell line.** RT-qPCR data demonstrated that treating the HT-29 cells with postbiotics effectively increased the expression levels of *RSPO2* (1.82-fold up, p-value < 0.01 while decreasing the expression of NGF (1.48-fold down, p-value < 0.05) and *MMP7* (1.9-fold down, p-value < 0.01) compared to untreated cells (Fig. 5).

DISCUSSION

Recent research shows intestinal microbiota composition can significantly impact cancer development and treatment. Probiotics are nonpathogenic microorganisms that can provide health benefits to the host and prevent cancer through various mechanisms (23). Many studies have shown that probiotic bacteria can effectively protect against cancer by producing postbiotic metabolites that induce apoptosis (24-30). Consistent with other studies, our investigation confirmed the anti-proliferative and anti-migration properties of two postbiotics obtained from *B. breve* and *L. rhamnosus* on HT-29 cell lines. The results revealed significant inhibition of cell growth in a dose- and time-dependent manner for both postbiotics. However, the IC_{50} of *B. breve* postbiotics was notably lower than that of *L. rhamnosus* postbiotics, indicating that probiotic anti-cancer ability could be genus-dependent. Similar to our study, Ardestani et

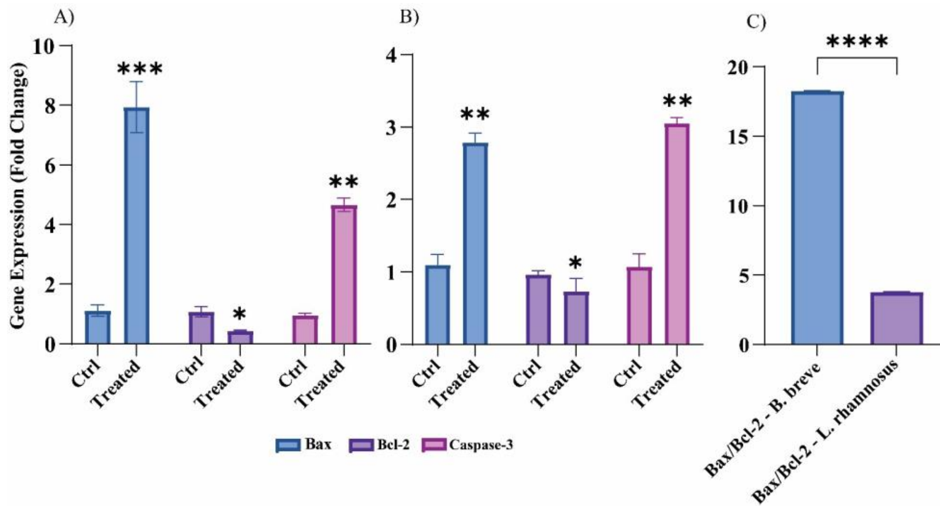

Fig. 3. *Bax, Bcl-2,* and *caspase-3* gene expression levels in HT-29 cell line in response to *B. breve* (A) and *L. rhamnosus* (B) extracted postbiotic treatment. The *Bax/Bcl-2* ratio in the cells treated by *B. breve* postbiotic compared with cells treated by *L. rhamnosus* postbiotic (C). The Y-axis represents the fold change in the expression of the target gene compared with the untreated group. *: significant difference compared with the untreate cells ($2^{(-\Delta\Delta ct)} = 1$ in untreated group). (p <0.05 ,p <0.01, p <0.001 and p <0.0001 respectively *, **, ***, **).

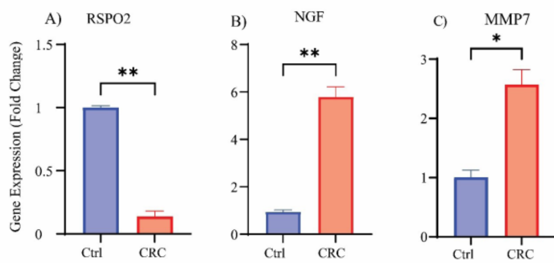

Fig. 4. Expression of *RSPO2, NGF,* and *MMP7* genes in colorectal cancer vs. normal adjacent tissues. Data (mean ± SE) are representative results derived from a minimum of three independent experiments. (p <0.05 and p <0.01, respectively *, **).

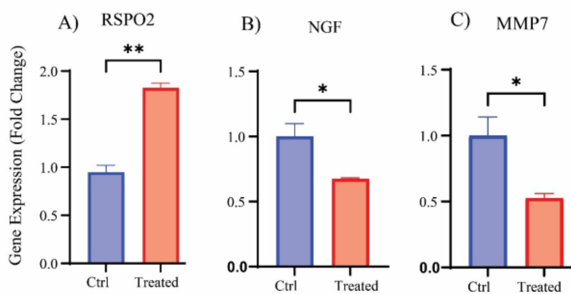

Fig. 5. Gene expression levels of *RSPO2, NGF,* and *MMP7* in HT-29 cell line in response to *B. breve* postbiotics treatment. The Y-axis represents the fold change in the expression of the target gene compared with the untreated group. (p <0.01 and p <0.05 respectively **, *).

al. reported that probiotic bacteria have anti-proliferative and anti-cancer properties, but the effectiveness varies depending on the specific genus and strain. For instance, they have shown that *Lactobacillus brevis* is more effective than *Lactobacillus paracasei* in inhibiting the growth of HT-29 cells and inducing apoptosis (31).

Moreover, we compared the IC_{50} values between HT-29 and HDF cells. We confirmed that the postbiotic concentrations required to elicit anti-proliferative and cytotoxic effects were considerably higher for normal cells than for the HT-29 cell line. Relative expression assays for *Bax, Bcl-2,* and *caspase-3* revealed an increased expression of *Bax* and *caspase-3* and a decreased expression of *Bcl-2*, which indicates that the postbiotics could exert their anti-cancer effects through the induction of apoptosis on the HT-29 cell line. Notably, the *Bax/Bcl-2* ratio in *B. breve*-treated cells was 4.1-fold higher than in *L. rhamnosus*-treated cells. This discrepancy suggests that postbiotics derived from *B. breve* may possess greater apoptotic effects on HT-29 cells than those from *L. rhamnosus.* The process of programmed cell death, known as apoptosis, is a natural occurrence in multicellular organisms. It involves regulating proteins such as *Bax, Bcl-2,* and *caspase-3.* Bax is a protein that supports apoptosis by encouraging the release of cytochrome c from mitochondria.

On the other hand, *Bcl-2* is an anti-apoptotic pro-

tein that inhibits apoptosis by preventing the release of cytochrome c from mitochondria. *Caspase-3* is a protease that breaks down many cellular proteins and is responsible for the changes in appearance associated with apoptosis. The induction of apoptosis through *Bax, Bcl-2,* and *caspase-3* can occur through various stimuli, including postbiotics (32, 33).

As part of our investigation, we conducted RT-qPCR experiments on tissue samples from twelve Iranian adenocarcinoma patients and their normal adjacent tissues to evaluate the expression of three key genes involved in colorectal cancer metastasis. The results showed that the expression of *RSPO2* was decreased while *NGF* and *MMP7* were increased in colorectal cancer tissues. Metastasis is the lethal feature of cancer and has been suggested to be associated with poor survival of patients with CRC (34). *RSPO2* is a member of the R-spondin family that inhibits Wnt/β-catenin signaling in the CRC cells (35, 36). Many studies have demonstrated that *RSPO2* inhibits CRC cell migration, invasion, and metastasis *in vitro* and *in vivo*, so reduced *RSPO2* expression is associated with tumor metastasis and poor survival in CRC patients (37-39). Nerve growth factor (*NGF*) is the founding member of the neurotrophins, responsible for the growth and developmental plasticity of neuronal populations (40, 41). *NGF* released by cancer cells can be a driver of tumor neurogenesis, and nerves infiltrated in TME release neurotransmitters, which might stimulate the growth and sustainment of tumor cells. It is reported that high expression of *NGF* is related to high incidence of metastasis in CRC because the binding of *NGF* to TrkA, phosphorylated TrkA, which activated MAPK/Erk signaling pathway increasing the expression NGAL to enhance the activity of *MMP2, MMP9,* and *MMP7*, promoted colorectal cancer metastasis (42-44). *MMP7*, also known as matrix metalloproteinase-7, is an enzyme that degrades extracellular matrix (ECM) proteins. It is highly expressed by epithelial tumor cells in invasive CRC compared to normal cells and is associated with distant metastasis (45). We also studied the expression of selected genes in the HT-29 cells treated with *B. breve* postbiotics as a potent anti-cancer metabolite. Our results demonstrated that *B. breve* postbiotics could increase the expression of *RSPO2* while decreasing the expression of *NGF* and *MMP7.*

Along with our investigation, various studies have shown that postbiotics have numerous physiological health benefits for their host. These benefits are achieved through various mechanisms such as apoptosis, anti-proliferative, anti-inflammatory, and anti-migration properties (46-49). In this way, several studies have validated the anti-apoptotic and anti-proliferative effects of *B. breve* and *L. rhamnosus* on different cancer cell lines and animal models. For instance, *L. rhamnosus* has been shown to effectively inhibit HT-29 cell growth through apoptosis induction, involving the release of cytochrome c and activation of the mitochondrial pathway (50). In another study by Gamallat et al. they investigated the impact of *L. rhamnosus* on CRC development. They revealed that treatment with this postbiotic decreases the expression of *β-catenin* and inflammatory mediators such as *NFκB-p65, COX-2,* and *TNF-α.* It also suppresses the anti-apoptotic protein *Bcl-2* while enhancing the expression of pro-apoptotic factors, including *Bax, caspase-3,* and *p53*, compared to control cells (51). Li et al. also demonstrated that *B. breve* can induce apoptosis in tumors and impede tumor growth in mice. This effect is attributed to the recruitment of intestinal dendritic cells (DCs) and the upregulation of *IL-12*, which promotes the recruitment of T cells to the tumor microenvironment. Their research further suggests that manipulating the gut microbiota by introducing exogenous *B. breve* may enhance its anti-tumor efficacy (52).

This study provides compelling evidence for anti-cancer properties of probiotics, highlighting their potential as therapeutic interventions in CRC and other cancer types. However, it is important to acknowledge that although our *in vitro* experiments with *B. breve* and *L. rhamnosus* postbiotics have shown potential anti-cancer effects on the HT-29 cell line, it is crucial to recognize that the behavior of CRC cells can vary widely. Therefore, the effects observed in this study may not necessarily extend to all types of CRC cells. Additionally, further research is needed to explore additional signaling pathways that contribute to the development of the cancer phenotype.

CONCLUSION

In this study, we confirmed the anti-proliferation and anti-migration effects of *B. breve* and *L. rhamnosus* postbiotics on HT-29 cell lines in a dose- and time-dependent manner. We concluded that these postbiotics have the ability to induce apoptosis by

upregulating the expression of *Bax* and *caspase-3* while simultaneously downregulating the expression of *Bcl-2*. Intriguingly, a comparative analysis between *B. breve* and *L. rhamnosus* postbiotics unveiled the superior anti-cancer properties of *B. breve*. It exhibited a more pronounced ability to inhibit the growth of HT-29 cells and induce apoptosis compared to *L. rhamnosus*. Moreover, our RT-qPCR results on tissue samples showed decreased expression of *RSPO2* genes and increased expression of *MMP7* and *NGF* in adenocarcinoma tissue samples compared to normal adjacent tissues. However, treating cells through *B. breve* postbiotics could upregulate the expression of *RSPO2* while downregulating the *NGF* and *MMP7* expression. These findings contribute valuable knowledge to exploring postbiotics from probiotic bacteria as potential agents against cancer growth and migration.

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